Safety Assessment of Transgenic Organisms

OECD CONSENSUS DOCUMENTS

Volume 2

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This book is published on the responsibility of the Working Group on Harmonisation of Regulatory Oversight in Biotechnology, which is a subsidiary group of the Chemicals Committee and Working Party on Chemicals, Pesticide and Biotechnology of the OECD.

FOREWORD

Genetically engineered crops (also known as transgenic crops) such as maize, soybean, rapeseed and cotton have been approved for commercial use in an increasing number of countries. During the period from 1996 to 2005, for example, there was more than fifty-fold increase in the area grown with transgenic crops worldwide, reaching 90 million hectares in 2005[1]. Such approvals usually follows a science-based risk/ safety assessment.

The environmental safety/ risks of a transgenic organism have been assessed based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/ risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/ safety assessment. This information is said to be mutually acceptable among member countries. To date, 25 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

This book is a compilation of those Biosafety Consensus Documents published before February 2006. It also includes two recently published texts: the first, entitled *An Introduction to the Biosafety Consensus Document of OECD's Working Group for Harmonisation in Biotechnology*, explains the purpose of the consensus documents and how they are relevant to risk/ safety assessment. It also describes the process by which the documents are drafted using a "lead country" approach. The second text is a *Points to Consider for Consensus Documents on the Biology of Cultivated Plants.* This is a structured checklist of "points to consider" for authors when drafting or for those evaluating a consensus document. Amongst other things, this text describes how each point is relevant to risk/ safety assessment.

This book offers ready access to those consensus documents which have been published thus far. As such, it should be of value to applicants for commercial uses of transgenic crops, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of this book are encouraged to provide any information or opinions regarding the contents of this book or indeed, OECD's other harmonisation activities. If needed, a short pre-addressed questionnaire is attached at the end of this book that can be used to provide such comments.

The published Consensus Documents are also available individually from OECD's website (http://www.oecd.org/biotrack) at no cost.

1. Clive James (2005), International Service for the Acquisition of Agri-biotech Applications (http://www.isaaa.org/)

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Part 3

CONSENSUS DOCUMENTS ON THE BIOLOGY OF TREES

SECTION 1
EASTERN WHITE PINE (*PINUS STROBUS* L.)

1. General Information

This consensus document addresses the biology of Eastern White Pine (*Pinus strobus* L.), referred to hereafter simply as Eastern White Pine (*pin blanc* in French Canada). Eastern White Pine is one of the most valuable tree species in eastern North America where its easily machined, uniform-textured wood is unsurpassed for doors, windows, panelling, mouldings and cabinet work (Mullins and McKnight, 1981; Farrar, 1995). The species played a major role in the settlement and economic development of New England and the Atlantic Provinces as England reserved all large Eastern White Pine suitable for masts under the "Broad Arrow" policy, starting in the late 1600's (Johnson, 1986). Eastern White Pine also responds well to nursery culture and is commonly used for reforestation, urban forestry and Christmas tree plantations.

The general biology of Eastern White Pine is described in the context of the species' role in natural forests and its domestication in planted stands. Taxonomic and evolutionary relationships with other *Pinus* species are described. Reproductive biology is described with a focus on aspects of mating system, gene flow, seed production and natural stand establishment. The current knowledge of genetic variation within the species is reviewed, highlighting the importance of geographic variation patterns and the potential for improvement by means of recurrent selection breeding strategies. The tremendous biological diversity and the complexity of ecological interactions with higher and lower flora and fauna are discussed. While Eastern White Pine has been commonly planted within its natural range, the extent of reforestation has been limited by susceptibility to white pine weevil (*Pissodes strobi*) and blister rust (*Cronartium ribicola*). Domestication and operational breeding activities are also reviewed. Crossing with other related white pine species offers some promise of producing hybrids with increased resistance to both the weevil and blister rust. While white pine reforestation is currently based on seed propagation, vegetative propagation techniques are available and research continues into regeneration from somatic embryos.

Canada was the lead country in preparation of this document. It is intended for use by regulatory authorities and others who have responsibility for making assessments of transgenic plants proposed for commercialisation, and by those who are actively involved with genetic improvement and intensive management of this species.

2. Taxonomy and Natural Distribution

A. Taxonomy and nomenclature

The genus *Pinus* L. (family Pinaceae) is widely distributed throughout the Northern Hemisphere, from the arctic circle south to Guatemala, the West Indies, North Africa and Indonesia, with as many as 100 species being recognised (Krüssmann, 1985). The genus was first classified on evolutionary characteristics by Shaw (1914), and taxonomists have since followed his general separation of the genus into two groups: *Haploxylon* Koehne, and *Diploxylon* Koehne; commonly called the "soft" (or "white") and "hard" pines, respectively, based on the presence of one or two vascular bundles in the leaves. Shaw's original subdivision of these groups has been reworked by different authorities (*e.g.*, Pilger, 1926; Duffield, 1952;

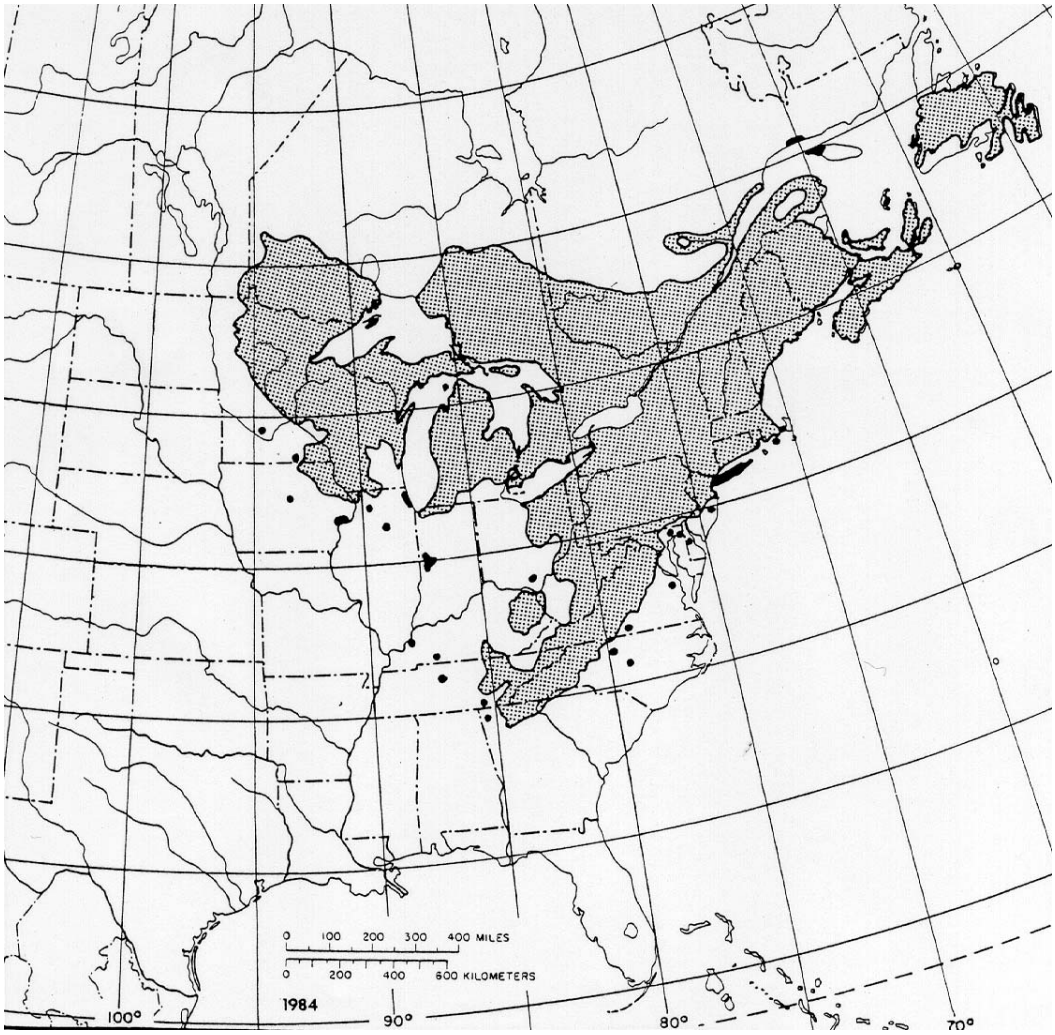
de Ferré, 1965; Landry, 1974b, 1978), but botanists in recent years have generally recognised the classification described by Little and Critchfield (1969, 1986), who place Eastern White Pine, *Pinus strobus* L., within the subgenus *Strobus* Lemm. (equivalent to subgenus *Haploxylon*), section *Strobus*, subsection *Strobi* Loud. Also known as northern pine and, in parts of Europe, as Weymouth pine, after Lord Weymouth, the species nomenclature has remained virtually undisputed since the publication of the *Species Plantarum* (Linné, 1753), although Provancher later referred to it as *Pinus alba Canadensis* Prov. (Landry, 1974a).

Several horticultural forms have been named, although none are currently recognised with varietal status (Krüssmann, 1985). Only one variety has been commonly described, *Pinus strobus* L. var. *chiapensis* Martinez, the Chiapas white pine, occurring in the mountains of southern Mexico and Guatemala. While similar morphologically, it is physiologically quite different (Wright, 1970) and now generally recognised as a separate species, *Pinus chiapensis* (Martinez) Andresen (Griffiths, 1994; Perry, 1991).

B. Natural distribution

Eastern White Pine has the largest range of any North American member of subgenus *Strobus*, and is the only species in the subgenus occurring on the eastern side of the continent. It extends from Newfoundland and Quebec, west to central Ontario and south-eastern Manitoba, south to Minnesota, north-eastern Iowa, northern Illinois, north-western Indiana, Ohio, Pennsylvania, and New Jersey, and south in the Appalachian Mountains to western North Carolina, northern Georgia, and Tennessee. Overall, the species spans a north-south range of over 1900 km, and about the same distance inland from the Atlantic coast (Critchfield and Little, 1966; Mirov, 1967; Wendel and Smith, 1990). The natural range of Eastern White Pine is illustrated in the map given in Figure 3.1.

Figure 3.1 The natural range of Eastern White Pine



Source : Wendel and Smith, 1990

C. Evolution and migrational history

Conifers probably originated around the periphery of the north Pacific basin (Li, 1953). Fossil records indicate that divergence of modern genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin, 1963), and *Pinus* is believed to be the oldest genus in the family (Miller, C.N. 1976, 1988). Opinion on whether the first pines were of subgenus *Strobus* or *Pinus* remains mixed, and the centre of origin of pines is uncertain. Millar and Kinloch (1991) describe the rapid spread of pines over dry, temperate paleolatitudes during the Cretaceous period, prior to the separation of North America from Europe, during which all sections and subsections appear to have originated. In the early Tertiary period, global climate changes favoured the spread of angiosperms, which adapted to the hot, humid conditions. These climate changes fragmented and displaced the pines into drier refugia at upper and lower latitudes, and scattered refugia at mid-latitudes, creating secondary centres of origin. Ancestors

of *P. strobus* and *P. monticola* were isolated in northern refugia from other species in section *Strobi* that were isolated in the south. The warm, tropical conditions changed rapidly at the end of the Eocene epoch and pines became re-established at middle latitudes. These abrupt changes in climate had drastic impacts on the gene structure of genetic variation of forest populations, with isolated populations continuing their short-term evolution (Critchfield, 1984).

3. Reproductive Biology

A. Reproductive development

Eastern White Pine is monoecious. Production of female strobili occurs as early as 4 years (Buckingham, 1963) while pollen production may not start for 10 to 20 years (Wright, 1970). As in other pines, development of the reproductive structures follows a 3-year cycle. Pollination occurs in the spring of the second year, with fertilisation delayed until the following spring, and seeds maturing in the fall of the third year (Owens and Blake, 1985). No other conifer genus has had its reproductive cycle described more often or more thoroughly, and Eastern White Pine was among the first pines to be studied in detail (Ferguson, 1901, 1904). Reproductive buds begin as axillary bud primordia within a complex long-shoot bud, consisting of a series of cataphylls initiated throughout the growing season. Many of the cataphylls support an axillary apex that first initiates a series of bud scales, then differentiates into a short (fascicular) shoot, seed or pollen cone, or lateral long shoot bud. Those axillary buds initiated at the base of the long shoot bud in the spring or early summer will differentiate into short-shoot or pollen-cone buds. Subsequent axillary buds differentiate only into short shoots. The distal axillary buds remain undetermined through winter dormancy of the long shoot bud, differentiating immediately the following spring into lateral long shoot or seed cone buds (Owston, 1969; Owens and Molder, 1977). While seed-cones generally develop on vigorous shoots in the upper portion of the crown, distribution of reproductive structures is often extremely variable.

Pollen development and meiosis does not occur until the spring of the second year as pollen cones resume their development. The ripening strobili turn light brown before releasing their pollen over a 1-week period. The seed cones also resume development in the spring, and are visible at the distal end of elongating long shoots. The developmental morphology of reproductive structures was well documented with colour photographs by Ho (1991). Wind-borne pollen grains landing on the receptive seed cone pass between the bracts and sift down to the surface of the micropylar arms, entering the micropyle by means of a pollen drop. The pollen germinates, but becomes dormant before the male gametes form (Owens and Molder, 1977). Fertilisation occurs about 13 months after pollination. Simple polyembryony in Eastern White Pine results from the fertilisation of 2 to 3 archegonia in each megagametophyte. The seed cones mature and seeds are dispersed in August or September of the same year (Krugman and Jenkinson, 1974).

B. Mating system and gene flow

Eastern White Pine is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system, although there are relatively few detailed studies. Isozyme studies of populations in Québec indicated a high rate of outcrossing, with most loci in Hardy-Weinberg equilibrium (Beaulieu and Simon, 1994, 1995).

Gene flow in *Pinus* is mediated by very small pollen grains, 40-60 μm at their widest point (Eisenhut, 1961), whose two air sacs and low density make them well-adapted for aerial transport (Di-Giovanni and Kevan, 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright, 1976). Nevertheless, conifer pollen may remain viable for several days and a substantial quantity may travel great distances (Lindgren *et al*., 1995; Lindgren and Lindgren, 1996). Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to

1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan, 1991).

C. Seed production

Eastern White Pine normally begins seed production at 5 to 10 years of age (Fowells, 1965; Sargent, 1965), although little pollen is produced during the early years of flowering (Wendel and Smith, 1990). The interval between heavy seed crops is usually 3 to 10 years (Krugman and Jenkinson, 1974; Wendel and Smith, 1990), becoming less frequent as trees become over mature (Horton and Bedell, 1960). A study in Germany recorded seed production as high as 73 kg/ha in a 90-year-old stand (Messer, 1956), while in Maine, a stand considered to be intermediate in density with a basal area of 28 m^2/ha produced over 4.4 million seeds per hectare in a "bumper" year (Graber, 1970). This corresponds to 69kg/ha seed.

Initiation of seed dispersal is weather and site dependent, and may be delayed by cool, moist weather. Most of the seeds are dispersed in the fall during a 4 to 8 week period (Horton and Bedell, 1960; Graber, 1970). The seeds are mature when cone moisture content decreases below 200% on a dry-weight basis, but cone specific gravity is not a reliable indicator of maturity (Barnett, 1988). A short "artificial ripening" period can increase yield and quality of seed from immature cones (Bonner, 1986, 1991; Barnett, 1988).

The seeds are winged and dispersal distances depend greatly on local and prevailing wind patterns (Rudis *et al*., 1978). The seeds may travel more than 60 m within a closed stand, and over 200 m in the open (Wilson and McQuilckin, 1963), although most of the seed will fall within a distance equivalent to the height of the seed tree (Horton and Bedell, 1960). The seeds themselves are smaller than those of most other "soft" pines, but similar to those of *P. monticola*, with average cleaned seed weight of about 17 g/1000 seeds (Krugman and Jenkinson , 1974).

D. Natural regeneration

Eastern White Pine seeds exhibit varying degrees of embryo dormancy that may be broken by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Krugman and Jenkinson, 1974; Nelson *et al*., 1980; Mittal *et al*., 1987; Downie and Bergsten, 1991). The recommended treatment for nursery sowing is stratification for 60 days at 1 to 5° C (Krugman and Jenkinson, 1974). Under natural conditions, over-winter stratification on the forest floor breaks seed dormancy and germination of most seeds occurs in late spring of the following year (Stiell, 1985).

Germination is epigeal. Moist mineral soil, polytrichum moss, and shortgrass cover of light to medium density are favourable seedbeds. Establishment on less favourable seedbeds, such as pine litter and lichen, will occur under partial shade and/or surface. Shelterwood harvesting systems provide good protection during initial establishment with sufficient light for subsequent growth of young stands (Wilson and McQuilckin, 1963; Corbett, 1994). Optimum conditions are provided when moist mineral seedbeds have greater than 20% of full sun, but where partial shade reduces surface temperatures and provides better moisture conditions (Lancaster and Leak, 1978). Low seedling densities are associated with competition from broad-leaved shrubs, herbaceous vegetation, tolerant conifer species and feather mosses (Carleton *et al*., 1996). White pine regeneration is usually associated with its proportion in the overstorey (Kittredge and Ashton, 1990), and under old-growth conditions is likely to become at least partially uneven-aged and self replacing, facilitated by local disturbances and continuous recruitment (Quinby, 1991; Ziegler, 1995). Older trees have increased their ability to recover from long periods of suppression (Abrams and Orwig, 1996).

E. Vegetative reproduction in nature

Eastern White Pine does not regenerate vegetatively under natural conditions (Wendel and Smith, 1990).

4. Crosses

Other members of subgenus *Strobus* do not occur within the natural range of Eastern White Pine, and introgressive hybridisation does not occur. Most artificial crosses among North American members of subsection *Strobi* have been successful, the exception being those involving *Pinus lambertiana* Dougl. (Critchfield, 1986; Critchfield and Kinloch, 1986). Successful crosses involving Eastern White Pine are summarised in Table 3.1. Only two of these hybrids have been widely field tested, including that with *P. monticola* Dougl. and its reciprocal, and with *P. wallichiana* A.B. Jackson (formerly *P. griffithii* McClell.) and its reciprocal (Kriebel, 1983). No successful hybrid crosses have been reported with species in other sections of *Pinus* (Critchfield, 1975).

Table 3.1 Summary of successful crosses with *P. strobus*

Species	Origin	References
P. monticola Dougl. ex D.Don.	western US and Canada	Wright, 1959, 1970; Kriebel, 1972b
P. wallichiana A.B. Jackson (syn P. griffithi McClell., P. excelsa Wallich ex D.Don), hybrid = P. ×schwerinii Fitschen	Himalayas	Wright, 1959, 1970; Kriebel, 1972b; Garrett, 1979; Zsuffa, 1979b; Blada, 1992
P. ayacahuite Ehrenb.	Mexico, Guatemala	Johnson and Heimburger, 1946; Wright, 1959; Garrett, 1979
P. parviflora Sieb. and Zucc. hybrid = P. ×hunnewelli A.G. Johnson	Japan	Johnson, 1952; Wright, 1959
P. peuce Griseb.	S.E. Europe	Fowler and Heimburger, 1958; Radu, 1976; Santamour and Zinkel, 1978
P. flexilis James (only one parent successful, may in fact be P. strobiformis Engelm.)	western US and Canada	Wright, 1959; Kriebel, 1972a

When hybrids are made successfully, they sometimes display hybrid vigour and out-perform the parent species (Wright, 1970; Kriebel, 1983). However, more important than increased vigour, hybrids with *P. wallichiana*, *P. peuce*, and *P. parviflora* have demonstrated potential resistance to blister rust (Heimburger, 1962, 1972; Patton, 1966; Zsuffa, 1979a), and those with *P. peuce* and *P. monticola* may be less susceptible to weevil attack (Heimburger and Sullivan, 1972a, b). While hybridity barriers within the hard pines are generally associated with pollen tube incompatibility, crossability barriers among the white pines are more often the result of embryo inviability (Kriebel, 1972a; Shafer and Kriebel, 1974).

5. Genetics

A. Cytology

Vegetative cells are normally diploid, with $2n = 24$ chromosomes (Saylor, 1983). Saylor and Smith (1966) reported that 4% of cells displayed meiotic irregularities such as precocious disjunction, lagging chromosomes, and inversion bridges.

B. Genetic variation

Population-level variability

While seed source testing of Eastern White Pine began in the United States in 1937 (Pauley *et al.,* 1955), provenance tests with range-wide sampling did not begin until the mid-1950's. Around this time, the USDA Forest Service initiated a large provenance test, in which 30 seed collections representing all parts of the natural range were established by co-operators in 13 test plantations in the United States and 2 in Ontario (Sluder, 1963; Wright *et al.,* 1963; Funk, 1965; Fowler and Heimburger, 1969b; King and Nienstaedt, 1969; Genys, 1977). Shortly after, another provenance test involving more seedlots on fewer test sites was started by the University of Maryland (Genys, 1968; Genys *et al*,. 1978). Encouraging early results from these tests, indicating the superiority of sources from the South Appalachians, led to intensive testing of these sources under the leadership of Michigan State University (Roth and Carson, 1976; Wendel and Cech ,1976; Wright *et al.*, 1976; Gall and Thor ,1977).

While correlations with latitude have sometimes been noted on a range-wide basis (Genys, 1987, 1991), relative differences in height and diameter between northern and southern sources diminish somewhat with age (Demeritt and Kettlewood, 1976; Demeritt and Garrett, 1996). Clinal patterns are often less distinct over shorter distances with the presence of non-clinal adapted ecotypes (Genys, 1968; Garrett *et al*., 1973; Thor, 1975; des Bordes and Thor, 1979; Funk, 1979). In Nebraska, seed sources from the southern Appalachians demonstrated correlations with latitude for needle length and reproductive phenology, a weak geographic pattern for variation in height, and none for survival (Sprackling and Read, 1976; Van Haverbeke, 1988). Ryu and Eckert (1983) investigated the genetic structure of 27 of these provenances for eight foliar enzymes coded by 12 loci and found four clusters of provenances, three of which may be representative of populations adapted to differing geographic and climatic conditions. The results of this study support the indication of ecotypic variation among three provenances in the southern Appalachians for growth performance and physiological variables, and suggest that these areas may have been isolated refugia during glaciation. Elsewhere in the northern part of the range, sources from the Atlantic coast outperformed those from further inland, while some exceptional sources originated from as far south as Georgia and Tennessee (Zsuffa, 1975; Abubaker and Zsuffa, 1991).

Southern provenances have heavier seeds (Genys, 1968), require longer periods of stratification before germinating (Fowler and Dwight, 1964; Graber, 1965) and longer chilling periods to break bud dormancy (Mergen, 1963), set bud later (Santamour, 1960) and are less cold hardy (Maronek and Flint, 1974). Wood specific gravity was negatively correlated with height and diameter, but differences among sources were small (Lee, 1974; Gilmore and Jokela, 1978; Olson *et al*., 1981). No variation could be detected for foliar monoterpene content, and no geographic pattern was evident for variation in cortical monoterpenes (Gilmore and Jokela, 1979).

Provenance tests have shown some variation in susceptibility to white pine weevil, but give little indication that resistant populations can be identified (Garrett, 1972, 1973; Connola and Beinkafner, 1976; Wilkinson, 1983b). Selective thinning of susceptible parents (dominant "wolf" trees) from a stand can increase the level of resistance in the progeny generation, and taller families tend to be more weevil resistant (Ledig and Smith, 1981). Although there is ample evidence of genetic control of susceptibility to weevil, the actual mechanism(s) of resistance remains uncertain.

Individual-level variability

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within-populations as the source of genetic gains. The partitioning of genetic variance among and within populations is greatly

influenced by the range of adaptive variation sampled by the tested provenances and the age at which the test material is assessed. Range-wide and regional studies have typically demonstrated strong heritabilities, sufficient to predict moderate to high genetic gains, although heritability tends to be lower for older material (Thor, 1975; Adams and Jolly, 1978; des Bordes and Thor, 1979; Olson *et al*., 1981). Hierarchical sampling of populations over a more limited range in Québec and Ontario showed that population differences were greatest for allozyme markers, where 98% of the variation was within populations (Beaulieu *et al.,* 1996). Growth traits, on the other hand, demonstrated variation within stands to be about half as great as that among populations (Li, P. *et al.,* 1997). Individual heritability for height declined from 0.547 at age 4 in the nursery, to 0.187 at age 10 in the field (Beaulieu *et al*., 1996). In an incomplete diallele cross experiment among individuals of a local provenance, Kriebel *et al.* (1972) found that narrow-sense heritability for height growth declined from 0.59 at age 1 to 0.16 at age 3, and that while dominance effects were small, maternal effects were rather large. By age 13, it was still possible to achieve substantial gains by family selection (Kriebel, 1978).

Significant genotype-environment interactions have been reported in Eastern White Pine, but the magnitude of the interaction variance is generally low (less than 2%). Genetic correlations between sites tend to be high, indicating that family ranks are stable across sites (des Bordes and Thor, 1979; Beaulieu *et al.,* 1996; Demeritt and Garrett, 1996).

The search for weevil-resistance has always been a driving force behind genetic testing in Eastern White Pine (Pauley *et al.,* 1955; Wright and Gabriel, 1959). Early studies indicated that selection for weevil resistance might be done indirectly by assessment of bark thickness (Kriebel, 1954; Gerhold, 1962, 1966) and/or leader morphology (Stroh, 1964, 1965), but when these are corrected for tree size, they appear to be of little value for effective selection (Wilkinson, 1983a, 1984). Other studies have identified that concentrations of various cortical oleoresin compounds are correlated with weevil susceptibility, but even these criteria leave much of the variation in weevil susceptibility unexplained (van Buijtenen and Santamour, 1972; Santamour and Zinkel, 1976, 1978; Bridgen *et al.,* 1979; Wilkinson, 1979, 1980, 1984, 1985).

C. Inbreeding depression and genetic load

Eastern White Pine is an outcrossing species that carries a fairly heavy load of deleterious recessive genes. Individuals are generally self-compatible, so that this genetic load is revealed by self-fertilisation (Fowler, 1965a; Fowler and Heimburger, 1969a). Although there is no reduction in numbers of filled seeds after selfing (Fowler, 1965b), selfed seedlings may be stunted, slow growing, chlorophyll-deficient and deformed (Johnson, 1945; Patton and Riker, 1958a; Fowler, 1965b). Simple polyembryony in Eastern White Pine results from 2 to 3 archegonia in each megagametophyte. As only one embryo normally germinates from the mature seed, it is likely that competition during seed development eliminates many weaker embryos, including those resulting from self-fertilisation (Willson and Burley, 1983). An isozyme study of populations in Quebec demonstrated a high outcrossing rate, with few loci deviating from Hardy-Weinberg equilibrium (Beaulieu and Simon, 1995). This study found evidence of family structure, with greater inbreeding in the filial than in the parental population, although few of the inbred genotypes were expected to reach reproductive age, due to natural selection.

D. Breeding programs

Eastern White Pine has been a candidate for tree breeding efforts throughout its native range. In the northern part of its range, throughout eastern Canada, the north-eastern US and the Lake States, planting programs have been limited by susceptibility to weevil and rust, so that seed orchards exist throughout this region (Zsuffa, 1985, 1986; Garrett, 1986; Miller, 1987; Eckert and Kuser, 1988; Lamontagne, 1992; Nielsen *et al*., 1995; Smith *et al*., 1997; *pers. comm.* R. Stine, Minnesota Tree Improvement Cooperative)

and, the level of effort reflects the restricted size of planting programmes. Pests are less of a problem for breeding programs in the Central States, where selection and hybrid breeding can focus on vigour (Kriebel, 1983). Outside of the natural range in Europe, selection within southern Appalachian provenances and crossing with other white pines, such as *Pinus wallichiana*, are used to develop fast-growing, rust-resistant hybrids (Kriebel, 1983).

Most seed orchards currently in production were established by grafting cuttings from plus-trees, and their establishment in cultivated field environments. Grafting success is usually very high. Flowering in field orchards can be enhanced by means of cultural treatments such as fertilisation (Hocker, 1962; Stephens, 1964). Flowering of young white pine grafts can also be stimulated by means of various cultural treatments, particularly those involving gibberellin $A_{4/7}$, and this has facilitated the turnover of breeding cycles (Ho and Schnekenburger, 1992; Ho and Eng ,1995).

E. Conservation of genetic resources

Domestication of a key species such as Eastern White Pine can influence diversity of genetic resources (1) indirectly, by the method of seed collection, extraction, and storage, and by nursery and plantation culture; and (2) directly, by intentional selection to increase the frequency of genes for desirable traits (Morgenstern, 1996). The inadvertent loss of genes by natural processes and human activity can have negative consequences on the adaptability of populations and the potential for future gains from breeding.

A long history of exploitation has resulted in white pine forest fragmentation and reduction of population sizes, particularly at the northern limits of the species range (Buchert, 1994; Buchert *et al*., 1997). Throughout most of the range of white pine, *in situ* conservation of genetic resources is practised by protection of ecological reserves, special areas, and parks (Pollard, 1995), and integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of isolated, small populations (Mosseler, 1995; Nieman *et al*., 1995).

Ex situ conservation, by cryopreservation of germplasm, by off-site maintenance of populations in arboreta, seed orchards and clone banks, and by multi-population breeding strategies (Eriksson *et al*., 1993; Namkoong, 1995), has been practised to a much lesser extent, although many provenances and families of Eastern White Pine are now represented in field tests and seed bank collections (Plourde *et al*., 1995). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller, 1992).

6. Ecology and Associated Species

Much of the information in this section originates from the excellent monograph on silvics of the species by Wilson and McQuilkin (1963). Other citations are given when appropriate when specific information is attributable to other sources.

A. Habitat

Climate

Eastern White Pine's natural range is cool and humid. July average temperatures are between 18 to 25° C, and annual precipitation varies from about 510 mm in northern Minnesota to 2030 mm in north-western Georgia, with at least half occurring between April and November. Average snowfall varies from less than 15 cm in the southern portion of the range to over 250 cm in the northeast (Wendel and Smith, 1990). There is a surplus of moisture in all seasons.

Soils and site type

Eastern White Pine grows on a wide variety of soils throughout its range, from dry sands and rocky ridges, to sphagnum bogs, although it grows best on moist sandy or loamy soils. Soils within the range are derived from granites, gneisses, schists, sandstones, and, to a lesser extent, phyllites, slates, shales and limestones. Eastern White Pine competes best on medium-textured, well-drained soils of moderate site quality, with surface pH between 4.0 and 7.5, and which are not sufficiently rich to support strong hardwood competition, or where competition is reduced during the establishment period, such as on old fields, burnt or blow-down areas (Horton and Bedell, 1960; Mader, 1986).

In the northeast portion of the range, Eastern White Pine generally occurs below 450 m above sea level, whereas in Pennsylvania, elevations vary between 150 and 600 m. In the southern Appalachians, stands generally occurs between 370 and 1070 m. Except in Pennsylvania and the southern Appalachians where stands are found on northerly aspects or in the shelter of stream bottoms. White pine sites are not generally restricted by slope or aspect.

B. Synecology and associated species

Eastern White Pine may form pure stands or occur as a major stand component of several stand types in association with other conifers and hardwoods such as: red pine (*Pinus resinosa*), balsam fir (*Abies balsamea*), black spruce (*Picea mariana*), White Spruce (*P. glauca*), red oak (*Quercus rubra*), sugar maple (*Acer saccharum*), red maple (*Acer rubrum*), hemlock (*Tsuga canadensis*), and chestnut oak (*Quercus prinus*). Eastern White Pine may also be found as a lesser stand component with jack pine (*Pinus banksiana*), pitch pine (*P. rigida*), shortleaf pine (*P. echinata*), sweet birch (*Betula lenta*), trembling aspen (*Populus tremuloides*), large-tooth aspen (*P. grandidentata*), black cherry (*Prunus serotina*), black oak (*Quercus velutina*), white oak (*Quercus alba*), and various hickories (*Carya* spp.) (Horton and Bedell, 1960; Eyre, 1980). The occurrence of associations depends on both site conditions and history of disturbance (Stiell, 1985).

Pure stands of Eastern White Pine usually support sparse cover of understory vegetation, but many species may be found under mixed stands, particularly those associated with hardwood associates. On drier sites, ground vegetation may consist of one or more species of blueberries (*Vaccinium* spp.), teaberry (*Gaultheria procumbens*), dwarf bush-honeysuckle (*Diervilla lonicera*), sweetfern (*Comptonia peregrina*) bracken fern (*Pteridium aquilinum*), clubmoss (*Lycopodium* spp.), and broom sedge (*Andropogon virginicus*). Richer, moist sites will often support ground cover of woodsorrels (*Oxalis* spp.), partridgeberry (*Mitchella repens*), wild sarsaparilla (*Aralia nudicaulis*), jack-in-the-pulpit (*Arisaema* spp.), and hay-scented fern (*Dennstaedtia punctilobula*). Intermediate sites may have varying amounts of the above species, together with dogwoods (*Cornus* spp.) and false lily-of-the-valley (*Maianthemum canadense*).

C. Competition and stand structure

Eastern White Pine is distributed over a larger area than any other North American white pine, and has demonstrated its capacity to grow and compete under a wide variety of environmental conditions (Stiell, 1978, 1985). While it is a long-lived successional species and may be a component of climax forest types, it is also well-known as a pioneering species on old fields in New England. Eastern White Pine is considered intermediate in its tolerance to shade, somewhat less tolerant than eastern spruces and more tolerant than its pine associates (Daniel *et al*., 1979). Vegetative competition for light and soil moisture is critical during seedling establishment, and remains important well into the life of the stand. Sites that have a high capability for productivity for pine tend to have greater competition. Competition problems are most severe on heavier, moist, rich soils, where Eastern White Pine will perform well, only if natural disturbance, such as fire, or silvicultural site treatments allow the pine to become established well ahead of

the hardwoods that normally occupy such sites (Horton and Bedell, 1960; Little *et al*., 1973; Stiell, 1985; Chapeskie *et al*., 1989).

D. Ecosystem dynamics

Several abiotic factors also interact with Eastern White Pine in forest ecosystems. While older trees have thick, heat-resistant bark, the thinner bark on exposed roots and younger stems is sensitive to fire. Even light fires can have a detrimental impact on seed supply, but may also reduce hardwood competition and leave a seedbed that is more conducive to the establishment of new germinants. Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils. Eastern White Pine is relatively wind firm, but may suffer storm breakage if the stand has been recently thinned. While it is widely held that Eastern White Pine is sensitive to ozone and sulphur dioxide pollution (Gerhold, 1977), recent data in the literature are somewhat contradictory and suggest that injury and growth losses may be strongly genotype and site dependant (Houston and Stairs, 1973; Genys and Heggestad, 1978, 1983; Townsend and Dochinger, 1982; Usher and Williams, 1982; Yang *et al*., 1982, 1983; Eberhardt *et al*., 1988; Rezabek *et al*. , 1989; Bartholomay *et al*., 1997; Hogsett *et al*., 1997).

The following table 3.2 shows species interactions with Eastern White Pine.

Table 3.2 Species Interactions with Eastern White Pine

Insects	
Common name	**Agent**
White pine weevil [Sullivan, 1961; Sun and Nigam, 1972; Sunandram *et al*., 1972; Berry and Steill, 1976; Sunandram, 1977; Stiell, 1979; Martineau, 1984; deGroot, 1985; Drooz, 1985; Gross, 1985a; Wallace and Sullivan, 1985; Stiell and Berry, 1985; Diamond and Bradbury, 1992; Katovich and Morse, 1992; Mielke, 1993; Humble *et al*., 1994; de Groot and Zylstra, 1996]	*Pissodes strobi* The most serious economic insect pest of white pine. Larvae tunnel down the inner bark of the shoot, killing the leaders.
Sawfly [Houseweart and Knight, 1986]	*Diprion similis.* Foliage damage
Pine false webworm	*Acantholyda erythrocephala* Foliage damage
White pine sawfly	*Neodiprion pinetum* Foliage damage
Jack pine budworm	*Choristoneura pinus* (when growing near jack pine) Foliage damage
Eastern pine shoot borer	*Eucosma gloriosa* Growing shoot damage
European pine shoot moth	*Rhyacionia buoliana* Growing shoot damage
Pine leaf adelgid	*Pineus pinifoliae* (when growing near red or black spruce) Causes shoot damage
White pine aphids	*Cinaria strobi* Can cause mortality in young trees
Seedling debarking weevil [Houseweart and Knight, 1986; Pendrel, 1990]	*Hylobius congener* Can cause seedling mortality
Warren's collar weevil	*H. warreni* Damages roots
Pine root collar weevil	*H. radicis* Damages roots
Pales weevil	*H. pales* Damages roots
Mound ants	*Formica* sp. Damages roots
Zimmerman pine moth	*Dioryctria zimmermani.* Damages sapling stems
Fir coneworm	*D. abietivorelle* reduces seed production
White pine cone beetle	*Conopthorous coniperda* reduces seed production
White pine cone borer [Wilson, 1977; Martineau, 1984; Rose and Lindquist, 1984; Syme, 1985]	*Eucosma tocullionana* reduces seed production

Fungi	
Disease	**Agent**
White pine blister rust [Patton, 1961; Van Arsdel, 1961; Charlton, 1963; Gremmen and Kam, 1970; Anderson, 1973; Lehrer, 1982; Lavalée, 1974, 1986; Robbins, 1984; Gross, 1985b: Stiell, 1985; Ostrofsky *et al*., 1988; Merril, 1991; Katovich and Mielke, 1993; Myren *et al*., 1994; Liebhold *et al*., 1995; Berube, 1996; Bowling and Niznowske, 1996; Hummer, 1997; La and Yi, 1976; Yokota and Uozumi, 1976; Stephan and Hyun, 1983]	*Cronartium ribicola*. the most serious fungal disease of white pine. Has alternate host from the *Ribes* species as well as Pedicularis and Castillija species.. Eradication of *Ribes* near white pine nurseries is a common control practice.
"Damping off" of emerging seedlings [Peterson, 1975]	*Fusarium* spp. *Pythium* spp *Rhizoctonia* spp. *Phytophthora* spp. *Cylindrocladium* spp.
Cytospora dieback	*Valsa* spp. disease of young seedlings
Tip blight	*Sphaeropsis sapinea* disease of young seedlings
Snow blight	*Phacidium infestans* disease of young seedlings
Rhizinia root rot	*Rhizinia undulata* disease of young seedlings
Needle casts	*Lopnodermium* spp. *Hypoderma* spp. *Cytospora* spp.
Brown spot needle blight	*Mycosphaerella dearnessiii* Disease of foliage
Sooty mold	*Catenuloxyphium semiovatum* Disease of foliage
Scleroderris canker	*Gremmeniella abietina* Disease of stems
White pine root decline [Hodges, 1986]	*Verticicladiella procera* Root disease
Armillaria root rot Belt fungus Tomentosus root rot Brown cubical root rot Black root stain [Syme, 1985; Hodges, 1986 and Myren *et al*., 1994]	*Armillaria mellea* complex *Fomitopsis pinicola* *Inonotus tomentosus* *Heterobasidium annosum* *Verticicladiella* spp.
Animals	
Common name	**Agent**
Moose	*Alces alces* use pine stands for cover
White-tailed deer	*Odocoileus virginianus*
Porcupine	*Erethizon dorsatum* may feed on bark
Snowshoe hares [Radvanyi, 1987; Bergerson and Tardiff, 1988]	*Lepus americanus* commonly feed on bark and buds of young trees
Eastern cottontail rabbit	*Sylvilagus floridanus* commonly feed on bark and buds of youg trees
Red squirrel [Syme, 1985]	*tamiasciurus hudsonicus* damages shoots in removing cones
Seed-eating birds	Many bird species commonly eat large quantities of seed

E. Symbiotic Relationships - Mycorrhizae

Field data indicate that ectomycorrhizae formed by *Pisolithus tinctorius* increase survival and growth of *P. strobus* and other southern pine species better than natural ectomycorrhizae on routine reforestation sites in the southern U.S (Marx *et al*., 1977). In Canada *Laccaria* sp., *Hebeloma* sp., *Tuber* sp. and *Thelephora terrestris* form ectomycorrhizas with *P. strobus* seedlings grown in pot cultures, while *Phialophora finlandia*, an unidentified ascomycetous "red-type" fungus, and the E-strain form ectendomycorrhizas (Schelkle *et al*., 1996; Ursic and Peterson, 1997).

Some ectomycorrhizal fungi can suppress root-rotting pathogens of conifers. A study of natural mycorrhizal colonisation and frequency of root rot on Eastern White Pine seedlings at a southern Canadian nursery revealed a negative correlation between *T. terrestris* and root rot. This suggested that the association of this ectomycorrhizal fungus with *P. strobus* roots might have some antipathogenic effects (Ursic *et al*., 1997). Additionally, removal of the basidiome of the ectomycorrizal fungus *Laccaria bicolor* associated with container-grown Eastern White Pine seedlings induces a very rapid decrease in both net photosynthesis and stomatal conductance of the host plant (Lamhamedi *et al*., 1994).

7. Domestication

Eastern White Pine has been an attractive species for planting within its range, with up to 40 million seedlings shipped yearly for fibre production and Christmas trees (Eckert and Kuser, 1988). The species has also been used for shelter-belts and urban plantings, and has been used on a small scale in some European countries. Despite its very high timber value, management difficulties with control of white pine weevil and blister rust in planted stands have discouraged its use. Eastern White Pine is thus a rather minor reforestation species, particularly in the northern parts of its range in Canada, where annual nursery shipments in Ontario, Quebec and the Maritimes are now well below 5 million. Nevertheless, the potential value of white pine planting and breeding is well recognised, and tree improvement programs for the species are maintained at some level throughout most of its range.

A. Deployment of reforestation materials

White pine has a long history as a species for reforestation, and nursery production techniques are well-established. In the early years, most planting stock were produced as bareroot seedlings (Coons, 1978), with 2+0 shipped from southern nurseries and 3+0 in the north, although 2+2 transplants have demonstrated superior performance in the field (Mullin and Howard, 1973, Mullin and Christl, 1982). Following developments in nursery technology, Eastern White Pine is now commonly produced from seed in containerised systems, in soil-less growing media. A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently (Landis *et al.,* 1989, 1990a, b, 1992).

Eastern White Pine planting stock can also be produced by means of vegetative propagation. Much of the research in this area has been motivated by possible clonal deployment of individual genotypes with putative resistance to white pine weevil and blister rust. While older trees are often difficult to propagate using long-shoot cuttings, those from 2- to 3-year-old seedlings have long been known to root easily (Deuber, 1942; Patton and Riker, 1958b; Zsuffa, 1973; Kiang *et al.,* 1974; Kiang and Garrett, 1975; Struve and Blazich, 1982). Propagation is also possible using fascicular shoots (Struve and Blazich, 1980, 1984). Growth and performance of rooted cuttings are comparable to planting stock raised from seed (Struve *et al*., 1984; Struve and McKeand, 1990).

Clonal propagation of Eastern White Pine can also be achieved through micropropagation of juvenile explant cultures derived from cotyledons, epicotyls and hypocotyls (Kaul, 1987, 1990; Webb *et al*., 1988). Techniques for the initiation of somatic embryos are also available, although whole plants have not yet been successfully recovered from these cultures (Becwar *et al.,* 1988; Finer *et al.,* 1989).

Some successful trials have demonstrated the potential of direct seeding as a regeneration technique for Eastern White Pine (Graber and Thompson, 1969; Horton and Wang, 1969; Graber, 1988), but stocking is often irregular (Torbet *et al,*. 1995). Operational use has generally been regarded as a failure and is not recommended (Waldron, 1974). Feeding losses to small mammals can be over 80%, unless the seeds are covered with soil at time of sowing (Graber, 1969).

B. Provenance transfer

Local seed sources are often not the preferred provenance for planting, and northerly transfers are often beneficial, except in the extreme. Sources from the southern Appalachians perform well in all but the most northerly locations, with high volume production and reduced branchiness (Sluder, 1963; Funk, 1971, 1979; Sluder and Dorman, 1971; Funk *et al*., 1975; Wendel and Cech, 1976; Wright *et al*., 1976, 1979; Kriebel, 1978; Williams and Funk, 1978; Funk and Jokela, 1979). However, faster-growing southern sources are not sufficiently hardy to thrive in the harsher continental climates above 41°N (Fowler and Heimburger, 1969b; King and Nienstaedt, 1969; Jeffers, 1977). The use of seed zone controls to limit the transfers within regions of adaptation have been recommended for the northern part of the species range in Québec (Li *et al*., 1997).

Tests in Australia indicated that the best provenances are from the southern part of the natural range, although none are as productive are *Pinus radiata* (Matheson, 1977; Wright *et al*., 1979). In the Lower Saxony region of Germany, Appalachian Mountain sources below 39°N perform consistently well, while those from north of 45° perform poorly (Stephan, 1974; Genys *et al*., 1978).

In most of Europe, North American pines are considered to be fast growing tree species. In Romania, *Pinus strobus* is the second most productive species after Douglas fir, and has the least variation in annual radial increment and the lowest wood specific gravity of any commercial species. It is recommended on rotations of 40-60 years for pulpwood and 60-80 years for saw timber (Radu and Radu, 1972). In contrast, despite the extensive introduction and promising performance of *P. strobus* in Bohemia and Moravia, its wood has been grossly underrated by the woodworking industry, largely as a result of premature felling (Vytiskova, 1970). Of the 20 exotic Pines (9 from North America) growing in the central chernozem region of south central Russia, *P. strobus* has the fastest growth rate. However, exotic pines grown in Russia are significantly inferior in growth rate and yield to the local *P. sylvestris* (Lutkin *et al*., 1974). In the Lower Saxony region of the former German Federal Republic, *P. strobus* is not recommended for pure stands, partly because of the poor price paid for its timber and the unsaleability of thinnings; however, because of its fast growth, pleasing appearance, windfirmness, hardiness and general adaptability, it is strongly recommended for mixtures and particularly for the rehabilitation of recreation forests (Schumacher, 1974). As well, in provenance tests established in 1960 in Lower Saxony, growth of the best provenances of *P. strobus* was comparable or superior to that of local P. sylvestris, contrary to the situation in Russia (Stephan, 1981). Additionally, at two sites in Lower Saxony, differences between a rangewide sample of North American provenances were observed in height growth and mortality and attack by *Chronartium ribicola* (Stephan, 1974). *P. strobus* is recommended for wet or periodically waterlogged sites in the lowlands and hills of medium to low fertility in the former German Democratic Republic, especially those of extreme frost hazard (Thomasius and Hartig, 1979).

8. Summary

Eastern White Pine is one of the most important tree species in eastern North America. It has the largest range of any North American species in subsection *Strobus*, the "white pines", and is the only representative on the eastern side of the continent. It is an outcrossing, wind-pollinated species that can transfer genes rapidly to neighbouring populations and to other related species. Eastern White Pine is regarded as intermediate in its tolerance to shade, and natural regeneration is favoured by silvicultural systems that encourage partial shade during establishment and initial development.

Eastern White Pine exhibits clinal variation patterns, generally correlated with latitude, although local seed sources are often not the best performers. Heritability estimates are moderately high at young ages and, while typically decreasing at older ages, are sufficient to predict considerable gains from recurrent

selection. Significant genotype-environment interactions have been reported, but family ranks are generally stable across environments.

Best production is on medium-textured, well-drained soils, in cool, humid areas. White pine can occur as pure stands, or in mixture with several other conifer and hardwood associates, depending on site conditions and history of disturbance. It is a long-lived, successional species, but can be an aggressive pioneer on old fields. The white pine weevil and white pine blister are serious pests and are the major challenge for management of both natural and planted populations.

Eastern White Pine is well-suited to artificial regeneration and it has a long history as a planted species throughout its natural range, both in forestry and urban applications. Tree breeding efforts have been targeted primarily at selection and interspecific hybridisation, in an attempt to produce varieties with resistance to the weevil and blister rust. Management difficulties have limited planting of Eastern White Pine, particularly in the north of its range, although seed orchards are maintained in all regions. Meanwhile, a long history of economic exploitation has resulted in fragmentation and reduction of population sizes in some areas, making genetic conservation of this species a growing concern.

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SECTION 2
EUROPEAN WHITE BIRCH (*BETULA PENDULA* ROTH)

1. General Description and Use in Forestry

A. Taxonomy

Taxonomy

European white birch, or silver birch (*Betula pendula* Roth), belongs to the genus *Betula*. There are some 40 *Betula* species, which are distributed throughout the northern temperate region. According to the systematics created by Regel, 1876 (in Natho 1957), the *Betula* genus is divided into two main sections, *Eubetula* and *Betulaster* (Table 3.3). The *Betulaster* section includes just a few birch species in Japan and China, in the subsection *Acuminatae*. The *Eubetula* section is further divided into three subsections, *Costatae* (yellow birches), *Albae* (white birches) and *Nanae* (dwarf birches). *Betula pendula* belongs to the *Albae* subsection, as does the other European treelike birch, *B. pubescens*.

In 1753 Carl von Linné defined the European arboriform birches as one species, *B. alba*. At the end of the century the German botanist, A. W. Roth, characterised the European white birch as its own species, *B. pendula* and this is the name used today, instead of *B. verrucosa* suggested by F. Ehrhardt (Raulo 1981). An Asian birch species, *B. japonica* is very close to *B. pendula*, since the hybrids of the two species produce fertile off-spring and the species are morphologically very similar (Johnsson 1945).

There are many variations and forms of *B. pendula* (Fontaine 1970). Most of the special forms of *B. pendula* var. *pendula* are grown as ornamental trees in parks and homesteads. An important variation economically is var. *carelica*, curly-birch. Its wood is strong and decorative and is used for making wooden ornaments. The wood is sold according to weight, and its price makes it more valuable than ordinary birch (Ryynänen and Ryynänen 1986).

Chemotaxonomy

The chemicals in birch stems are useful in recognition of different birch species. Julkunen-Tiitto *et al*. (1996) compared concentrations of 12 secondary metabolites in birch seedlings and saplings. Of phenolic compounds, dehydrosalidroside was found specifically in *B. pendula*. Platyphylloside was also a typical component in *B. pendula* and was found also in *B. papyrifera* but not in *B. pubescens*. Triterpenes are found in the resin glands on the surface of young birch stems. *B. pendula* contained mainly papyriferic acid (as did *B. papyrifera* and *B. platyphylla*) accompanied with deacetylpapyriferic acid. The secondary product composition in *B. pendula* differs markedly from that of, for example, the morphologically similar *B. pubescens* by the presence of platyphylloside and terpenoids. On the other hand, *B. platyphylla* (Japanese white birch) and *B. resinifera* show a moderately close phenolic and terpenoid relationship with *B. pendula*. Besides the chemical composition of the stem, foliar chemistry is also used for recognition of birch species. The end product of the ellagitannin pathway, 2,3-(S)-HHDP-glucose accumulates in the leaves of *B. pubescens* and *B. nana*, whereas it is present only in trace amounts in the leaves of *B. pendula* (Salminen *et al*. 2002). These results show that, together with exomorphic and cytological features, chemo-

taxonomical comparison of secondary components can be used to distinguish between species or varieties (Julkunen-Tiitto *et al*. 1996).

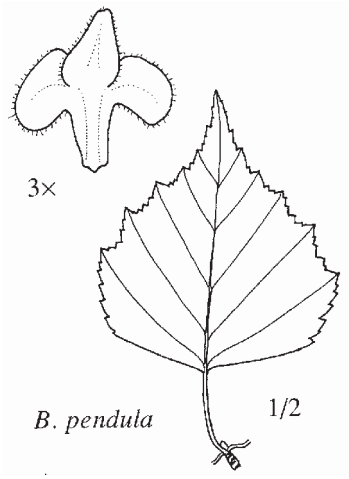
Table 3.3 The taxonomy and distribution of the 44 species in the genus *Betula* (Fontaine 1970; Raulo, 1981). The taxonomy of the genus Betula is under dispute (Flora Europaea, 1993)

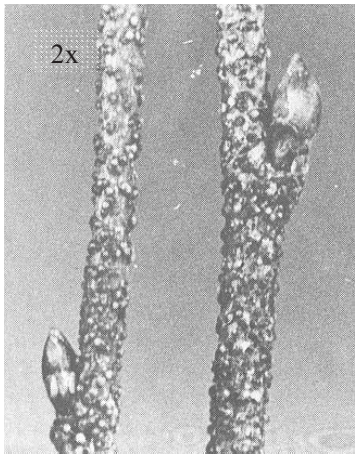
Genus	*Betula*			
Section	*Eubetula*			*Betulaster*
Subsection	*Costatae*	*Albae*	*Nanae*	*Acuminatae*
Species	*B. costata* Trautv. [1] *B. globispica* Shiras [1] *B. medwediewii* Regel [1] *B. utilis* D. Don [1] *B. ermanii* Cham. [1] *B. chinensis* Maxim. [1] *B. schmidtii* Regel [1] *B. potanini* Shirai [1] *B. albo-sinensis* Burk [1] *B. alleghaniensis* Britt. [2] *B. lenta* L. [2] *B. nigra* L. [2] *B. grossa* Sieb. & Zucc[1]. *B. jacquemonti* Spach[1] *B. coryifolia* Regel & Maxim.[1]	*B. japonica* Sieb. [1] *B. mandshurica* Nakai [1] *B. davurica* Pall. [1] *B. papyrifera* Marsh. [2] *B. populifolia* Marsh. [2] *B. pendula* Roth [1,3] *B. pubescens* Ehrh. [1,3] *B. platyphylla* Sukatchev[1] *B. caerulea-grandis* Blanchard[2] *B. occidentalis* Hook. [2] *B. minor* Fern.[2] *B. concinna* Gunnarsson[3] *B. coriacea* Gunnarsson[3] *B. microphylla* Bge[1] *B. turkestanica* Litvinoff[1]	*B. nana* L. [1,3] *B. fruticosa* Pall. [1,3] *B. exilis* Sukacz. [1,2] *B. middendorffii* Trautv. & Mey [1] *B. pumila* L.[2] *B. michauxii* Spach [2] *B. glandulosa* Michx. [2] *B. humilis* Schrenk[1,3] *B. glandulifera* Butler[2] *B. hallii* Howell[2] *B. uber* (Ashe) Fern.[2]	*B. maximowicziana* Regel [1] *B. alnoides* Buch.-Ham.[1] *B. luminifera* Winkl. [1]
Distribution	Asia [1] North America [2]	Asia [1] North America [2] Europe [3]	Asia [1] North America [2] Europe [3]	Asia [1]

B. General description

Betula pendula grows up to 30 m high, with usually one stem. It can become up to 100 years old, under favourable conditions even 150 years. The bark is smooth and silvery white and exfoliates in long, thin strands. The basal parts of the trunks of old trees are black and vertically fissured; in young trees (up to 10 years old) the bark is light brown. Older branches are often pendulous, whereas young branches are spreading or ascending. Twigs are glabrous, with peltate resin glands on the younger twigs. Buds are acute, not sticky. Leaves are 2.5 – 5 cm long, ovate-deltate, apex acuminate, base truncate or broadly cuneate. Leaf margins are double serrated with the primary teeth very prominent and curved towards the leaf apex; petioles are 10 – 18 mm long. The scales are short, with a broad cuneate base, broad lateral lobes spreading and curving towards the base, and a deltoid, obtuse middle lobe. The achene is glabrous, 1.5 – 2.4 mm long, 0.8 – 1.3 mm wide; the width of the fruit is 3 – 5 mm, the upper edge of the wings surpassing the stigmas by 0.5 – 1.5 mm (Atkinson 1992; Figure 3.2.).

Figure 3.2 The leaf, scale, seed, stem, buds and female and male catkins of *Betula pendula* (Raulo 1981, Hämet-Ahti *et al*. 1992).





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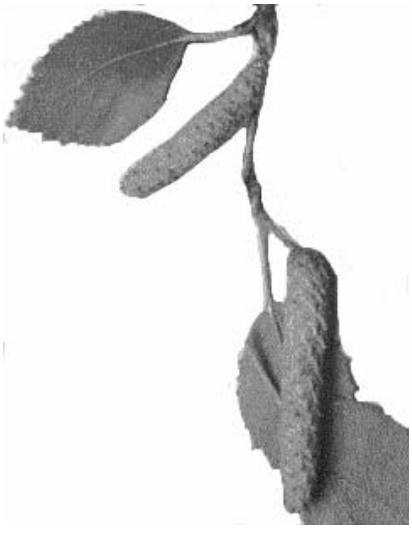
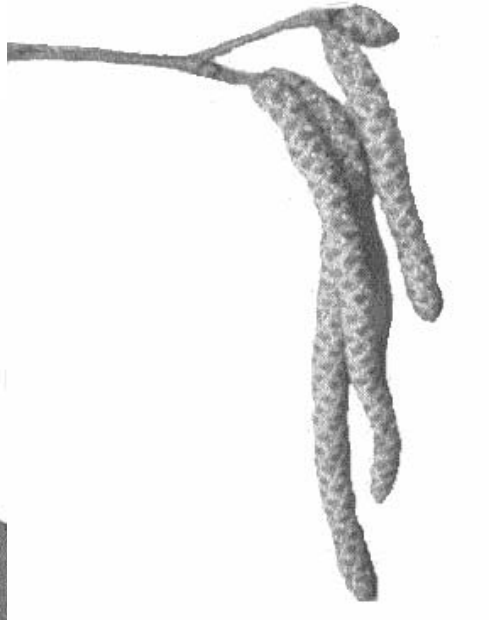




C. Use of European white birch

Firewood

Birch has been an important source of energy, and earlier it was mostly used as firewood. The effective thermal value of birch is about 20 MJ/kg when absolutely dry, and about 15 MJ/kg with 30 % humidity. The high content of volatile oils makes the burning properties good (Salmi 1987).

Plywood production

In plywood production, the value of a birch log used for planks or veneer is influenced by its diameter, stem taper, and number and size of knots and branches. Logs of required dimensions can usually be produced with proper planting densities and thinning treatments.

Pulpwood

Birch fibres are relatively long and thin-walled and have a high content of hemicelluloses. As a result, birch pulp has good strength properties, low light-scattering ability and high density. The main product is bleached sulphate pulp. Coated and uncoated wood-free fine papers, which contain 50 to 90 % of bleached birch sulphate pulp, are especially important (Tammisola *et al*. 1995).

Curly-birch, Carelian birch

Curly-birch (*B. pendula* var. *carelica*) is a special form of *B. pendula*, and it occurs throughout Northern Europe and parts of Central and Eastern Europe. The wood is strong and decorative and is used in wooden ornaments and furniture. The genetic background of the curly-grained trait is not known. Curly-birch is self-sterile, and in controlled crossings between two individuals a maximum of 75 % of the progeny can be curly-birch. It has been suspected that the trait is homozygotically lethal, or that it is a genetical disease caused by a micro-organism or a virus (Ryynänen and Ryynänen 1986). The establishment of a plantation is an uncertain process, because it takes 10 years to see whether an individual will become a curly-birch. Normal birches have to be removed in order to prevent the curly-birches becoming suppressed. The propagation of curly-birches from cuttings has been attempted but the results have not been promising. Micro-propagation techniques have been developed for propagating curly-birches with the optimum stem form (Ryynänen and Ryynänen 1986).

2. Forestry Practices

A. Reproductive methods

Flower induction

In order to speed up the induction of flowering, and consequently, breeding, the Foundation for Forest Breeding in Finland built the first flower induction hall in 1976. It was equipped to provide good growing conditions for breeding material all year round. In the plastic greenhouses continuous illumination (24h day length) was kept at 10,000 – 20,000 lux by mercury and high pressure sodium lamps. Temperature was controlled by thermostats and fans. CO_2 concentration was increased by burning propane gas and relative humidity was kept above 60 %. Required nutrients were provided automatically via continuous drip irrigation. The best results were obtained from birch: the daily growth was 7 cm per day, male flower buds appeared in 77 days and the first seed crop was collected 8 months after sowing (Holopainen and Pirttilä 1978).

B. Vegetative propagation

Birch can be propagated vegetatively, *i.e.* cloned, by grafting, rooting or by means of tissue culture. Production of grafts is quite expensive and grafts are used mainly for clonal collections and seed orchards (Ryynänen 1987). Also, rooting of birch cuttings is not much used in birch propagation as the percentage of rooted cuttings is low (10 – 50 %) for *B. pendula*, whereas Kling *et al.* (1985) found rooting rates of 80 % for a closely-related *B. platyfylla*.

Micro-propagation

Much research on tissue culture of birches has been done and effective micro-propagation techniques have been developed. Leaf callus from young seedlings can be used (Simola 1985) but with mature trees, vegetative buds are used as tissue material (Ryynänen and Ryynänen 1986). Shoots and roots can be specifically induced on different aseptic culture media, after which the plantlets can be potted on peat and moved into the greenhouse. The development of micro-propagation methods made an effective propagation of selected adult trees possible. Micro-propagated plants have proved viable in test plantings under field conditions (Meier-Dinkel 1992; Jones *et al.* 1996). A review on micro-propagation of juvenile and mature birches including results on field trials and cold storage was compiled by Meier-Dinkel (1992). Some clonally propagated birch plants have been used for practical forest cultivation in Finland (Viherä-Aarnio 1994a). Large-scale production of micro-propagated *B. pendula* seedlings was terminated in 1994, as it was considered unprofitable (Viherä-Aarnio and Velling 2001). Today, micro-propagation is used in commercial scale for curly-birch cloning. Micro-propagated plants can also be used instead of grafts in greenhouse seed orchards, when specific genotypes are required (Viherä-Aarnio and Ryynänen 1995).

Viherä-Aarnio (1994a) studied the field performance of micro-propagated birch plants in a small-scale field test with three clones and three seed-born lots. The best two lots of the experiment as regards the height and diameter growth at the age of six years were clones, but the weakest lot was also a clone. This indicates that the selection for clonal propagation should be done carefully. It is also important to test the clones in field trials before wide scale propagation. In fact, further studies revealed that micro-propagated clones do not differ from seed-born seedlings in terms of growth and resistance against pests and herbivores (Viherä-Aarnio and Velling 2001).

C. Reproductive materials used

Seed collection stands

Seed collection stands are natural birch stands of outstanding growth and quality. The best individuals in these stands have been selected as plus trees. A part of the seed used in nurseries is collected from seed collection stands (Viherä-Aarnio and Ryynänen 1994).

Seed orchards

As birch responds positively to intensive cultivation in a greenhouse, today most birch seed used for seedling production is obtained from polythene greenhouse seed orchards, a method which was developed by the Foundation for Forest Tree Breeding in Finland in 1972 (Huhtinen & Yahyaogly 1974). Most seed orchards are multi-clonal orchards with some 30 to 50 clones. Bi-clonal orchards have also been established in order to produce desired full-sib families (Viherä-Aarnio and Ryynänen 1994). Besides greenhouse orchards, open seed orchards in the field are used for seed production.

Seed production in greenhouse seed orchards begins three years after planting and continues for 5 to 7 years, until the trees grow too big to be kept inside. Flowering and seed ripening take place 1 to 2 weeks earlier in the greenhouse than in natural conditions. As a result, growing birches in greenhouses also

prevents unwanted background pollination. A seed orchard can be established with grafts of tested plus trees, seedlings selected from the best progenies or micro-propagated clones (Viherä-Aarnio and Ryynänen 1995).

Cultivation

In the Nordic countries and Europe, Finland has the longest experience in birch cultivation. The main emphasis in birch planting has been the afforestation of former agricultural land (Ferm *et al*. 1994). When a field is abandoned, it is first colonised by annual species, which then give way to perennial herbs and grasses. As field vegetation competes with tree seedlings for water and nutrients, success with field afforestation requires effective weed control during the establishment year. Soil preparation also affects the survival of trees. In 20-year-old experiments the best growth was gained when bare-rooted, large transplants of silver birch were planted on a ploughed and tilled field and weeds were properly controlled (Torpo 1991). Weed control improves the nutrient status of tree seedlings and increases growth. Birch seedlings with efficient weed control were 40 – 50 cm taller after two years than control seedlings, and they had bigger leaves (Ferm *et al*. 1994). Vegetation control reduces the risk of cicada (*Cicadella viridis*) wounding and consequently infection by pathogenic fungi, and is also effective against vole damage (Ferm *et al*. 1994).

D. Breeding

In the breeding programme for birch, outstanding stands for seed collection were selected and the best individual trees in these stands were selected as plus trees. The most important selection criteria were fast growth and good stem quality. After selection of plus trees, controlled crossings have been carried out, and progeny tests with both full-sib families from controlled crosses and half-sib families from open pollinated lots have been established (Koski 1991). Plus tree selection is still being continued in order to improve the geographical coverage of the breeding material (Viherä-Aarnio 1994b).

Planted birch stands of improved material can reach the size of final cutting by as early as 40 years and produce over 400 m^3/ha (Viherä-Aarnio 1994b). Examples of realised genetic gain cannot yet be shown, but a potential gain of 20 to 30 % in volume growth has been suggested (Koski 1991) and in first generation seed orchards, the growth rate of the best selected family is 89 % higher than the control (Wang 1996).

Provenance transfers

Raulo and Koski (1977) reported on the first geographical transfers of silver birch. According to their study, seed transfers of 200 km northwards or southwards in central and southern Finland had no effect on the mortality or growth rate of the progenies; on the other hand they did not recommend long-distance transfers because of the risk of lower survival.

E. Conservation of genetic resources

Genetic diversity of forest trees

The cultivation of forest trees can be thought to endanger the genetic diversity in general. Maintaining genetic diversity in forest tree populations also means maintaining adaptability to changing environments. In naturally generated areas there is no concern about the loss of genetic diversity but large monocultures of a single clone would reduce genetic variability. However, forestry based on monoclonal blocks is not common, but cultivation of single clones is mostly applied to small plantations of special forms or variations, *e.g.* curly-birch (Viherä-Aarnio and Velling 2001). Moreover, real monocultures are impossible to maintain, as other trees can not be prevented from growing in the plantations.

Most forest trees are cross-fertilising and pollinated by wind and consequently there is crossing both between individuals in a stand and between stands. Even if small stands were to be cultivated with only one clone, effective wind-pollination will secure the mixing of genes between populations. Also, real monocultures are impossible to maintain, as other trees can not be prevented from growing in the plantations. Cultivation of single clones will probably mostly apply to small plantations of special forms or variations, *e.g.* curly-birch. The danger from planting large areas with a single clone arises when the adaptability of the clonal genotype is exceeded by adverse conditions. To avoid multiplication of poorly adapted or extremely susceptible genotypes, trees for micro-propagation are selected from old stands. Another option is the use of clonal mixtures which are buffering environmental risks (Kleinschmit 1998).

It has been estimated that a population of 500 trees is big enough to contain all possible alleles of different genes (Koski 1995), and this is the amount usually used in breeding populations. The seed from seed orchards is usually a result of cross-fertilisation of tens of plus tree clones from different localities, and it has been shown in studies with genetic markers that the genetic diversity is as large as that of natural seed collection stands (Koski 1995).

Gene reserve forests

Ongoing breeding activities may cause losses of genetic diversity, especially if population size is reduced from one generation to the next. Random allele loss is problematic for advanced-generation elite populations, and this has raised concerns about the maintenance of genetic diversity in forest-tree breeding programs.

Gene reserve forests have been established in order to conserve representative samples of the natural gene pool of forest trees. They have to be big enough (preferably tens of hectares) for the pollination to occur inside the forest and for the reserve to contain most of the local genetic diversity. Management and harvesting are allowed, but the forests are regenerated naturally or by sowing with seed from inside the forest or by planting seedlings grown from local seed. Gene reserve forests should consist of a network of forests in the area of the natural distribution of the species, in order to contain the diversity between provenances and localities. In Finland the gene reserve forests are situated in northern Finland and are part of a network of gene reserve forests covering all Europe (Parviainen *et al*. 2000).

Advanced breeding strategies

Two advanced models have been suggested in order to prevent random allele loss in breeding programs: HOPE (Hierarchical Open-Ended Breeding System) and MPBS (Multiple-Population Breeding Strategy) (Eriksson *et al*. 1993).

The HOPE system is composed of a hierarchy of breeding populations with successively higher performance levels. A large base population is maintained and is open to new material. Higher level populations are more stringently selected and from these, selections are made for commercial production. Under MPBS, a breeding population is divided into independent subpopulations which represent different sources or selection criteria. There is differentiation among subpopulations both in their source of germplasm and in their traits and environmental adaptabilities. There can be random allele loss in some subpopulations but, on average, gene frequencies remain fairly constant (Eriksson *et al*. 1993).

Of these, the MPBS seems to be a better choice for birch because it combines the highest possible genetic gains and the highest possible genetic diversity. It also gives the breeder more options for changing breeding goals with changing environment and markets (Eriksson *et al*. 1993).

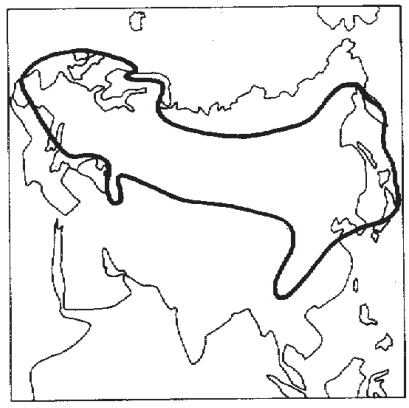
3. Centres of Origin / Diversity

A. Natural distribution and origin

Natural distribution

B. pendula is distributed throughout Eurasia (Figure 3.3.). It extends in Europe into the mountainous regions of Spain, Greece and Italy in the south, throughout Asia all the way to the Sea of Okhotsk and the Sea of Japan in the east and to Caucasus, Crimea and the mountains of Central Asia in the south. In the north it extends to about 65°N (Hämet-Ahti 1963; Atkinson 1992).

Figure 3.3 The natural distribution of *Betula pendula*



Source : Hämet-Ahti *et al*., 1992

The northern limit of the range of silver birch appears to be determined by protection from cold north-easterly winds. The southern limits approximate to the line of an average of 10 mm July rainfall (Atkinson 1992). There are different views of the eastern limit of the range, 60º by Jalas & Suominen (1976) and 103º by Hämet-Ahti *et al*. (1992).

Total distribution

In North America *B. pendula* is used as an ornamental and is a naturally spreading escape from cultivation for example in Ontario, Canada (Catling and Spicer 1988). The corresponding species of *B. pendula* in North America is *B. populifolia* (grey birch) which is used in re-vegetation of mine spoils, and is economically important as a source of fuel, sugar and fibre for corrugating pulp (Catling and Spicer 1988).

Migration to Fennoscandia

Birch migrated to Fennoscandia in the late glacial with pine, spruce and alder. The last remnants of the Scandinavian ice cap may have disappeared at around 8,000 years ago and the early postglacial (pre-boreal) forests in Fennoscandia were dominated by birch. In the boreal (6,800 – 5,000 BC), the climatic conditions were drier and cooler, and pine replaced birch especially in the north. At around 5,000 BC, the climate became warmer again, and birch and alder spread to new areas. At around 1,000 BC, as the climate became cooler again, spruce replaced birch and pine in many areas (Hämet-Ahti 1963).

4. Reproductive Biology

A. Sexual reproduction

Birches are monoecious (male and female flowers on the same tree) and diclinous (flowers unisexual). Male inflorescences are 1 –2 cm long, 4 mm wide while over-wintering; 2 – 6 cm long, 6 mm wide at anthesis. There are 2 – 4 male catkins together at the ends of small shoots. Female inflorescences are erect, 1.5 – 3.5 cm long, 7 mm wide (Atkinson 1992). They are pale green when immature, turning brown in the late autumn.

Flowering

Flowering usually starts when trees are between 5 and 10 years old but some single trees can be induced to flower within one year after germination (Elo *et al*. 2001). The female flowers emerge on the short-shoots at the same time as the buds open. The female flowers usually develop one day before male flowers on the same tree shed pollen. Flowers remain receptive for 4 days and after the sixth day are blackened and dead (Sarvas 1952). *B. pubescens* flowers about a week later than *B. pendula*.

Ripening of the flowers is closely related to the temperature conditions of the spring. The arrival of the first heat wave of daily temperatures above +10°C usually triggers flowering. Most pollen release occurs within 2 or 3 days of anthesis. Pollen shed is highest in the late afternoon and near zero from about midnight to early morning (Sarvas 1952). *B. pendula* pollen can remain viable for at least 20 days at room temperature in the dark. If the pollen is exposed to sunlight for 8 hours, however, ultraviolet rays and other factors reduce viability (Perala and Alm 1990).

Flowering is polygenically inherited and female and male flowers are governed by different sets of genes. Weather conditions have a strong influence on female flowering whereas they do not play a role in male flowering (Eriksson and Jonsson 1986).

Three cDNAs of *B. pendula* representing ADS-box genes BpMADS3, BpMADS4 and BpMADS5 are active during the development of both female and male inflorescence and the respective genes are involved in the determination of the identity of the inflorescence or flower meristem. BpMADS3 shows highest expression at late developmental stages. BpMADS4, besides regulating inflorescence, is expressed in roots and stem. Expression of BpMADS5 is inflorescence specific and continues during seed development. Ectopic expression of BpMADS3, BpMADS4 or BpMADS5 with CaMV 35S promotor in tobacco results in extremely early flowering (Elo *et al*. 2001).

B. Pollination

Male meiosis and pollen development

The male catkins develop in the autumn but do not release pollen until spring the following year. The principal events during micro-sporogenesis are the initiation and growth of the male buds, generation and growth of the sporogenous tissue, pre-meiotic maturation of the PMCs (pollen mother cells), meiosis, the tetrad stage, androgenesis and anthesis. The species in *Betulaceae* reach the tetrad phase from late July to mid-August (Luomajoki 1986). In *Betulaceae* the heat sums seem to have less connection with the development stage of micro-sporogenesis than in conifers, as the northernmost stands of *Betula* species develop first and the southernmost last. The timing of meiosis in *Betula* is more dependent on day length, and a hypothetical critical day length of 15 hours can quite accurately predict the onset of the tetrad phase (Luomajoki 1986).

Pollination

Pollination is anemophilous, *i.e.* birch is pollinated by wind. There is an intense maximum at the immediate start of flowering lasting 2 – 3 days during which 70 – 80 % of the total pollination takes place. Prolonged cold and wet conditions are likely to reduce the total amount of pollen (Sarvas 1948).

Pollen flow

As an evidence of long-distance pollen transport, *Betula* pollen concentrations in Fennoscandia can be relatively high before the local flowering period. The pollen is transported by south-eastern air-masses from eastern central Europe and the Baltic countries, with travelling times for pollen grains in the range of 9 – 20 hours (Hjelmroos 1991).

In northern Europe, birch pollen allergy is one of the most common reason for spring-time human rhinoconjunctivitis and asthma. In Sweden, about 8 – 9 % of the population suffers from allergies related to birch pollen (Hjelmroos and van Hage-Hamsten 1993). The major birch pollen allergen Bet v I is highly homologous to pathogenesis-related plant proteins. Another well described allergen is profilin, an actin binding protein, which is predominantly expressed in the pollen of different plant species. The birch profilin is recognised by IgE antibodies in 10 % of individuals allergic to birch pollen and seems to be an intermediate or major allergen in individuals allergic to pollens of grasses and weeds (Valenta *et al*. 1991). Another birch pollen allergen, Bet v III, representing a class of Ca 2+ binding proteins, has also been characterised (Seiberler *et al*. 1994).

C. Mating system

Cross-fertilisation

B. pendula is an outcrossing species. There is cross-fertilisation both between trees standing close in the stand and among distant individuals in the same or nearby stands. Hagman (1971) studied incompatibility in *B. pendula* and *B. pubescens* and found partial incompatibility in both cross-pollinated and self-pollinated trees. When trees from the same population were cross-pollinated some combinations were incompatible. Incompatibility occurs in the style as retarded growth of the pollen tube and is probably based on a one-locus multiple allele system. Incompatibility reaction is also influenced by environmental conditions: compatibility is increased at low temperatures.

Self-fertilisation and inbreeding depression

In the studies of Hagman (1971), self-incompatibility was observed after self-pollination. Self-incompatibility was not complete and the filled seed frequency was highest when the pollinations took place at low temperature.

Wang (1996) studied inbreeding depression in *B. pendula* and the possibility of using heterosis in breeding. He found that inbreeding depression in survival and in stem volume of progenies in three successive generations of selfing was significant. The mean survival rates were 88 %, 37 %, 28 % and 42 % for outcross controls, S1, S2 and S3 selfed progenies, respectively. Inbreeding depression for stem volume also increases with advancing generations of selfing. The mean stem volumes were 5.31, 3.15, 2.66 and 2.12 dm^3 for outcross controls, S1, S2 and S3 selfed progenies, respectively.

D. Seed crop

Seed crops

The percent of germinating seed is directly related to the amount of pollen shed: seed crops with the highest viability will be produced during years of abundant flowering (Sarvas 1952). Each strobile contains about 450 seeds. In the average year in ordinary regeneration areas, seed crop is some 340 filled seeds m^{-2}, with 2 300 seeds m^{-2} in a good seed year. In a good seed year in a pure birch stand the best annual result has been 53 200 seeds m^{-2}, *i.e.* 128 kg per hectare. A crop amounting to 20 % of the best seed crop occurs only every third year, and intervening crops may be only 5 – 10 % of a good year (Sarvas 1948). Cold springs can lead to massive loss of male catkins. Birch produces empty fruits in the absence of pollination.

Dispersal range of birch seed is limited by the small seed size. The fall rate for silver birch seed is 0.52 m s^{-1} (Sarvas 1948). Most seeds fall within 40 – 50 m of the source, with a maximum amount of seeds at 10 – 12 m from the tree. The amount of seeds is still 5 % of the maximum at 100 m from the source, but after 70 m most seeds are empty. In *B. pendula* seeds are also dispersed throughout the autumn and winter, so there is probably also secondary dispersal of seed over the surface of snow by wind. With *B. lenta*, seeds were distributed over an area 3.3 times greater than by initial seed fall, and further dispersal by melting water in the spring could be some 50 km (Matlack 1989).

E. Natural regeneration

Germination and establishment in the field

A non-fluorescent water-soluble substance in the seed coat has germination-inhibiting properties. This inhibitor apparently increases the oxygen and light requirement of the embryo. Un-chilled, intact seeds require light for germination, but breaking the seed coat allows rapid entry of oxygen and hastens germination in darkness (Perala and Alm 1990). A long-day photoperiod or illumination with red light induces germination, whereas blue or far-red light inhibits germination. Stratification (chilling) or temperatures above 20°C override the photoperiod requirement. Fluctuating temperatures within the range of 20 – 30°C are more favourable for germination than steady temperatures (Perala and Alm 1990). Stratification also eliminates the effect of temperature on germination rate (Atkinson 1992). In natural conditions in the northern parts of the range of birch, the cold requirement is usually met.

In the autumn, after seed shed, short days and low temperatures inhibit germination and help seed survival over the winter. In the spring, after a long enough chilling period, dependence of germination on day length disappears and seeds germinate when temperature rises and there is enough moisture in the ground (Nygren 1987). Birch seeds may also germinate soon after seed deposition in the summer, if there is enough moisture in the ground. As the individual seeds are small and contain small amounts of reserve food, the radicle of the birch seedling remains short with the result that the plant frequently withers away and dies and seedlings only grow up on spots which contain enough moisture.

The percentage of germination of *B. pendula* seed in southern Finland (60° N) is about 61 % and it decreases towards the North, being about 53 % in central Finland (62° N). The opposite is true for *B. pubescens* with germination of 63 % and 70 %, respectively (Sarvas 1948).

In natural conditions the seeds are concentrated in the upper parts of the soil profile indicating a rapid turnover. Hill and Stevens (1981) showed that 80 % of the seeds were found in the litter layer and showed no long-term survival. In studies where *B. pendula* and *B. pubescens* seed were sown to two forest sites in northern Sweden, only 6 – 9 % of the seeds remained viable after one year. The depletion rate was slower in the two successive years (Granström and Fries 1985). This might be due to litter-fall and growth of mosses altering the environment, making the seeds less liable to germinate. When the seeds were buried

beneath the litter layer, the viability of the seeds did not fall consistently over a five-year period even though after three years the pericarps were more or less degraded exposing naked embryos (Granström 1987).

80 – 90 % of birch seedlings die within the first year, perhaps mainly as a result of drought. Establishment is best on bare mineral and humid soils and cushion Sphagnum. These surface types have the most favourable moisture conditions (Kinnaird 1974).

F. Vegetative reproduction in the field

Sprouting

Sprouting by basal buds occurs as a response to fire as well as other damage such as felling and grazing. The sprouting ability of *B. pubescens* is stronger than that of *B. pendula*. Sprouting ability differs seasonally, and is generally higher for dormant trees. Felled *B. pendula* produces the lowest frequency of stump sprouting in summer and the highest in fall and spring. The frequency of sprouting and shoot size (but not the number of shoots) depends on light intensity and temperature. No stumps were observed to sprout at temperature lower than 10°C (Perala and Alm 1990). Sprouting depends also on the soil type: sprouting is more frequent in moist forests and on peat land than on dry heath and woodlands. Sprouting also depends on the method of regeneration. It is most frequent on clear-cut areas and least frequent on group selection cutting areas, forest fire areas and burnt-over clear-cut areas because of damage to the basal buds (Sarvas 1948).

5. Crosses

A. Inter-specific crosses

Natho (1957) found several natural crosses between *B. pendula* and other birch species in Germany, *i.e. B. pendula* x *B. pubescens*, *B. pendula* x *B. humilis* and *B. pendula* x *B. pubescens* x *B. humilis*. Moreover, the author showed that there is a continuum from *B. pendula* via *B. pubescens* to *B. humilis* in leaf shape. This and the triple hybrid indicate that there is gene flow among the three species.

Johnsson (1945) found large variation among experimental crosses of *B. pendula* and five other birch species. The percentage of fruits containing seed were 53.2 %, 8.8 %, 0.9 % and 0.4 % for *B. pendula* x *B. japonica*, *B. pendula* x *B. papyrifera*, *B. pendula* x *B. ermanii* and *B. pendula* x *B. maximowicziana*, respectively. The hybrid *B. pendula* x *B. pubescens* was infertile in this experiment.

Clausen (1970) carried out inter-specific crosses between 12 *Betula* species (Table 3.4.). He observed that high-ploidy female x low-ploidy male usually gave low seed germinability. Crosses within a subsection did not give more germinable seeds than crosses between species from different subsections. Crosses with *B. ermanii* as female gave more germinable seeds than any other cross. Each species was represented by 1 – 13 individuals, so definite conclusions can not be drawn from these results. However, it is the most comprehensive study of its kind to date.

Table 3.4 A summary of the results on seed germination in different crosses betwwen *Betula* species

		COSTATAE				ALBAE				NANAE			
		len.	*nig.*	*erm.*	*all.*	*pen.*	*pop.*	*pub.*	*pap.*	*gla.*	*hum.*	*nan.*	*pum.*
lenta	2n		0	0	***	-	*	-	0	0	0		0
nigra	2n	*		***	*	**	*	**	*	*	**	**	**
ermanii	4n	***	*		0	***	*	***	***	0	***	0	***
alleghaniensis	6n	*	*	*		0	*	*	***	*			*
pendula	2n	*	*	**	*		***	*	***	**	*	*	*
populifolia	2n	*	*	*	*	*		*	*	*	*		*
pubescens	4n	0	*	*	*	*	*		*	*	***	0	*
papyrifera	4-6n	**	*	**	**	***	*	***		0			***
glandulosa	2n		0	-	0	0	0	***	-			-	-
humilis	2n	0	0	***	*	*	0	***	**	0			***
nana	2n	-	*	0		0	*	**	0	**			0
pumila	4n	0	0	*	0	*	*	*	*	0	*	0	

Germinability %: <10 low (*), 10—30 moderate (**), >30 high (***), no seed set (-)

Hagman (1971) studied incompatibility in *B. pendula* and *B. pubescens*. Incompatibility between *B. pendula* and *B. pubescens* is more pronounced when *B. pendula* is the male parent. Moreover, the *B. pendula* pollen tubes do not seem to be able to penetrate the style of *B. pubescens*. Low temperature gave the highest frequency of successful hybridisations. The following natural inter-specific hybrids of *Betula* species have been found in Finland (Kurtto and Lahti 1987):

- B. pendula x pubescens; B. nana x pendula; B. nana x pendula x pubescens.
- B. nana x pubescens.

B. Intra-specific crosses

Raulo and Koski (1977) studied long-distance crosses (distances between parent trees > 100 km) in Finland in order to find out if inter-provenance crosses would result in hybrid vigour, that is, in a heterosis effect. In these experiments crosses between Finnish provenances did not result in a heterosis effect.

Wang (1996) studied inbreeding depression and long-distance crosses in *B. pendula* and the possibility of using heterosis in breeding. He found heterosis both in hybrids of inbreeding progenies and in provenance hybrids. The growth performance of the long-distance crosses (central Finland x Estonia, northern Finland x Latvia, Germany x southern Finland, central Finland x Austria) significantly exceeded that of controls. The southern long-distance crosses also outperformed short-distance crosses (between southern and central Finland, southern Finland and Estonia, and southern and northern Finland), but did not significantly differ from the local inbred crosses. Hybrids of selfed progeny gave a significantly better growth performance than controls of either stand origin or conventional full-sib family origin: the mean stem volume of hybrids was 52 % higher, and the volume of the best hybrid family was 112 % higher than the mean of improved full-sib family controls. These results suggest that hybrids of inbred lines or provenances could be used in breeding of silver birch for superior yield.

5. Genetic Variability

A. Genetic background

Chromosome number

The birches are characterised by their small chromosomes which are only a few micrometers long. A double-staining method is useful in counting the number of chromosomes in *Betula* and in other hardwood species with chromosomes of small size and large number (Hömmö and Särkilahti 1986).

Betula pendula is a diploid with 28 chromosomes; *B. pubescens* is a tetraploid with 56 chromosomes. Trees morphologically resembling tetraploid but with intermediate chromosome number (2n=42) are sometimes found. Because the chromosomes tend to lie in groups of seven and because of small numbers of quadrivalents in meiosis in the 28 and 56 chromosome plants, the original basic chromosome number is thought to be seven rather than fourteen (Eriksson and Jonsson 1986).

Natural polyploidy is very frequent in the genus *Betula* and the number of ploidy levels differ between the four subsections of the genus. *Costatae* has diploid (2X=28), tetraploid (4X=56) and hexaploid (6X=84) species and in addition to these, three ploidy levels. *Albae* has also a pentaploid (5X=70) species, *Nanae* has both diploid and tetraploid species and *Acuminatae* only diploid ones. *Betula* is a young species from the evolutionary point of view which explains the polyploid nature and the occurrence of various ploidy levels (Särkilahti and Valanne 1990).

The possibility of using induced polyploidy to speed up the natural evolution and breeding of *Betula* arose in the 1960s (Särkilahti and Valanne 1990). The polyploidisation experiments were performed by treating seeds with colchicine during germination. Of the 687 polyploid trees produced by these experiments in Turku, Finland, 287 were still alive in 1990. Moreover, a series with ploidy levels ranging from diploid to dodecaploid (2X – 12 X) consisting of both natural and induced polyploids is available (Särkilahti and Valanne 1990).

Identification of polyploid trees of *B. pendula* is possible visually on the basis of leaf morphology, with polyploid trees having larger leaf blades, thicker leaf petioles and a rougher network structure on the abaxial leaf epidermis than normal trees. The mortality of colchicine polyploid trees is typically high in every growth phase, growth rate is slow and growth habit more or less abnormal. Thus, colchicine-induced autopolyploid trees are not of great value as such but they can be used for studying the effect of the ploidy level on growth, breeding, adaptability and evolution of *Betula*. In natural conditions polyploids are said to possess greater ecological and genetic amplitude and, therefore, exhibit greater variability than related diploids (Särkilahti 1990). Ploidy manipulation seems to produce mainly sterile trees but micro-propagation can be used for multiplication of polyploid material.

B. Variability within and between populations

Long-lived species such as forest trees are subject to conditions varying greatly from year to year. Thus, they have a large within population variation so that there are always genotypes well adapted to the varying conditions at regeneration. For example, in studies of five year heights of *B. pendula* progenies Velling (1985) stated that the within-population variation was as large as that between populations originating from latitudes 60 – 63° N. Raulo and Koski (1977) found that intra-group variation within provenances and between individuals was large compared to variation between stands or localities. Birch stands are not closed populations and gene exchange between stands is successful as a result of pollen dispersal by wind. A large within-population variation was also reported, for example, in two separate studies (Johnsson 1951 and Langhammer 1982, cited by Eriksson and Jonsson 1986).

Differences in qualitative traits; marker techniques

Raulo and Koski (1977) reported a large variation in growth and stem quality between progenies from different plus trees. Many progenies attained a stem volume that exceeded the mean value of the test by more than 40 %, and good progenies could be found among both open-pollinated and cross-pollinated families. Variation in wood density is generally smaller than that in growth and stem quality characteristics, but significant differences can be found between progenies (Velling 1979a). Nepveu and Velling (1983) studied the inheritance in wood quality characteristics. The inheritance in basic density and shrinkage was fairly strong, but volume growth and pulp yield showed low heritability.

As attention has focused on the external quality of the stem for plywood production, no active breeding or selection of wood quality has so far been applied. The properties of birch pulpwood could be improved if the bark content could be reduced, the size and/or number of branches reduced and the carbohydrate content of the wood increased. Increasing the number of fibres without affecting the fibre length would improve the optical properties and bulk of the pulp. Tammisola *et al*. (1995) studied tree-to-tree variation to determine if there is variation in the pulpwood properties of *B. pendula* that can be used in breeding. They found that significant variation occurred between individual trees in the properties studied, and high significance levels give support for underlying genetic differences.

If DNA markers closely associated with pulping and papermaking properties were found, it would be possible to select the most desirable trees within a progeny more quickly and cheaply compared to the more or less destructive analyses of mature trees (Tammisola *et al*. 1995). Altogether 157 nucleotide sequences and 177 proteins of *B. pendula* are listed in the database of NCBI. Molecular biology is the most active research area within biology and the number of known nucleotide sequences and proteins is increasing rapidly. The databases at http://www.ncbi.nlm.nih.gov/ have the most updated information about genetic markers. For an old review on genetic markers in *Betula*, see (Hattemer *et al*. 1990).

C. Adaptivity to climatic conditions

Growth cessation and winter hardening

In cool and temperate regions the annual temperature rhythm is the main regulating factor of the environment. Forest trees are adapted to the variation of the growing season between years as well as to the long-term average.

The timing of growth cessation and the subsequent process of winter hardening is determined by a joint effect of heat sum and night length (Koski and Sievänen 1985). In Punkaharju, Finland (61°48’ N), growth ceased at the cumulative temperature sum of 800 degree-days and night length of 7.5h. Koski and Sievänen (1985) predicted that an adapted provenance of *B. pendula* will cease growth by the accumulation of about two-thirds of the total heat sum for a normal growing season. If northern provenances were moved southwards, growth would cease with two thirds of the original local heat sum, but due to longer nights, growth would cease a few days earlier, that is, with a smaller heat sum than at the original locality. A northern provenance from Punkaharju (Finland) moved to Suwalki (Poland) would cease growth three weeks earlier than the local one. The opposite transfer northwards of southern provenances would lead to growth cessation three weeks later than the local ones. The heat sum characteristic of southern provenances would not be reached until the beginning of September, but long nights would induce growth cessation 10 days earlier, thus adapting the trees for the local growing season (Koski and Sievänen 1985).

Breaking the dormant state is affected by chilling temperatures during winter. The effect of chilling is cumulative increasing up to a threshold when the buds are released from dormancy. Bud burst and growth

start in spring occur after the accumulation of a certain heat sum above a specific base temperature (Myking and Heide 1995).

Growth start

As *B. pendula* is geographically widely distributed, it has by latitude and altitude determined ecotypes with different optimum and critical chilling temperatures and durations. The ecotypes of northern origin have the earliest bud burst with variation in the requirement for duration of chilling but not for chilling temperature. A longer chilling requirement is found in southern ecotypes. A clinal difference in the base temperature for growth among the ecotypes is also found. In the studies of Myking and Heide (1995) the north Norwegian ecotypes flushed 2.5 months earlier and developed faster and had a lower base temperature for growth than ecotypes from southern Scandinavia. The Danish birches have adapted to a milder and more variable winter climate by developing greater dormancy stability involving both a longer chilling requirement and a higher base temperature. Night length had no effect on bud burst after the chilling requirement was fully met. After full dormancy release, time of bud burst in birch depends solely on the temperature regime in late winter and spring (Myking and Heide 1995).

The upper temperature limit for normal dormancy release in birch is probably slightly above 12°C. Chilling deficit is thus unlikely to occur in Scandinavia and in other areas where the chilling requirement is far exceeded, even with a climatic warming of 7 – 8°C above the current normal winter temperature. The likely effects of a climatic warming include earlier bud burst, a longer growing season and increased risk of spring frost injury, especially in northern ecotypes (Myking and Heide 1995).

Growth capacity of different provenances

In provenance trials with *B. pendula* at four sites within the latitudinal range of 56 – 64°, Johnsson, 1977 (cited by Eriksson and Jonsson 1986) reported that long-distance transfers both southwards and northwards tend to result in growth reduction. The longer the transfer northwards, the more is the growth period prolonged for the material from the south and the higher is the risk for dieback of the leaders. Short-distance transfers (< 250 km) do not influence the height growth considerably. Kleinschmit and Svolba (1982) reported on three-year heights of populations from central Europe to Finland. The Finnish and Swedish populations moved to Germany performed poorly as a consequence of the long-distance transfer southwards. In a study of growth of seedlings (Velling 1979b) a dependence between height and latitude of the origins was found. Seedlings of different provenances (from Latvia, 56°31'N, to central Finland, 61°48'N) were grown in central Finland and the more southern the origin, the greater the height of seedlings. A correlation was also found between the degree of leaf yellowing and the origin of seedlings. While the Finnish origins had turned completely yellow in the autumn, the Latvian origins still retained their green colour. The colouring was connected with survival, with poor winter resistance causing increased mortality in the southern origins.

7. Ecology and Physiology

A. Dynamics of regeneration

Birch is a pioneer species, and it quickly colonises bare areas and does not tolerate shading. Young birches can not survive fire because of their thin bark, but some mature trees may, because the thin forest floor under birches can not support intense and persistent surface fires. Post-fire pioneer successions are often dominated by birch. Abundant seed production enhances the pioneering character of birch. Without fire or human intervention birches would be replaced in succession by more shade-tolerant and longer-lived species (Perala and Alm 1990).

Light

Seedling density is independent of canopy cover, *i.e.* germination is unaffected by light, but the ability of birch seedlings to penetrate the canopy is low and they cannot establish in even the lowest vegetation. Most birch seedlings are only about 5 – 12 cm tall after the first year, and competing vegetation on fertile sites can easily overgrow and subdue them. The birches are also sensitive to chemical interference (allelopathy) by other plants (Perala and Alm 1990). Moreover, shaded birches are a preferred host by insect herbivores (see *e.g.* Ruohomäki *et al*. 1996).

Shading by neighbouring trees has a profound effect on shoot growth. In a short-term experiment shaded seedlings were higher than those reared in the simulated sun-light (Aphalo and Lehto 1997). Fewer buds are initiated and a higher proportion die in zones of heaviest shading (Atkinson 1992). Optimum sunlight for silver birch height growth was found to be 43 % of full sunlight for weeded seedlings, but 24 % for seedlings competing with weeds. Silver birch has also been found to grow less when sunlight decreases from 56 to 16 %. In greenhouse experiments with conifers, birch was more sensitive to both its own canopy and root competition than to competition by conifers (Perala and Alm 1990).

The Eurasian birches can endure as much as 90 % shade by adapting leaf structure. As sunlight diminishes, the light intensity for photosynthetic saturation, maximum photosynthetic rate, leaf mesophyll thickness, and chlorophyll concentration all diminish. The maximum photosynthetic efficiency for silver birch is at 10 – 50 % full sunlight, much higher than for shade-tolerant plants (Perala and Alm 1990).

Temperature

The birches are adapted to cool climate and grow best at about room temperature. Once the soil temperature reaches 2 – 3°C, the growth of silver birch depends more on air temperature than on soil temperature. Seedling shoots grow in direct proportion to heat sums, gradually diminishing as photoperiod shortens. New shoots can tolerate growing season temperatures of -3 to -5°C (Perala and Alm 1990).

Water requirements

Assimilation of silver birch is fastest at about -5 x 102 kPa and water use diminishes in wet conditions. The birches are sensitive to both drought and flooding. Seedling mortality increases at water potentials below -1.6 x 102 kPa. However, adaptability of birches to anaerobic conditions by oxygen transfer from the shoots to the roots, reduces the effects of flooding. Fertilisation improves water uptake and drought resistance (Perala and Alm 1990).

The birches use water inefficiently. Silver birch seedlings maintain turgor at high soil water potential only by closing stomata, which partially close at about -15 x 102 kPa. In large trees daily transpiration per unit of foliage mass is about 514 kg water/kg foliage. Extreme transpiration demand reduces growth even on moist soil because transpiration and photosynthesis have partly separate control systems. Drought depresses photosynthesis more than it does transpiration (Perala and Alm 1990).

Nutrients

To achieve maximum productivity, white birch requires all necessary nutrients, an optimum ratio of nitrogen sources NO_3^- and NH_4^+ in a rhizosphere and an optimal total nutrient solution (Ingestad 1971). In general, *B. pendula* is a nitrogen-limited species. Low-nitrogen conditions reduce growth and increase amount of condensed tannins, whereas in high nitrogen conditions *B. pendula* grows faster and the content of flavonoids in foliage is higher (Keinänen *et al*. 1999). The effects of nitrogen fertilisation on herbivore resistance are not clear. Nitrogen fertilisation did not affect preference of mammalian herbivores on *B. pendula* seedlings. On the other hand, the autumnal moth grew larger on fertilised seedlings (Mutikainen *et*

al. 2000). The limiting effects of other nutrients on the birches are not as well defined. At optimum levels, both zinc and manganese stimulate seedling growth, but toxicity problems have also been reported. There is a wide genetic variation in zinc tolerance and uptake. *B. pendula* has a high requirement for sulphur and it readily takes up boron. It sometimes suffers from manganese toxicity on poorly drained peat. The pH optimum for *B. pendula* and *B. pubescens* is between 4 and 5. *B. pendula* can tolerate some soil salinity (Perala and Alm 1990).

Effects on soil

B. pendula grows on fertile mineral soils and on drier and lighter soils than *B. pubescens*, which grows commonly on both peat and mineral soils (Gimingham 1984). The birches usually improve soils by efficiently cycling nutrients. First generation birch stands on former *Calluna vulgaris* (heather) heathland have increased earthworm activity, higher soil pH, greater total P, higher base status, faster rates of N mineralisation and cellulose decomposition and more diverse ground flora. Surface soil N, P, K, Ca, Mg and Mn is increased. Silver birch on Sphagnum peat soils accelerates microbial decomposition, accumulates dead woody roots and increases soil bulk density (Perala and Alm 1990).

Photosynthesis

CO_2 exchange variables showed considerable genetical variance in *B. pendula*. 54 % of variation in net photosynthesis, 36 % of variation in stomatal conductance and 45 % of variance in intercellular CO_2 were assigned to family (Wang *et al.* 1995). Photosynthesis related traits are modified by environmental factors as well. Defoliation reduces leaf area and biomass, but not photosynthesis, since *B. pendula* is able to compensate the damage by increasing photosynthetic activity. In fact, in nitrogen-rich environments damaged leaves have higher photosynthetic activity than the undamaged controls (Ovaska *et al.* 1993). The degree of compensation is dependent on the source of damage. The compensative response of *B. pendula* after artificial damage does not differ from that of the autumnal moth (Ovaska 1993), whereas the response was relatively weak after the damage by the alder beetle *Agelastica alni* (Oleksyn *et al.* 1998).

Root development

Birch trees exploit soils efficiently by developing both an extensive and dense surface root system to intercept precipitation and sinker roots to penetrate dense 'pans' and exploit deep water. The tap-root becomes horizontal after about 30 cm or is overtaken by side roots. Narrower roots may penetrate to a considerable depth. The extreme length of horizontal roots can be 25 m on sandy soils. In anaerobic conditions, *B. pendula* and *B. pubescens* roots elongate more than in aerobic conditions. Birch roots penetrate deep into poorly drained soils such as peats, and benefit inter-grown conifers by oxygenating the soil. On the other hand, birch small-root biomass may be twice that of the conifers offering intense root competition (Perala and Alm 1990).

B. Mycorrhizae

Early mycorrhizal infection is an important factor in the successful establishment of birch seedlings especially on nutrient-poor soils. The rates of nitrogen mineralisation of many forest litters are so slow that nitrogen can become the key growth limiting element. Some ectomycorrhizal fungi (*e.g. Amanita*, *Boletus*, *Paxillus*, *Suillus* and *Thelephora*) have proteolytic activity and thus the potential to mobilise nitrogen from proteins, peptides and amino acids and make it available to the plant. Some fungi, like *Laccaria laccata*, lack this activity and are dependent upon mineralisation processes initiated by other organisms (Read 1991). The assimilation of mycorrhizal amino compounds also provides supplementary carbon as well as nitrogen to the host plant. Abuzinadah and Read (1989) showed that up to 9 % of the carbon assimilated by the host plant (*B. pendula*) over a period of 55 days was derived heterotrophically from the protein by

mycorrhizal fungi. This is thought to be an adaptation to shade stress in young trees which spend the early part of their lives under the canopy of mature trees and which need to supplement their carbon budgets by heterotrophic assimilation. Grellier *et al.* (1984) showed that association with a mycorrhizal fungus (*Paxillus involutus*) practically doubled the growth of *in vitro* grown birch seedlings compared to non-mycorrhizal seedlings. Soil fauna have also been found to positively influence nutrient uptake and net production of birch seedlings (Setälä and Huhta 1991). Mycorrhizae can be inhibited by acute phosphorous deficiency, but can be encouraged even by a light application of phosphorous. Mycorrhizae like *Paxillus involutus* increase zinc tolerance by adsorbing zinc to the hyphae and slowing its transport to the shoots (Perala and Alm 1990).

Because little growth is possible on the nutrient reserves available in the seed, the seedlings are dependent on an external nutrient supply by the production of the first pair of leaves. Newton and Pigott (1991) report that ectomycorrhizal infection was indeed recorded by the time the first pair of leaves had expanded. Two groups of mycorrhizal fungi have been recognised: "early-stage" fungi (*Inocybe* spp., *Hebeloma* spp. and *Laccaria* spp.) can inoculate roots of seedlings from basidiospores or from added inoculum whereas "late-stage" fungi (*e.g. Lactarius pubescens* and *Leccinum roseofractum*) infect by hyphal connection. In natural circumstances the "late-stage" fungi are the most vigorous colonists of birch seedlings (Read 1991).

C. Diseases

Firm Rot

Black coloured firm rot around the pith is a serious cause of concern in young cultivated birch stands. Several different pathogens (fungi and bacteria) are responsible for the defect. Their entrance is, obviously, facilitated by frost cracks, insect exit holes (possibly the exit holes of *P. betulae*) and especially vole or moose browsing. The defect is especially harmful in plywood industry if the black colouring spreads outside the peeler core (Uotila 1987).

Birch rust

Birch rust (*Melampsoridium betulinum*) is the most common leaf disease of *B. pendula*. The rust causes yellowing and premature falling of the leaves, but it does not affect buds or the wood. If epidemics appear in several consecutive years, the growth of young seedlings can be decreased because of the shortened assimilation period (Uotila 1987; Vuorinen 1992). There are clear genetic differences in susceptibility to rust among birch clones, and screening clones for resistance could be used in breeding. A leaf-disc bioassay was used for determining the field rust resistance of birch clones in the study of Poteri and Rousi (1996).

Stem spotting

Stem spot disease caused by a group of fungi (*Godronia multispora*, *Botrytis cinerea*, *Fusarium avenaceum*, *Cylindrocarpon* sp., *Alternaria* sp.) is common in nurseries and can be very detrimental to young birch seedlings. The small necrotic lesions in the bark produced in the autumn enlarge during winter, and seedlings may die before spring. These fungi infect trees through mechanical wounding sites, frost cracks or insect (especially cicada, *Cicadella viridis*) wounding sites (Juutinen *et al.* 1976). Stem spotting is common in birch trees grown in unsuitable sites, *e.g.* in waterlogged soils (Uotila 1987).

Rot fungi

B. pendula is mainly rotted by *Polyporaceae* fungi, which start the rotting in the heartwood in the inner part of the trunk, and then proceed to the living sapwood. Especially timber is susceptible to decay.

Fomes fomentarius and *Inonotus obliquus* are the most common rot fungi and they attack living trees through wounds. *Ochroporus igniarius* also infects living trees.

Piptoporus betulinus is less common and attacks old or dead trees, destroying both heartwood and sapwood at the same time. The best way to prevent rot damage of living trees is good forestry practice: thinning should be done in time and only dead branches pruned, since branch scars act as a route for rotting fungi (Uotila 1987). Other fungi that rot dead trees and timber are *Stereum sanguinolentum*, *S. purpureum*, *Pycnoporus cinnabarinus*, *Cerrena unicolor*, *Trametes multicolor* and *T. hirsuta* (Uotila 1987).

D. Insect herbivory

Insect herbivory affects birch growth. For instance, in 1965 – 1967 the autumnal moth (*Epirrita autumnata*) defoliated over 5000 km^2 of birch forest in Finnish Lapland (Lehtonen and Heikkinen 1995). On the other hand, birches have a large community of invertebrate herbivores (Annila 1987), which may damage seedlings and trees locally, but at the stand scale birch is able to compensate the damage. Of geometrid defoliators living on *B. pendula*, *Opheroptera brumata* is among the most voracious species (Tikkanen *et al.* 1998, 1999, 2000). The species is common in Baltic countries, southern Scandinavia and central Europe. The damage may be severe after the insect outbreak but usually climatic conditions enable birch to compensate the damage by re-growth. Beetles may damage trunks of mature trees, and thereby reduce the economical value of the timber. For instance, *Hylecoetus dermestoides* and *Trypodendron signatum* bore tunnels in logs and timber. *H. dermestoides* can attack also living trees and a simultaneous infection by rot fungi can be fatal to the tree (Raulo 1981). Larvae of the birch cambium fly, *Phytobia betulae*, mine in the birch wood near the cambium layer and cause 1 – 4 mm wide brown streaks in the wood. The streaks decrease the value of birch wood used in plywood and furniture industry. The damage is aesthetic; the mechanical strength of the wood is not affected. The resistance mechanisms of birch and the biology of *Phytobia betulae* are not known. Ylioja *et al.* (1995) studied the susceptibility of European (*B. pendula*) and Japanese white birch (*B. platyphylla*) to *Phytobia* damage. Birch progenies which had *B. platyphylla* in their ancestry included more pith flecks than pure *B. pendula* progenies. Fast growing birches were also more susceptible to *Phytobia* attack. Differences in susceptibility to *Phytobia* attack between birch clones and progenies of plus trees could be used in resistance breeding.

Besides the genuine seasonal change in foliar phenolics (Salminen *et al.* 2002), insect grazing or artificial damage increases the level of phenolics in the leaves of *B. pendula*. In the study of Hartley (1988), increases in phenolic compounds did not affect further feeding, either by a natural birch-feeding herbivore (*Apocheima pilosaria*) or a polyphagous non-birch feeding insect (*Spodoptera littoralis*). Hence, there is no evidence that the tree's responses were specific defences against further attack by insect herbivores.

E. Mammalian herbivores

In Scandinavia, trees of the genus *Betula* are important winter food for herbivorous animals, especially voles (*Microtus*, *Clethrionomys*), hares (*Lepus*) and moose (*Alces alces)* (Rousi *et al.* 1989, 1990; Jia *et al.* 1997). They usually destroy woody plants in wintertime when alternative food plants are under snow cover. The variation of resistance among origins and families and even among individual seedlings within a genus can be very large. The centres of origin of cultivated plants are thought to be the best places to find resistances to diseases and herbivores. In these centres, plants have been exposed to selective pressure from local pathogens and herbivores for a long time, and have consequently developed resistance to them. Bryant *et al.* (1989) indicated that *Betula* and *Salix* species from Pleistocene refuges (Alaska and Siberia) were more resistant to mountain hare than species from regions that were glaciated during the Pleistocene. Likewise, birches from Iceland, where there were no browsing mammals before the

Norse colonisation, were more susceptible than birches from regions with more browsing mammals, that is, Alaska, Siberia and Finland (Bryant *et al.* 1989).

The bark of seedlings, young shoots and twigs of *B. pendula* contain resin droplets that consist of papyriferic acid and other triterpenoids. The juvenile resistance is accounted for by the resin, which is synthesised in and excreted by glands that are active only during the season when the primary apical growth of the shoot takes place (Taipale *et al.* 1993). On the other hand, phenolic substances are present in winter-dormant birches of all growth stages, but are rapidly metabolised by the plant in the spring when leaves emerge. Of these, platyphylloside is shown to exhibit repellent and anti-nutritional effects in mountain hares (Palo *et al.* 1992).

In *B. pendula* there is a sharp decrease of resistance after the tree has reached certain dimensions and the tree is no longer within reach of the herbivores. In the feeding trials with hare, Rousi *et al.* (1989) found that 1-year-old seedlings were less palatable than twigs taken from 7-year-old saplings of the same origin.

Vole

Voles cause considerable damage to forest plantations, especially during the peak years of density fluctuations. Birch plantations are especially vulnerable, since they are often afforestations of old fields, which are habitats favoured by *Microtus* voles (Rousi *et al.* 1990). The vole destroys birch seedlings under snow cover and it can eat the bark of the seedlings until the basal diameter reaches 4 cm (when the seedling is about 5 years old). Triterpenes in young birch seedlings seem to be deterrents of vole feeding. However, as the resin droplets are mainly situated in the top parts of the seedlings, and voles usually feed at the base of the seedling, they avoid the deterrent substances of the resin droplets. In field tests of Rousi *et al.* (1990), there were no clear differences in resistance between European white birch families in field tests with voles, but the Japanese white birch (*B. platyphylla*) turned out to be especially resistant to vole feeding. Rousi *et al.* (1990) suggest that hybrids between *B. pendula* and *B. platyphylla*, or Finnish-Siberian crosses of *B. pendula*, could be used to increase vole resistance.

Hare

Hares feed on the upper branches of young birch seedlings (of 40 – 70 cm) and especially in winter the damage can be fatal for the seedlings. Hares are discriminating feeders and determination of palatability is guided to a large extent by olfactory stimuli, and the resistance of young birch seedlings is tied to the production of papyriferic acid. For example, in the feeding experiments of Rousi *et al.* (1991), for the mountain hare (*Lepus timidus*) the palatability of birch seedlings and saplings was strongly and negatively correlated with the number of resin droplets on the bark. The Japanese white birch (*B. platyphylla*) turned out to be the most resistant of different birch species.

Rousi *et al.* (1991, 1996) also tested how the growth of birch correlates with resistance and whether fertilisation lowers the resistance of seedlings to browsing. Contrary to predictions of growth-defence trade-off theories, no trade-offs were found in the resistance and growth rate. Fertilisation stimulated growth but did not affect the palatability of the seedlings. Consequently, fast growing birch species and families should not be more susceptible to damage by herbivores, and fast growth can be promoted parallel with herbivore resistance by means of breeding.

Moose

Moose browsing on young birch seedlings occurs throughout the year. During winter only twigs are browsed, but during summer both leaves and young twigs are browsed. In order to feed on the young twigs of the crown, moose often break the main stem of the saplings. Moose can cause serious damage to birch

plantations. In Finland, plantations established in 1976 – 1977 were studied in 1985 (Heikkilä and Raulo 1987). Only one third of the plantations were found to be undamaged. Half of the total area had been damaged slightly and 15 % seriously. At the time of the establishment, though, the moose density was very high, 3 – 8 animals/1 000 ha. With a density of 2 – 3 animals/1,000 ha moose are not a serious threat to growing birch. Also, establishing plantations close to built-up areas or main roads can considerably prevent the risk of moose damage.

Heikkilä *et al*. (1993) showed that after stem breakage, the recovery during the first two years was fairly good. If the stems were at the leader shoot of the previous year, re-growth was weaker than in unbroken trees, the angle of crookedness was stronger and wound healing weaker. Injuries to the wood commonly become discoloured, and there was discoloration in 80 % of the sample trees. The significance of discoloration and decay depends on their distance from the wood surface, and the effect of discoloration on the quality of logs, when used for saw timber or veneer, can only be determined after a longer growth period. Bergström and Danell (1987, 1995) simulated winter browsing and summer browsing of moose, and studied the effects on the morphology and biomass of *B. pendula*. The birches responded to simulated winter browsing by growing fewer but larger and more branched shoots. There was also a slight decrease in viable seed production (Bergström and Danell 1987). In the experiments of Danell and Huss-Danell (1985) birches browsed by moose had more ants, psyllids, leaf-galls, leaf-miners and other leaf-eating insects. The leaves of browsed trees were larger and heavier, appeared greener and contained more nitrogen and chlorophyll. A decrease in resin content was observed. The trees seemed to allocate most of the nutrients and energy to growth in order to grow above the browsing line (Danell and Huss-Danell 1985). No induced defence in juvenile trees has been found; those trees that had been browsed during the previous winter were more palatable than previously un-browsed trees (Danell *et al.* 1985). Defoliation (simulated summer browsing) resulted in an overall decline in biomass and reduction in height and diameter growth. The long-shoots produced on defoliated trees were smaller and suffered more from tip-drying than shoots on control trees (Bergström and Danell 1995).

F. Abiotic damage

Abiotic damages are caused by too high ground water, drought, frosts and frost cracks (Uotila 1987). Snow load can cause stem breakage or permanent bending in young trees (Hannelius *et al.* 1989). Mechanical wounding increases fungal infections. UV-radiation does not seem to affect the growth, morphology or specific leaf area of *B. pendula* seedlings. The absorption by the secondary metabolites (phenolic glucoside, phenolic acids and flavonoids) provides the main part of the total UV absorbance of birch leaves. Plant secondary metabolism responds to enhanced UV-radiation by increasing synthesis of the above mentioned compounds that are the most effective UV-protectors (Lavola *et al.* 1997). The growth of *B. pendula* does not seem to be adversely affected by acid rain (Ashenden and Bell 1988). Instead, there was a stimulation in the height of birch seedlings with increasing acidity. A slight chlorosis of leaf margins after exposure to 2.5 pH rainfall was observed. Soil characteristics might have an influence on the sensitivity to acid rainfall.

Ozone causes physical damage on leaf surface, *i.e.* chlorosis, decoloration, black spots and necrotic areas and finally, leaf shedding (Maurer *et al.* 1997). Moreover, it changes the balance between CO_2 assimilation and stomatal conductance, which may severely limit plant's ability to repair ozone damage at the cellular level (Zhang *et al.* 2001). Ozone activates biosynthetic pathway, and thus the production of phytohormone ethylene (Kangasjärvi *et al.* 1997). Sequences of the cDNA of 1-aminocyclopropane-1-carboxylate oxidase (ACO), the enzyme catalysing the last step in ethylene biosynthetic pathway, as well other possible ACO-homologue fragments have been submitted to the Plant Gene Register (EMBL accession numbers X97993, X97992, X97994 and Y10749). Interestingly, *B. pendula* is among the most ozone-resistant woody plants in central Europe. The gas exchange traits differ by factor or two when compared to more vulnerable woody plants (Zhang *et al.* 2001).

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SECTION 3
NORWAY SPRUCE (*PICEA ABIES* (L.) KARST)

1. General Description and Use in Forestry

A. Taxonomy

Norway spruce (*Picea abies* (L.) Karst) belongs to the genus Picea, which includes at least 36 different species that all have their distribution in the northern hemisphere (Schmidt-Vogt 1977). The species *P. abies* has been designated by a large number of synonyms, partly caused by classification to different species or subspecies due to its great number of varieties and forms. The best known of these is the classification of the Siberian spruce in a separate species, *P. obovata*, based on the shape of its cone scales. These variations, however, should be considered as normal patterns of variation within a widespread species and should at most be used to designate different climatic varieties (Schmidt-Vogt 1977, 1978).

B. Use of Norway spruce

Norway spruce is the economically most important conifer tree species in Europe. It has a long history of cultivation in central Europe, and has been seeded and planted very intensely since the middle of the 19th century. This has changed natural forests into artificial ones and has led to the species' introduction far outside its natural range, both in countries where it occurs naturally (*e.g.* Germany and Norway) and in new countries such as Denmark, Belgium and Ireland. To some extent, Norway spruce has also been planted in North America.

Norway spruce has shown good yield and quality performance under very different site conditions, and this favoured the species over a long period. In some areas, where maladapted provenances were used, damage and reduced yield have occurred. In the last two decades the species has suffered severely during the forest decline in central Europe, resulting in stands with high percentages of trees with needle loss (Wolf 1995) or in completely destroyed stands (Vancura 1995). The health problems of the central European spruce forest, and reduced possibilities for recreation in young spruce stands, have to some extent reduced the popularity of this species for reforestation, particularly outside its natural range.

2. Forest Practices for Norway Spruce

A. Breeding of Norway spruce

Usually the goal of breeding programmes is to produce superior material for reforestation by managing genetic variability. Breeding of Norway spruce was initiated in several European countries in the late 1940s (Danell 1991, Mikola 1993). In some countries the work started with the selection of phenotypically superior trees ("plus trees") in natural stands (Skrøppa 1982b, Mikola 1993, Gabrilavicius and Pliura 1993). Mature trees that had superior height and diameter growth, and stem and branch quality, compared to neighbouring trees in the stand were selected. They were grafted in multiple copies on rootstocks in clonal archives or seed orchards. Each grafted seed orchard is composed of a rather large number of selected clones (50-500), with the intention of seed production for one geographic region. The

seed orchard will generally start to flower 10-15 years after grafting. However, both the regularity and amount of flowering are very dependent on climatic conditions at the orchard site. To promote flowering, seed orchards have often been located to sites with a warmer climate than that from which the selected parents originate and where the orchard seed is intended for use.

It was soon realised that the selection of plus trees in natural Norway spruce stands is not an efficient method of selecting superior genotypes. It is necessary to test the genetic value of each parent, based on an evaluation of its offspring. In Norway this is done in progeny tests planted at several sites, where measurements are made of survival, height and diameter growth, and quality traits. The progeny tests are sometimes supplemented with tests in which seedlings are grown under controlled conditions in growth chambers and measurements are made of physiological traits. On the basis of several traits, a subset of the original parents is selected for further breeding. Seeds for practical planting can be collected in the orchard from the selected parents, the orchard can be thinned, or a new orchard can be established based on the selected group.

In other countries, breeding programmes were based on selected materials from populations with high adaptive potentials observed in comparative provenance trials. According to performances measured in progeny trials with families from these populations, the best individuals within progeny were selected either directly to produce seeds in seed orchards, or to create a breeding population through controlled crosses. Some of these programmes were also aimed at mass production of rooted cuttings of tested and selected clones (Birot 1982, van de Sype and Roman-Amat 1989, Kleinschmit 1993). Of major concern in the development of breeding strategies have been the breeding objectives; the sizes of the breeding and seed producing populations required to maintain genetic diversity; design and efficiency in testing; and identification of suitable regions where the orchard seed should be recommended for use. Based on test results, gains from the original plus tree selection have been estimated, at 6% genetic improvement in height growth at stand closure (Danell 1991). An additional 10% advance in the selected trait can be expected after progeny testing and further selection.

In Norway, Sweden and Finland several thousand selected spruce trees have been grafted in seed orchards or clonal archives. A large number of these have been progeny tested. Most seed orchards, however, consist of the original set of non-tested parents. In some orchards selective harvests have been made on the basis of progeny test results. The main selection criteria have been traits characterising the annual growth rhythm, height growth, and the avoidance of damage in field tests. Artificial freezing tests are being used to determine autumn frost hardiness, particularly for materials intended to be planted at high altitudes or far north. Each seed orchard is intended to produce seed for a specific region, which is determined by the origin of the parents and the growth rhythm and hardiness performance of the orchard offspring.

Seed orchard breeding is also the most common technique being used for spruce in central and eastern Europe (*e.g.* Dolgolikov 1993, Gabrilavicius and Pliura 1993, Kleinschmit 1993). Special types of orchards have been established, based on materials from provenance trials, in order to reconstitute provenances that probably no longer exist or are untraceable (Giertych 1993). Clonal breeding programmes based on rooted cuttings have been developed in Germany (Kleinschmit 1993) and Sweden (Karlsson 1993).

B. Reproductive methods

Flower induction

Application of growth regulators, primarily giberellins, has become an efficient method of regulating flowering in forest trees. In the case of Norway spruce grafts, treatments during the shoot growth period (with giberellic acid alone and in combination with heat) have been efficient in promoting female

flowering (Johnsen *et al*. 1994). Heat treatment alone will induce male flowering. Of mechanical flower stimulation techniques attempted in Norway spruce seed orchards, only thinning is useful on a larger scale (Schneck *et al*. 1995). The problem of shortening the juvenile phase and obtaining regular flowering at an early age remains unsolved (Chalupka 1991).

Vegetative propagation

Norway spruce has for decades been propagated by grafting. Scions to be grafted are taken from the crown of mature trees and grafted onto a rootstock, which is a young spruce seedling. This technique is being used to establish clonal seed orchards and clonal archives.

Vegetative propagation with rooted cuttings is easily achieved with young spruce seedlings and can be applied on a large scale (Kleinschmit *et al*. 1973). The propagation can be made either in early spring before bud flushing, or after shoot growth terminates at the end of the summer. Both the rooting capacity and the growth form of the cutting depend on the age of the cutting donor, but they can be improved in a proper rooting environment. A large genetic variation has been observed in rooting capacity and subsequent growth habit of the cutting (Johnsen and Skrøppa 1992). Several systems of propagation have been developed in order to keep the plant material at a juvenile stage (Roulund 1981, Dekker-Robertson and Kleinschmit 1991). Clonal tests with rooted cuttings have provided valuable information for Norway spruce genetics research, in addition to providing clonal material with high genetic value intended for commercial use (*e.g.* van de Sype 1989, Kleinschmit and Svolba 1991, Isik *et al*. 1995).

Cell and tissue culture techniques for micropropagation have recently been developed for conifers. The most promising method for Norway spruce appears to be somatic embryogenesis, obtained for the first time by Hakman *et al*. (1985). Somatic cells are stimulated to develop into somatic embryos, somewhat analogous to that of a zygotic embryo. The somatic embryo can be stimulated to mature and develop into a plant which will be of the same genotype as the zygotic embryo used for induction of this embryogenic cell line (Hakman 1993, von Arnold *et al*. 1995, von Arnold 1996, Egersdotter and von Arnold 1998). However, in *P. abies* somatic embryogenesis has also been initiated from explants other than zygotic embryos (Wescott 1994, Ruaud 1993). The somatic embryos can be propagated in unlimited numbers on a large scale and can be formed as artificial seeds. The method works only for a limited number of genotypes and requires controlling plant quality. Therefore, further developments are needed before somatic embryogenesis can be used for practical forestry purposes (Mo 1993). Norway spruce also belongs to the few spruce species with advanced *in vitro* regeneration, and thus could have possibilities for genetic transformation. There has recently been a breakthrough in production of stably transformed Norway spruce, based on *Agrobacterium tumefaciens* mediated transformation (Wenck *et al*. 1997) and on biolistic transformation (Walter *et al*. 1997). Also, genetic transformation of Norway spruce pollen and the use of transformed pollen in controlled pollinations is in progress (Häggman *et al*. 1997).

C. Reproductive materials used

The regeneration of Norway spruce forests is based on both natural regeneration and planting, with an emphasis in many countries on natural regeneration where that is a feasible method. The largest proportion of Norway spruce seeds being used world-wide are collected in natural or planted stands. Each seed lot be identified by the geographic origin of the stand. In several countries it is required that the seed stand be selected for superior performance (EEC 1966, Muhs 1986). The relative amounts of stand and seed orchard seed being used vary considerably between countries, and between regions within countries. Clonal forestry based on rooted cuttings, which was initiated to some extent in the 1970s in Germany and Sweden, occurs at present only on a small scale.

In the Nordic countries, stand seed accounts for the largest proportion of seed used. The percentages of stand seed used in 1994 were: 89% (Denmark), 84% (Sweden), 81% (Norway) and 80% (Finland) (personal communication Bjerne Ditlefsen, Lennart Ackzell, Gunnar Haug, Hannu Kukkonen). The percentage from seed orchards varied between 10 and 20%. Sweden is the greatest importer of spruce seed, 35% of which is stand seed. Rooted cuttings account for less than 1% of the total number of Norway spruce plants.

D. Provenance transfers

The first provenance transfers were more or less random and unrecorded, with the result that in large areas of central Europe and southern Scandinavia most spruce stands are of unknown and mixed origins. Subsequently, provenance trials have provided information about the effects of transferring provenances from different parts of the range of the species and have identified provenance areas which generally have both a high adaptability potential and high growth capacity. Examples are provenances from southern Poland and Romania, which have been planted with success in several countries (Lacaze 1969, Krutszch 1992, van de Sype 1998). Some other provenances present a low adaptation potential and exhibit huge variations in performance for survival rate and growth ability according to trial location (*e.g.* van de Sype 1998).

Provenance transfers have been used deliberately in several cases where materials with specific adaptive properties were sought. An example is the transfer of late-flushing Norway spruce provenances from Byelorussia and the Baltic countries to sites exposed to late spring frosts in southern Sweden. These provenance transfers have reduced frost damage and improved growth (Werner *et al*. 1991, Werner and Danell 1993). In other cases, however, maladaptation has occurred, particularly after transfers of provenances from a warm to a colder climate. In south-eastern Norway, spruce provenances from Austria and southern Germany were extensively planted during a 20-year period starting in the 1950s. A survey of 79 planted stands of central European origin and 21 of local origin, all at the age of 27 years, was recently made in Østfold County, Norway (Skrøppa *et al*. 1993). In the stands of local origin, 30% of trees were classified as having saw timber qualities, while only 7% of those in the central European stands obtained the same quality classification. This provenance transfer generally had a negative effect on timber quality and a small positive effect on volume production, even if a few of the stands planted with southern provenances had both high volume production and good stem quality. It is not known to what extent the pollen cloud from these stands may cause reduced fitness in offspring from the seed in neighbouring stands of local origin.

E. Conservation of Norway spruce genetic resources

In many countries the combination of natural regeneration and planting of materials with a broad genetic diversity is the basis for conservation of genetic resources of Norway spruce. In addition, several specific conservation measures are taken. *In situ* activities include national parks, nature reserves and specific conservation or gene reserve forests. The grafted clone banks and seed orchards and provenance and progeny tests constitute the *ex situ* gene conservation, together with long-term seed storage. Co-operation at the European level is achieved through the recently established European Forest Genetic Resources Programme (EUFORGEN). Norway spruce is one of the species for which a co-operative network has been formed (Turok *et al*. 1995, Turok and Koski 1997, Koski *et al*. 1997).

3. Centres of Origin and Immigration History

A. Natural distribution and origin

The natural distribution of *P. abies* can, according to Schmidt-Vogt (1977), be divided into three areas: the central and south-eastern European, north-eastern European and Siberian spruce areas. The first covers mainly mountainous and sub-alpine regions and is separated from the second by a spruceless area in Poland. The north-eastern European area includes the Baltic, Nordic and Russian spruce. The Siberian area covers all spruce east of the Urals.

The total natural distribution of *P. abies* covers 31 degrees of latitude from the Balkan Peninsula (latitude 41°27'N) to its northernmost extension near the Chatanga River, Siberia (latitude 72°15'N). Longitudinal range is from 5°27'E in the French Alps to 154°E at the Sea of Okhotsk in Eastern Siberia. The vertical distribution is from sea level to altitudes above 2 300 m in the Italian Alps. Outside this area the species has been widely planted, particularly in central Europe (Ellenberg 1988) and in Scandinavia.

It is thought likely that *P. abies* had its prehistoric origin in east Asia, from which it migrated to Europe through Siberia and the Urals (Schmidt-Vogt 1977). During the last Ice Age, the species is assumed to have survived in refugia in four European regions: north-central Russia, the Carpathians and Transylvanian Alps, the Dinaric Alps and the Apennine peninsula. From these refugia, *P. abies* migrated to its present natural areas. The central and south-eastern European spruce originate from the last three refugia, while the north-eastern European spruce migrated from Russia.

In central Europe, *P. abies* could probably be found only in two small areas in the present Czech Republic about 11,500 BC (Vancura 1995). It migrated from the Beskids over the Sudeten to the Ore Mountains in Germany, which were reached about 6500 BC. Spruce established in the Harz Mountains about 4000 BC, while the Black Forest in south-western Germany was reached about 1500 BC. In Switzerland, Norway spruce established in the eastern and south-eastern part of the country about 8000 years ago and expanded to the valleys of the Alps within 3500 years (Bonfils and Sperisen 1997). The expansion was much slower North of the Alps.

The migration of Norway spruce to Fennoscandia took place from the Russian refugium. It advanced through the forest area at the present border between Russia and Finland and passed through Finland and northern Sweden into Norway in the period 3500-500 BC (Moe 1970, Schmidt-Vogt 1977, Hafsten 1991, 1992a, b). The advance was quite rapid; it has been estimated at an average speed of 9 km every ten years (Moe 1970).

4. Reproductive Biology

A. Sexual reproduction

P. abies is monoecious, having both male and female flowers on the same individuals but on separate organs. The male flowers are in most cases located at the base of the preceding year's shoot, while the female strobili are at the tip of the shoot, directed upwards. The reproductive buds are initiated during the growth season the year before.

Male meiosis and pollen development occur after the winter dormancy in late winter or early spring and are regulated by the temperature conditions (Luomajoki 1993). A male flower may contain as many as 600 000 pollen grains, which are released at anthesis and dispersed by wind.

Female meiosis usually starts before the female strobili become receptive (Sarvas 1968) and occurs close to or during the period of pollination. Pollen grains are accommodated in a pollen chamber which has

a limited volume and, on average, contains three to five pollen grains. They germinate and pollen tubes grow towards the female gametophyte (egg cell) in the ovule, where fusion of the male and female gametes takes place. Under natural conditions this normally occurs five to six weeks after pollination. The fertilised egg develops rapidly into a seed. Several egg cells may be fertilised in each ovule, but only one will develop into a seed. Empty seeds may result from lack of pollination or from abortion.

Climatic conditions play an important role during several stages of the reproductive process. High temperature during the growth season is one of the main factors favouring floral initiation and the development of reproductive buds which will flower the next spring. Dry conditions and moderate to high temperatures during the flowering period the following year are necessary in order to obtain sufficient pollination. Seed development and maturation require high accumulated temperature sums during the summer and early autumn. Such specific weather patterns during two successive years occur rather seldom, particularly far north and at high latitude regions, which may explain why seed crops are both rare and irregular in these areas.

B. Mating system and gene flow

The mating system of Norway spruce falls into the mixed mating category. That is, the largest part of the seeds are produced through cross-fertilisation and the rest through self-fertilisation. Cross-fertilisations are both between trees that are close together in a stand, and among distant individuals in the same or nearby stands.

Spruce pollen is able to move over long distances. Andersson (1955) found that the amount of pollen at 2 500 m distance was 47% of the amount at the edge of the forest in one stand, and 15% in another stand. In two Norway spruce stands in Finland, Koski (1970) estimated the background pollination to be approximately 60% of the total pollen catch. Lindgren *et al*. (1991) reported results from studies of the dispersion of Norway spruce pollen artificially released in seed orchards outside the local pollen shedding season. These results showed a rapid drop-off in the pollen dispersion curve over distance from the source, with the largest amount of pollen being deposited less than 50 meters from the pollen source. However, large variation patterns most likely due to wind turbulence were found between pollen catches on individual days, indicating that a curve showing a smooth movement in the wind direction is not a good model for individual pollen dispersion events (Lindgren *et al*. 1991).

Both local and long-range pollen movements will determine the natural pollination patterns and actual gene flow of Norway spruce. On average, most pollinations will be with local pollen or pollen from nearby populations (Koski 1970), with exceptions in some years. In an allozyme marker genetic study in an experimental Norway spruce plantation, Xie and Knowles (1994) estimated the proportion of ovules fertilised with own pollen, pollen from trees within the plantation and that from trees outside the plantation to be 0.09, 0.75 and 0.16, respectively. The proportions varied considerably among parent trees. Wind-pollinated forest trees are known to have a high number of migrants per generation compared to other plant species (Govindaraju 1988, 1989). No good estimates of migration rates are available for Norway spruce, but Koski (1970) and Finkelday (1995) both concluded that the gene flow between nearby populations by means of pollen dispersal is effective and may play an essential role in the population genetics structure of Norway spruce.

The actual rate of self-fertilisations in natural populations may vary considerably between trees. It was found by Müller (1977) to vary between 7 and 18% in a study of five trees. Koski (1973) estimated the mean proportion of self-fertilisations at 10%, but concluded that only 1% of the filled seeds originate from self-fertilisations.

C. Inbreeding depression

Most spruce trees will produce some filled seed after self-pollinations, but the seed yield is much reduced (Skrøppa and Tho 1990). The main reason for this is embryo abortions caused by lethal or deleterious genes that become harmful when they occur as recessive homozygotes in the selfed individuals (Koski 1971). Inbred spruce trees generally have reduced fitness compared to their outbred relatives. They have lower survival in the field, their growing season is shorter, and they grow more slowly (Langlet 1940, Eriksson *et al*. 1973, Skrøppa 1996). The inbreeding depression varies both among populations and among individuals within the same population (Skrøppa 1996). For a trait such as height growth at age ten years, it may vary in the range of 10 to 50% among selfed offspring of trees from the same population.

D. The seed crop

Norway spruce trees undergo a rather long juvenile period, during which they will not flower and set seeds. In the open stand, sexual maturity will generally be reached after 20-30 years, while it occurs later in the closed stand (Schmidt-Vogt 1978). However, a substantial flowering and seed set may occur considerably earlier than 20 years from planting (Skrøppa, unpublished) if the temperature conditions are favourable for floral induction. In exceptional years, female flowering has been observed on eight- to ten-year-old and male flowering on twelve- to 15-year-old Norway spruce trees.

Under central European conditions, Norway spruce will flower several times in a decade (Schmidt-Vogt 1978). In the boreal forest, cone harvests occur less frequently and at irregular intervals. There were no cone crops in the lowlands of southern Norway between 1976 and 1983. Since then, however, substantial flowering and seed crops have occurred in 1983, 1987, 1989, 1993 and 1995. In northern Norway, successful seed harvests have been obtained only three times during the last 40 years (1958, 1970 and 1981).

In central European seed orchards, flowering has occurred less frequently than was expected (Kleinschmit 1993).

E. Natural regeneration

The Norway spruce seeds are dispersed mainly by wind and partly by birds and animals (Sokolov *et al*. 1977). Most seeds will be dispersed close to the mother tree, but some may also be dispersed over larger distances.

Natural regeneration also very much depends on the species composition of the bottom and field layer. The most productive spruce forest appears to be the most problematic for natural regeneration, for example in “spruce forest with tall herbs” (*Melico-Piceetum aconitetosum*) and “spruce forest with tall ferns” (*Eu-Piceetum athyrietosum*). The optimal habitat for natural regeneration is the “spruce forest with small ferns” (*Eu-Piceetum dryopteridetosum*). Regeneration in our most common and widespread spruce community, “spruce forest with bilberry” (*Eu-Piceetum myrtilletosum*), is greatly hampered by a thick raw humus layer, especially at higher altitudes (Mork 1944, 1945, 1968).

The Norway spruce seedlings are very shade-tolerant and can survive for decades under a closed canopy (Siren 1955). They grow slowly during the first years, and the height growth increases after five to ten years (Sokolov *et al*. 1977, Nikolov and Helmisaari 1992). *P. abies* is often associated with grey alder (*Alnus incana*) on river plains in the boreal zone and may tolerate occasional flooding (Sokolov *et al*. 1977).

F. Dynamics of regeneration

Boreal forests undisturbed by human activity have dynamic properties, with complex succession stages which influence habitats and create possibilities for regeneration. The most important natural disturbance factors are fire, storms, and pathogens such as bark beetles (Tømmerås 1994). The fire tolerance of *P. abies* is very poor (Drakenberg 1981). Mainly due to its shallow root system, spruce is intolerant to windthrow (Sokolov *et al*. 1977). Storms can blow down many trees, particularly in wind-exposed areas, where occasionally almost all the trees in a stand may blow down. Bark beetles (*Ips typographus)* can from time to time become a serious pest and kill trees (Christiansen and Bakke 1988). Together with gaps created by the death of individual old trees, these disturbances open up the canopy and lay the foundation for regeneration.

G. Vegetative reproduction in nature

Under certain conditions, Norway spruce will naturally reproduce vegetatively through the lowest branches, which may come into contact with the soil and differentiate roots and new shoots (layering). This occurs particularly in alpine areas, where the climatic conditions prohibit sexual reproduction, and is therefore important for species distribution (Skoklefald 1993). Examples of such vegetative reproduction can be seen above the timberline where scattered clonal groups occur.

5. Crosses

A. Crossability with other species

Crossability of spruce species can be judged both from introgressive hybridisation and from attempted controlled hybridisation. The only known natural hybrids involving Norway spruce are *P. abies* var. *obovata* with *P. jezoensis* and *P. koraiensis* (Schmidt-Vogt 1977) in eastern Asia. Successful artificial hybridisations are reported with eight other spruce species (*P. asperata, P. glauca, P. mariana, P. montigena, P. omorica, P. orientalis, P. rubens* and *P. sitchensis)* (Kleinschmit 1979). Rather few of the potential hybrid crossing combinations have been attempted.

B. Interprovenance hybridisation

No crossing barriers are present between spruce trees from different provenances. Provenance hybrids are therefore produced naturally in regions with both indigenous populations and introduced provenances or cultivars. The extent and implications of these hybridisations depend on many factors, such as the characteristics of the provenances discussed in section VI.

Experimental results with inter- and intraprovenance crosses have demonstrated that the hybrids are intermediate between the two parents for most traits (*e.g.* Ekberg *et al*. 1982, 1991, Kaya and Lindgren 1992). Hybrid vigour therefore does not appear to be present to any large extent.

6. Genetic Variability

The number of chromosomes of Norway spruce is 2n = 24 (Schmidt-Vogt 1977 and references cited therein). Tetraploidy has occasionally been observed in Swedish and German provenances (Kiellander 1950) and has also been induced by colchicine treatments (Johnsson 1975). Trees with irregular chromosome numbers appear in general to have reduced fitness.

The genetic variability of Norway spruce has been studied using a large number of methods and at different genetic levels. More than 100 years ago, experiments replicated at several locations with seed lots from different origins (provenances) were carried out in Austria, Germany and Switzerland (Langlet 1971).

In these first genecological experiments, traits such as height increment, needle morphology, growth habit, time of growth initiation and frost damage were studied. Later, both national and international provenance experiments were established, some of these organised by the International Union of Forest Research Organisations (IUFRO), and often including large numbers of provenances planted on multiple test sites in several countries (Krutzsch 1992). The traits studied are related, in particular, to forestry cultivation of Norway spruce. They characterise climatic adaptation, growth potential and quality. They are typically quantitative, having a continuous phenotypic distribution, and are strongly influenced by the environment. The same type of traits have been measured in numerous experiments with offspring from both natural and artificial populations, in most cases established with the intention of testing breeding materials. The field trials have been supplemented by tests under more controlled conditions, in which variability in specific physiological traits has been studied. Since biochemical markers became available, several population genetics studies have characterised the intra- and interpopulation variation and genetic structure of the species (*e.g.* Lagercrantz and Ryman 1990, Müller-Starck *et al*. 1992, Konnert and Maurer 1995). Recently, molecular DNA marker techniques have been developed for Norway spruce (Bucci and Menozzi 1993, Binelli and Bucci 1994).

A. Overall variability

Large genetic variability exists within the extensive range of the natural distribution of Norway spruce. The most pronounced adaptive patterns relate to populations' responses to climatic conditions. Across the European range of the species these patterns of variability can often be related to latitude and altitude of origin, and with degree of continentality, and will sometimes vary clinally. In central Europe, however, differences among populations from the same geographic region are in many cases large and reflect several generations of Norway spruce cultivation. This fact often blurs patterns of variation of adaptive traits. Traits that characterise the annual growth cycle, particularly onset of growth in the spring and termination of growth and development of frost hardiness in late summer, show the most pronounced provenance variability (Langlet 1960, Krutzsch 1975, Dormling 1973, Beuker 1994, Beuker *et al*. 1998). However, observations of these and of growth performance traits are made in common garden field tests comparing provenances that are transferred unequal distances. Such provenance transfers may affect provenances differently. Provenance differences must therefore always be interpreted relative to the planting site conditions.

Populations with an early growth start, often expressed as bud burst or bud flushing, originate from high latitudes in northern Scandinavia, Finland and Siberia and from high altitudes in the central European Alps (Langlet 1960, Krutzsch 1975, Holzer 1993, Beuker 1994). The eastern and more continental provenances generally have a late growth start, and the latest flushing populations come from Byelorussia, north-eastern Poland and the interior of the Baltic Republics. The variation in bud flushing and initiation of shoot growth of Norway spruce provenances are assumed to be regulated both by differential responses to accumulated temperature sums in the spring and by conditions during acclimation the proceeding year (Heide 1974b, Schmidt-Vogt 1977, Dormling 1982).

Photoperiod is the environmental factor that initiates the cessation of growth and development of frost hardiness (Dormling 1973), but with some modifications caused by temperature (Heide 1974a). Under controlled growing conditions in growth chambers, seedlings of provenances from the northernmost latitudes will respond with a terminal bud set at a night length of two to three hours, compared to eight to nine hours of darkness for south-western European origins (Dormling 1973). The northern Scandinavian and Finnish provenances, and those from high altitudes in the Alps, have the earliest cessation of shoot growth (Skrøppa and Magnussen 1993). The latest growth cessation occurs in provenances from southern Poland and the eastern Carpathians.

At the provenance level, strong relationships are generally present between traits that characterise the timing and duration of the growth period, the lignification of the annual ring, and the development of autumn frost hardiness (Skrøppa and Magnussen 1993, Ekberg *et al*. 1994). These traits are components of an annual sequence of developmental events which describe the inherent annual rhythm of trees of the same provenance (Sarvas 1972, Skrøppa and Magnussen 1993).

Resistance to late spring frost is closely related to the time of growth start; the late flushing provenances from eastern Europe suffer less damage than native Nordic provenances during spring frost events in Scandinavia (Werner *et al*. 1991). A similar but somewhat weaker relationship is present between the timing of growth cessation and resistance to autumn frost. Provenances with early termination growth will normally enter dormancy (develop frost hardiness) earlier than those extending their growth period late in the summer, and thus be less damaged by early autumn frosts. In the Alps, high altitude provenances will be more resistant to early autumn frosts than those of lowland origins (Holzer 1993).

The growth capacity of different provenances is closely related to the duration of their growth period (Holzer 1993, Skrøppa and Magnussen 1993). Northern provenances, or those from high altitudes that are adapted to a short growth season, will therefore have a poorer growth potential than those adapted to a longer season. Two provenance regions with particularly high growth potential have been identified (Schmidt-Vogt 1978). One covers parts of the post-glacial advance of spruce from the Russian refugium, including the Baltic Republics, north-eastern Poland and northern parts of Byelorussia. The other region covers the eastern Carpathian and Bihor Mountains and parts of the Beskids.

Wood quality traits, such as basic wood density and its determining components, have been shown to vary considerably among different provenances (Mergen *et al*. 1964, Worrall 1970, Schmidt-Vogt 1986). They are also influenced by the extent of provenance transfer. Strong relationships exist between these traits and annual growth rhythm characteristics.

Provenance variation has been shown for a number of other traits, such as nutrient demands, respiration activity and shade tolerance (Schmidt-Vogt 1977). Provenance differences are present in crown form, related to snow and ice break resistance, with the resistance increasing according to altitude (Holzer 1964, Schmidt-Vogt 1977).

Enzyme genetic marker studies reveal a great genetic variability within Norway spruce populations (Müller-Starck *et al*. 1992, Goncharenko *et al*. 1995), and also in populations close to the climatic margin of the species (Tigerstedt 1973, 1979). In the most comprehensive isozyme study on Norway spruce (Lagercrantz and Ryman 1990), only 5% of the total genetic diversity was explained by differences among provenances. Some differentiation occurs among populations derived from different glacial refugia and appear to reflect their post-glacial evolutionary history (Lagercrantz and Ryman 1990). Populations from the same region show little genetic differentiation (Bergmann 1973, Lundkvist and Rudin 1977, Lundkvist 1979, Konnert and Franke 1991). Central European provenances appear to have reduced genetic diversity, expressed by a reduced level of average heterozygosity compared to those from eastern Europe and Scandinavia (Lagercrantz and Ryman 1990, Goncharenko *et al*. 1995).

Patterns of provenance variation show geographic variability on a large scale. However, genetic variation may be present between offspring from populations within the same provenance, and this variation is often larger than that between provenances. Dietrichson (1973) sampled three populations from the same altitude (620-750 m) in each of five provenances, covering an area of 200 km north-south and 250 km west-east in southern Norway. Measurements were made of heights at two and four years, growth initiation and cessation, and lignification of the annual ring. Several traits showed a larger variation among populations within a provenance than among provenances. In studies of clones sampled from several populations from the same provenance region and from different provenances, variation among clones has

been demonstrated for a large number of traits (Sauer *et al*. 1973, Sauer-Stegmann *et al*. 1978, Kleinschmit *et al*. 1981, 1981).

B. Variability within populations

The large within-population genetic variation demonstrated by genetic markers has been confirmed in studies of quantitative traits. Genetic variability has been found within all natural Norway spruce populations studied, as well as for traits that show clinal variation at the provenance level (Dietrichson 1971, 1973, Eriksson 1982, Skrøppa 1982a, Ekberg *et al*. 1985, 1991). Traits that show such variation characterise germination, early and later height and diameter growth, the timing and duration of the shoot growth period, autumn frost hardiness, survival in the field, and branch, crown form, stem and wood quality (Schmidt-Vogt 1977, Skrøppa 1991, 1993, Hylen 1997). The range of variation may sometimes be as large as that found between geographically distant provenances.

C. Resistance to fungi and insects

Studies investigating Norway spruce's resistance to root rot (*Heterobasidion annosum*) have been carried out at both the provenance and clonal level (Dimitri and Kliefoth 1980). Treschow (1958) found no variation in growth of *H. annosum* among trees of different provenances. In an inoculation experiment with *H. annosum* on 98 Norway spruce clones, differences among clones were found in lesion length and fungal growth in sapwood (Swedjemark and Stenlid 1996). This indicates the presence of genetic variation among individual clones in degree of resistance to the fungus, and indicates that progress in resistance can be achieved through selection. Similar variation among clones has been found in resistance to bark beetle fungus infection (*Ceratocystis polonica*) (Christiansen and Berryman 1995, Brignolas *et al*. 1995).

Differences have been demonstrated among provenances in respect to infestation by spruce aphids (*Adelges* ssp.) (Balut and Sabor 1993), and also among families and clones from the same population (Skrøppa, unpublished). Little information is available on genetic variation in resistance to attacks by other insect species.

D. Factors influencing the genetic variability

The great genetic variability of the Norway spruce forests is influenced by a large number of factors: ancient origin and immigration history, natural selection, an extensive gene flow caused by pollen dispersal, genetic drift due to small population size, and human activities.

Different factors may cause specific variation patterns to be present in parts of the range of the species. As an example, in some areas the existence of frost pockets or different slopes and exposure aspects may have resulted in selection of different annual growth rhythms. Patterns of spacial differentiation may therefore be the result of complex interactions of gene flow and selection (Finkelday 1995, Krutovskii and Bergmann 1995). In central Europe the species has been cultivated for more than 300 years, partly with seed material transferred from other regions. Differences in performance between provenances from the same region therefore may not exclusively express adaptational differences. Recent experimental results indicate that phenotypic provenance variation in traits characterising climatic adaptation is not only regulated by classical (Mendelian) gene frequency differences, but also by other mechanisms (*e.g.* gene regulation). These mechanisms appear to be triggered by environmental influences during the generative reproductive process (Skrøppa and Johnsen 1994, Johnsen and Skrøppa 1996, Johnsen *et al*. 1995, 1996).

7. Ecology

A. Synecology and associated species

Spruce forests are found in many different habitats. They usually belong to the acidophilous order *Vaccinio-Piceetalia* and to a lesser extent to the *Fagetalia* (Ellenberg 1988, Fremstad 1997).

Forests of *P. abies* play a dominating role in the boreal zone in Fennoscandia and northern Russia. They belong to three associations, according to Kielland-Lund (1981, 1994):

- *Eu-Piceetum* (EP). This is the most common forest association. EP is the typical climax community on nutrient poor to medium rich, podzolic soil types. EP is subdivided into sub-associations, *e.g. myrtilletosum* (= "spruce forest with bilberry"), *dryopteridetosum* (= "spruce forest with small ferns") and *athyrietosum* (= "spruce forest with tall ferns"), with increasing soil richness and soil humidity (Dahl *et al*. 1986, Kielland-Lund 1994).

- *Melico-Piceetum* (MP). According to Kielland-Lund (1994), MP (= "spruce forest with low herbs") occurs in warmer localities and on more calcareous soils than *Eu-Piceetum*, mainly in the boreonemoral and south boreal zones. MP has three main sub-associations: *pinetosum* (= "calcareous low-herb woodland") on dry limestone soils, *typicum* (= "spruce forest with low herbs") and *aconitetosum* (= "spruce forest with tall herbs"). Most *P. abies* forests have a trivial vascular plant flora, except *Melico-Piceetum pinetosum,* which houses, among others, rare and in some areas threatened orchids, *e.g. Ophrys insectifera* and *Cypripedium calceolus*.

- Kielland-Lund (1981, 1994) has described the association *Chamaemoro-Piceetum* on clay or thin organic soils. This community is called "spruce swamp forest".

Norway spruce forest communities in the superhumid parts of central Norway differ from those described (Kielland-Lund 1981, 1994) in having frequent oceanic species: in the field layer, *e.g. Blechnum spicant* and *Cornus suecica*; and in the bottom layer, the sub-oceanic bryophytes *Plagiothecium undulatum* and *Rhytidiadelphus loreus*.

The most common types of central European spruce forest are the montane and the sub-alpine. Spruce also occurs in wide areas in lowlands with mixed woodland communities. It can be dominant at both the sub-montane and planar levels where there is low competition, *e.g.* around the edges of raised bogs, in acid marshy ground and on waterlogged soils. Four montane and sub-alpine *Vaccinio-Piceetalia* associations are described:

- *Piceetum montanum* (PM). According to Ellenberg (1988), PM occurs in the montane zone of the valleys of the Alps. PM has two main sub-associations: *galietosum* and *melicetosum* (= *Melico-Piceetum)*. The slightly humid *galietosum* is found where the substrate is rich in bases. It has many herbs, predominantly *Galium rotundifolium*. The dry montane *melicetosum* is poorer in species. This type also occurs on bedrock, which is poor in bases, and even on dry slopes with relatively base-rich and loamy soils.

- *Veronico urticifoliae-Piceetum* (VP). VP is a special association of a more productive montane spruce wood type (Ellenberg 1988). It develops on acid soil where the water supply is somewhat better than that where *Piceetum montanum melicetosum* woods are found.

- *Piceetum subalpinum* (PS). In contrast to the montane spruce woods, the sub-alpine woods are as a rule poorer in species (Ellenberg 1988). These are more constant and may appear in large

numbers, *e.g. Oxalis acetosella, Vaccinium myrtillus, V. vitis-idaea, Calamagrostis villosa* and *Hylocomium splendens.* The spruce treees are mostly stunted because of severe winters. The type is common in central European highlands.

- *Sphagno-Piceetum* (SP) or *Piceetum subalpinum sphagnetosum*. This type, which has plants associated with wet conditions such as the genus *Sphagnum*, is found on waterlogged soils in all acidophilous spruce woods, especially in the sub-alpine region. It is frequent in high precipitation areas along the perimeter of the Alps.

The symbiotic relationship between the roots of Norway spruce and mycorrhiza fungi is important for spruce forest ecosystems. The importance specifically concerns Norway spruce in dry habitats, in habitats where soil moisture is variable, or generally in habitats with marginal growing conditions. In optimal growing conditions for Norway spruce, mycorrhizae are not so well developed. In dry habitats, mycorrhizae facilitate water uptake. Hundreds of species of mycorrhizae are described on Norway spruce.

B. Norway spruce as a key species

Norway spruce's importance for a very large number of species is due to its ability to change the soil, and to create essential structures owing to the size of individuals and their distribution in large continuous forests, as well as its dominance in creating dynamics in the landscape. In addition, Norway spruce as growing tree and decaying wood provides "home and food" for hundreds of species. In Fennoscandia and northern Russia, it dominates the forest landscape as the region's key species. In Norway, an estimated 20 000 species (~ half the number of species in the country) are associated with forests, a major part to spruce forests. Almost half the threatened species in Norway, Sweden and Finland (898, 695 and 717, respectively) live in forests (data from Nord 1994).

C. Special lichens

The spruce forest (called "boreal rain forest" or "coastal rain forest") of the west central part of Norway is very rich in mosses, fungi and lichens. The latter group includes 40-50 species belonging to the so-called "Trøndelag element". The lichen species either have their only known occurrence in Europe in this forest type, or have their main occurrence here (Holien 1996). A good indicator group for the occurrence of rare epiphytic lichens in the coastal rain forest is the *Lobarion* community, for which *Lobaria pulmonaria* is a characteristic species. In the alliance *Lobarion,* the epiphytes *Pseudocyphellaria crocata* and *Ramalina thrausta* are considered among the vulnerable Red List species of the coastal rain forest, whereas *Pannaria ahlneri* and "trønderlav" *(Erioderma pedicellatum)* are highly endangered. "Trønderlav" was classified as extinct in Europe until the summer of 1994, when it was found in two extremely small populations.

D. Special bryophytes

Norway spruce forests have a wide range of microclimatic and edaphic niches for bryophytes. Logs of Norway spruce at various stages of decay are habitats for many more or less specialised and very often rare communities and species of liverwort. Liverwort's occurrence on Norway spruce logs depends mainly on two factors: (i) the stage of decay (or length of time since the tree was felled) and (ii) the size of the log. Different species have different preferences. Larger logs have more species and larger populations. Typical Red List species living on decaying logs are *Lophozia ascendens* and *Calypogeia suecica.* The former is a vulnerable species with a preference for large young logs, while the latter prefers large old (heavily decayed) ones. Both occur mainly in the superhumid spruce forest of central Norway.

E. Interaction between planted Norway spruce forests and other forest types

The general consequences of artificial introduction of *P. abies* into deciduous forests are well known, especially from western Norway and central Europe (Ellenberg 1988, Fylkesmannen i Rogaland 1993). The high shade tolerance of *P. abies* gives it a competitive advantage over nearly all deciduous species, and over ground flora if the temperature regimes are favourable for Norway spruce. The microclimate becomes more humid and oceanic after introduction of *P. abies*. In addition, the humus becomes rawer and more acid. Changes in microclimatic and edaphic conditions result in sparse ground flora and fauna (Børset 1985). Under natural conditions there is a balanced dynamic in the competition between *P. abies* and trees and communities. However, when spruce stands are introduced outside their natural range of occurrence, the species can show unpredictable invasiveness.

F. Elements of boreal spruce forest ecosystems

Due to Norway spruce's dominant role in Fennoscandia and northern Russia, there should be a strong focus on the significance of the structure and dynamics of boreal spruce forests (Hansson 1992), especially in these areas.

G. Tree species

Different tree species are normally dominant at different succession stages. There are exceptions, however, in a few vegetation types in the boreal zone where the same tree species is both the pioneer and climax species. Spruce forests go through a deciduous stage (mainly birch, rowen, aspen, *Salix* sp. and alder in Fennoscandia) as the first step before the spruce becomes dominant. The biodiversity of the spruce forest depends on its succession stage (reviewed in Tømmerås 1994).

H. Deciduous trees in conifer forests

Deciduous trees lose their dominance in spruce forests after the pioneer period, but some of these trees are always part of the forest stand. The richer the vegetation type, the more deciduous trees are present. Old deciduous trees and large dead ones are very important for numerous lichens, insects and birds.

I. Period of rotation

Plant and animal species adapt to many niches, in a complicated pattern, during the forest's successive stages. Species that require very specific conditions may be dependant on the presence of burned trees, dead wood, small seedlings, or old but living trees. The effects of disturbances in the rotation cycle on species diversity are not well known (Hansson 1992).

J. Layers

From an ecological point of view, the existence of more than one vertical layer in forests is a key factor in determining an area's biodiversity. This layer structure is most dominant late in the pioneer and later stages.

K. Old trees

Spruce in natural forests may reach an age of 200-300 years. Usually some individuals become old and are of great size. These trees are habitats for many forms of life, such as woodpeckers, lichens, bryophytes and insects.

L. Dead trees

Dead wood results from various causes, including storm felling, fire, pathogens and normal ageing. This leads to a wide spectrum of types of dead wood being found in a spruce forest. Over a thousand plant and animal species take part in the process of decomposing dead spruce; a large proportion are specialists at a particular stage. It is estimated that more than 700 Norwegian beetle species are dependent on the dead wood of boreal forest tree species (Tømmerås, unpubl).

M. Continuity

Some areas in boreal forests are free from natural disturbances such as fire and storm felling. These forest areas can cover 20-40% of a forest landscape, and are often widespread on humid soil and in steep valley areas. Many cryptogams and invertebrates are dependent on continuous forest structure.

N. Importance of structural conditions and dynamic processes for plants and animals

The natural dynamics of spruce forests, in which a special mosaic landscape changes over time, leave some parts very little affected by disturbances (continuous forest) and other parts dominated by, for example, fires once every 100 years. Plant and animal communities adapt to these conditions. Many species are dependent on the stable structural conditions of a continuous forest, while others need disturbances such as fire (*e.g.* the threatened ortolan bunting *Emberiza hortulane* L., many insects and fungi) or storm felling. Finally, many species are dependent on the mosaic combination at the landscape level. Among these are the three species of forest hens: black grouse (*Tetrao tetrix* L.), willow grouse (*Lagopus lagopus* L.) and hazel grouse (*Bonasa bonasia* L.).

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SECTION 4
POPLAR (*POPULUS* L.)

1. Forestry Practices

It has been estimated that more than 90% of poplar cultivation throughout the world is concentrated on species and hybrids in section *Aigeiros* (Thielges 1985). This is due to the ease of intrasectional hybridisation and intersectional crosses with species in the *Tacamahaca* section, the broad adaptability of these species and hybrids in the temperate and sub-tropical zones, and the ease of vegetative propagation. Section *Turanga* has been increasing in importance, and the scale of some planting programs is enormous: the Three North Shelterbelt System is a 35.6 million hectare shelterbelt project across the desert border in northern China, where poplars make up 60% of the effort, much of it *P. euphratica* and hybrids *Populus simonii* × *P. nigra* (Weisberger *et al*. 1995, Wang 1996).

A. Deployment of reforestation materials

Seedling propagation of reforestation stock is most commonly used for difficult-to-root species in section *Populus*, although there are no significant reforestation efforts with species in this section in Canada (PCC 1996b). When practised, primarily for growing out controlled crosses, seed propagation is easily carried out using standard greenhouse propagation techniques (Burr 1986, Stanton and Villar 1996).

For the most part, stem cuttings from species in the *Tacamahaca* and *Aigeiros* sections root easily, while rooting is usually poor for those in sections *Populus*, *Leucoides* and *Turanga* (Zsuffa 1975). The rooting ability of *P. deltoides* varies, while that of *P. nigra* and *P. balsamifera* is very high. These latter species transmit their better rooting ability to hybrids with *P. deltoides* (Zsuffa *et al.* 1993). Vegetative propagation techniques for poplars in nursery culture can be divided into two groupings: autovegetative propagation, including cuttings, layering, etc., and heterovegetative propagation, including grafting, budding, etc. (Fröhlich and van der Meiden 1979).

For easy-to-root varieties, stem cuttings are normally taken from ripened one-year-old shoots during the dormant season, usually from plants in nursery stool beds, but sometimes from epicormic branches of older trees. Such varieties are often planted directly as unrooted cuttings or as pre-rooted stock on well-prepared planting sites. For harder-to-root varieties, more intensive rooting procedures must normally be used, often using greenwood material, rooting hormones and mist chamber techniques. Poplars from section *Populus*, that are notoriously difficult to propagate from stem cuttings, are more commonly propagated from root suckers, root cuttings and layers (Benson and Schwalbach 1970, Zsuffa 1971, Dirr and Heuser 1987, Hall *et al*. 1989).

Grafting and budding of difficult-to-root varieties is possible, and some species combinations demonstrate that intersectional compatibility is present, for example *P. tremula* on *P. trichocarpa* (Dirr and Heuser 1987), and *P. alba* or *P.* × *canescens* on *P. lasiocarpa* (Fröhlich and van der Meiden 1979) is possible: the most extensive application of grafting as a propagation technique has likely been in China where *P.* × *tomentosa* (*P. alba* × *P. adenopoda*), a variety that roots poorly from stem cuttings, is grafted onto *P. simonii* or one of its hybrids. If the grafted plant is planted with the union below the soil surface, it is felt that the scion forms its own roots over time (Zsuffa *et al*. 1996).

Poplars are also amenable to propagation by tissue culture and various protocols and explant materials have been used. The difficult-to-root species from section *Populus* have been propagated *in vitro* by enhanced axillary branching of shoots. Other cultivars of *P.* × *canadensis* (*P. deltoides* × *P. nigra*) and *P. yunnanensis* are propagated *in vitro* using dormant buds as the explant source (Dirr and Heuser 1987). In vitro techniques offer the most likely means for vegetative propagation of species and hybrids in section *Populus* (Fröhlich and Weisgerber 1985, Ahuja 1987). The use of somatic embryogenesis has proved successful with *P. alba* × *P. grandidentata* (Michler and Bauer 1991).

Embryo culture (also referred to as embryo rescue) techniques have been developed to improve the recovery rates of hybrid genotypes where crosses often exhibit high frequencies of aborted or immature embryos (Stanton and Villar 1996). While it has been possible to culture individual embryos or whole ovules removed several weeks after pollination (Kouider *et al*. 1984, Savka *et al*. 1987), subsequent developments have allowed the culture of half capsules or individual carpels, followed by subculture of germinated embryos (Raquin *et al*. 1993).

Poplars are deployed not only in production plantations, but are also important species for protection planting, especially as windbreaks on the plains for North America, and for other landscape use. Plantations may be deployed as monoclonal stands, mosaics of monoclonal blocks or clonal rows, and as intimate single-tree mixtures of various genotypes (Zsuffa 1993).

While Canada presently has no regulations governing the control and certification of planting stock, a certification service has been designed and introduced by the Poplar Council of Canada. The service provides certification of: (1) varietal (clonal) identity; (2) quality and type; and (3) sanitary condition. In addition the service maintains a Canadian register of clones and varieties *not* recommended for planting (Zsuffa 1993, PCC 1996a). In Europe, the 130 cultivars registered for commercial use in any country of the European Union (EU) can be marketed within the EU since 1966, and their circulation became free in 1993 with the opening of the single market (Pinon and Valadon, 1997). In Germany, *Populus* reproductive materials are regulated by the Gesetz über forstliches Saat-und Pflanzgut (FSaatG).

B. Provenance transfer

Although large geographic variation has been observed among provenances in morphology, growth and wood properties, the results to date indicate that possibilities for gain "vary from encouraging to confusing to bleak" (Farmer 1996). Limited studies in various *Populus* species do suggest that productivity gains might be achieved through provenance transfer (*e.g.*, Nelson and Tauer 1987), but the focus on hybrid breeding and clonal selection has precluded serious consideration of provenance transfer as an improvement strategy.

C. Breeding programs

Poplar breeding has been characterised by two main features: interspecific hybridisation and clonal selection (Bisoffi and Gullberg 1996). The spontaneous occurrence of natural hybrids among sympatric and introduced species was recognised early this century and suggested a logical starting point for breeding programs. The more often assumed rather than proven superiority of F1 hybrids has been based on performance of selected clones usually attributed to heterosis, although recent studies have confirmed the existence of hybrid vigor (Stettler *et al*. 1988, Bradshaw and Stettler 1995). The incorporation of clonal selection as a characteristic of poplar breeding programs is largely due to the relative ease of vegetative propagation, compared with sexual reproduction (Mohrdiek 1983, Thielges 1985).

Interest in the breeding of polyploids followed the discovery of fast-growing triploid aspens in the 1930s (Einspahr *et al*. 1963, Einspahr and Winton 1976) but largely declined in the 1970s, with the

exception of some molecular genetics studies (Bradshaw and Stettler 1993). Renewed interest in aspen breeding in western Canada and the Lake States has focused on hybrid vigour of crosses between *P. tremuloides* and *P. tremula*, followed by clonal selection and deployment (Li and Wyckoff 1991, Li *et al*. 1993, Li 1995).

Important long-term breeding programmes have a long history in Europe (Italy, France, Belgium, Netherlands). More recently, co-operation between universities and pulp industries has lead to a very intensive effort in the Pacific Northwest region of North America concentrating on *P. trichocarpa* × *P. deltoides* hybrids, and to a lesser extent those of *P. trichocarpa* × *P. maximowiczii*, *P. trichocarpa* × *P. nigra*, and *P. deltoides* × *P. nigra* (Stettler *et al*. 1996b, Zsuffa *et al*. 1996). In Bavaria in Germany cross breeding between *P. maximowiczii* × *P. trichocarpa* and *P. maximowiczii* × *P. nigra* has also been conducted. Intensively managed, short-rotation (5-8 years) plantations are geared toward pulp production, and over 30,000 ha are under cultivation as part of this programme between southern Oregon and British Columbia (Zsuffa *et al*. 1996).

Since the modern techniques of molecular biology are now being successfully applied to *Populus* it can be anticipated that novel traits, such as herbicide resistance, insect resistance and modified wood characteristics, will be introduced into these species.

D. Conservation of genetic resources

The history of poplar breeding and intensive culture spans a 70-year period. The International Poplar Commission (IPC) was established in 1947 to assist in the direction and co-ordination of this effort and to promote conservation and exchange of germplasm in 35 member nations (IPC 1996). In 1992, the IPC formally requested its member countries "to adopt appropriate measures to ensure that existing genetic resources of poplar and willow species, in natural and man-made stands, be properly preserved, stressing the role that fast-growing species may play in reducing pressure on delicate and endangered natural environments world-wide" (IPC 1992).

Some species of *Populus* are near the point of disappearing in some parts of their natural range, for example, *P. nigra* in Western Europe, while other species are still in full evolution, for example, *P. deltoides*. IPC nations have thus been encouraged to develop strategies for *in situ* conservation. Such efforts started in North China with the *ex-situ* conservation of *Populus simonii* due to three FAO co-ordinated poplar projects (Weisgerber *et al*. 1995), and in Europe with the conservation of *P. nigra* by the EUFORGEN network (Turok *et al*. 1997, Cagelli and Lefèvre, 1996). For broadly distributed species like *P. deltoides*, the recommended focus of *in situ* conservation is on small, isolated populations at the limits of the species range, as a source of adaptive variation. Once widely distributed, much of the original genetic resource for *P. nigra* was lost when natural regeneration was excluded by human activity (Steenackers 1996). The disappearance of *P. nigra* from middle Europe may also have resulted from the backcrossing of hybrids, especially of *Populus* × *canadensis* and *P. nigra*.

Other poplar species have a restricted natural range and require special protection. *P. suaveolens* (syn. *P. maximowiczii*) is well protected in natural reserves at different altitudes in the mountains of Hokkaido and can freely regenerate from seed in these areas. *P. heterophylla* is another species with restricted distribution, growing on soils that are too wet for *P. deltoides*; a special strategy is required to protect this species *in situ* (Steenackers 1996).

Despite the long history of domestication, *ex situ* conservation efforts have been limited and rarely involve seed materials. A notable exception is the *P. trichocarpa* seed bank created by the Netherlands in the 1970s. Promotion of *ex situ* conservation efforts has been recommended as an imperative to the IPC, as

has the need for guidelines for the global management and conservation of poplar genetic resources (Steenackers 1996).

2. Taxonomy and Natural Distribution

Poplar species (*peuplier* in French) are members of the genus *Populus* L., in the family Salicaceae (willow family) and the order Salicales. The genus is traditionally subdivided taxonomically into sections. Five of these sections are widely recognised: *Turanga*, *Leucoides*, *Aigeiros*, *Tacamahaca*, and *Populus* (known synonymously as *Leuce*) (Zsuffa 1975). Periodically, taxonomists have been inclined to add a sixth single-species section to resolve classification problems. For example, Browicz (1966) proposed section *Tsavo*, to include the east African species *P. ilicifolia*, a species not even recognised by some taxonomists and included by others in section *Turanga.* Section *Ciliata* has been proposed to include the Himalayan species *P. ciliata* Wall. Ex Royle, formerly included in *Leucoides* (Khosla and Khurana 1982), an apparent mistake that others have suggested be resolved by classifying the species under *Tacamahaca*. Still another section, *Abaso*, has been proposed to accommodate *P. mexicana* that seems weakly related to other species of section *Aigeiros*, in which it has previously been placed (Eckenwalder 1996). The dispute over the sectional classification of poplars will no doubt continue; meanwhile, it is generally accepted that three of the sections are represented in Canada: *Populus*, *Aigeiros*, and *Tacamahaca* (Krüssmann 1985, Farrar 1995).

Disagreements over the species classification of poplars show no sign of abatement. The wide distribution of many poplar species, frequent introgressive hybridisation, a long history of cultivation and ease of vegetative propagation has led to much confusion in the nomenclature of poplars. Numerous synonyms exist, and hybrids and cultivated varieties have often been named as species (Zsuffa 1975). Thus, species counts for the genus range from the low 20's to over 80, depending on the authority. The classification suggested by Eckenwalder (1996), which enjoys the transitory advantage of being the most recently published, recognises 29. This classification is presented, with synonyms recognised by Zsuffa (1975), in Table 3.5. Polymorphisms of DNA add new data to this "conventional" classification (Cervera *et al*, 1997).

Section Turanga *Bge*

The three species in this section are native to northeast Africa and Asia. The most important is *P. euphratica* which, although not commonly cultivated in the past, can tolerate poor soils, extreme heat and soil salinity, and is now a key species for anti-desertification purposes in the large Three North Shelterbelt project in northern China (Wang 1996).

Section Leucoides *Spach – Large-leaved Poplars*

While no members of this section are native to Canada, swamp cottonwood (*P. heterophylla*) is a secondary species on wet sites in the central and eastern United States. Other examples of this section, *P. lasiocarpa* and *P. glauca*, are native to temperate regions of China.

Section Tacamahaca *Spach – Balsam Poplars*

North American members of this section, found in both Canada and the United States, are balsam poplar – *peuplier baumier* (*P. balsamifera*), black cottonwood – *peuplier de l'Ouest* (*P. trichocarpa*), and narrowleaf cottonwood – *peuplier à feuilles étroites* (*P. angustifolia*). This group includes the commonly planted Simon poplar – *peuplier de Simon* (*P. simonii*) from eastern Asia. Other important members from Asia include *P. laurifolia*, and *P. suaveolens.*

Table 3.5 Suggested classification, nomenclature and occurrence of *Populus* species (Eckenwalder, 1996) and synonyms given by an earlier classification (Zsuffa, 1975) in square brackets

Section	Scientific name & synonyms	Common names	Occurrence
Abaso Ecken.			
	P. mexicana Wesmael		Mexico
Turanga Bge.			
	P. euphratica Oliv.	**Euphrates poplar**, bahan	Spain, NE Africa, Asia
	P. ilicifolia (Engler) Rouleau		E. Africa
	P. pruinosa Schrenk		E. Eurasia
Leucoides Spach		**large-leaved poplars**	
	P. lasiocarpa Oliv.	**Chinese necklace poplar**	China
	P. glauca Haines [*P. wilsonii* Schneid.]		China
	P. heterophylla L.	**swamp cottonwood**, swamp poplar,	USA
Tacamahaca Spach		**balsam poplars**	
	P. angustifolia James	**narrowleaf cottonwood**, narrowleaf balsam poplar	southern Sask. And Alberta to southwestern US
	P. balsamifera L.	**balsam poplar**	North America
	P. ciliata Royle		Himalayas
	P. laurifolia Ledeb.	**laurel poplar**	eastern Asia
	P. simonii Carr.	**Simon poplar**	eastern Asia
	P. suaveolens Fish. [*P. cathayana* Rehd. *P. koreana* Rehd *P. maximowiczii* A.Henry]	**doronoki**, Japanese poplar	NE China, Japan
	P. szechuanica Schneid.		E. Eurasia
	P. trichocarpa Torr. & A.Gray	**black cottonwood**, western balsam poplar	western Canada and US
	P. yunnanensis Dode		E. Eurasia
Aigeiros Duby		**Cottonwoods and Black Poplars**	
	P. deltoides Marsh. [*P. sargentii* Dode, *P. wislizenii* Sarg.]	**eastern cottonwood** (ssp. *deltoides*), **plains cottonwood** (ssp. *monilifera*), **Rio Grande cottonwood** (ssp. *wislizenii*	Quebec, Ontario Prairie Provinces to Texas SW USA
	P. fremontii S.Wats.	**Fremont cottonwood**	SW USA
	P. nigra L.	**black poplar**, European black poplar	Europe, western Asia
Populus L. [***Leuce*** Duby]		**aspens**	
	P. adenopoda Maxim.		
	P. alba L.	**white poplar,** silver poplar	central and southern Europe to N. Africa, central Asia
	P. gamblei Haines		E. Eurasia
	P. grandidentata Michx.	**largetooth aspen**, bigtooth aspen, aspen, poplar, popple	eastern North America
	P. guzmanantlensis Vasq. & Cue.		Mexico
	P. monticola Brand		Mexico
	P. sieboldii Miq.	**Siebold aspen**, Japanese aspen	Japan
	P. simaroa Rzed.		Mexico
	P. tremula L. [*P. davidiana* (Dode) Schneid.]	**European aspen**, tremble, Zitterpappel	Europe, northern Africa, north-eastern Asia
	P. tremuloides Michx.	**trembling aspen**, quaking aspen	North America

Section **Aigeiros** *Duby – Cottonwoods and Black Poplars.*

This section includes the "true" cottonwoods (a term also associated with *Tacamahaca*). In North America the section is represented by eastern cottonwood – *peuplier deltoïde* (*P. deltoides* ssp. *deltoides*), and plains cottonwood – *peuplier deltoïde de l'Ouest* (*P. deltoides* ssp. *monilifera*), found both in Canada and the United States, and by Fremont cottonwood (*P. fremontii*) and Rio Grande cottonwood (*P. deltoides* ssp *wislizeni*) as secondary species in the southwestern United

States. The black poplar – *peuplier noir* (*P. nigra*) is an important species native to North Africa, central and western Europe, and the cultivar known as Lombardy poplar – *peuplier noir d'Italie* (*P. nigra* cv. 'Italica') is commonly planted as a hardy ornamental in North America.

Section Populus L. (syn. Leuce Duby) – Aspens.

This section is further subdivided into two subsections, ***Albidae*** and ***Trepidae***, containing the white poplars and aspens, respectively. The North American representatives of this section are both members of *Trepidae*: trembling aspen – *peuplier faux-tremble* (*P. tremuloides*), and largetooth aspen – *peuplier à grandes dents* (*P. grandidentata*). *P. tremula* is an important and highly variable aspen from Europe, while *P. sieboldii* is a recognised species from Japan. However, aspens throughout Eurasia are now thought to be races of a single, highly polymorphic species, viz. *P. tremula* (Barnes and Han 1993). While no white poplars are native to North America, the European white poplar – *peuplier blanc* (*P. alba*) was among the first species introduced from Europe.

Natural hybridisation has been reported between almost all sympatric poplar species, and between introduced and native poplars, both in North America and Europe (Schreiner 1974, Demeritt 1990). Natural hybridisation generally occurs between species in the same section to the limited extent that the parent species overlap (Brayshaw 1965, Eckenwalder 1977), although intersectional hybrids also occur. Species in different sections, though broadly sympatric, are ecologically isolated from one another, so that hybridisation occurs over large geographic areas but within a relatively narrow ecological range of overlap (Eckenwalder 1984a, c). Complicated natural hybrid populations may also form where three or more species are sympatric (Rood *et al*. 1986).

Eastern cottonwood was introduced into France from southeastern Canada in the late 1700's. In southern Germany, since the early 1970's, cross-breeding of *P.* × *canadensis* has been replaced by *P. trichocarpa* × *P. deltoides* or *P. trichocarpa* × *P.* Subsequent natural hybridisation with the native black poplar produced the hybrid which was named *P.* × *canadensis* in 1789 (Mühle Larsen 1960, Wright 1976). Clones of this hybrid are now widely planted across Europe. This hybrid was also the first poplar hybrid produced by controlled pollination, by Englishman A. Henry (Larsen 1956). Artificial hybridisation has been used in North America since the 1920s and 30s (Stout and Schreiner 1933, Heimburger 1936). Several of the more important hybrids occurring naturally in North America are listed, together with synonyms and common names, in Table 3.6.

3. Centres of Origin/Diversity

A. Natural distribution

The genus *Populus* is widely distributed throughout the Northern Hemisphere, in both the temperate and subtropical zones. Representative species are found from Alaska and Labrador south to northern Mexico, as well as Europe, North Africa, the Himalayas, mainland China and Japan (Schreiner 1974). Some species are very widely distributed. *P. tremuloides*, for example, is the most broadly distributed tree species in North America, spanning 110° of longitude and 47° of latitude, and the second most widely distributed in the world (Jones 1985, Barnes and Han 1993).

B. Evolution and migrational history

It was long felt that *Populus* was one of the oldest contemporary angiosperm genus, originating in China and Japan during the Triassic; however, these fossil records are now associated with other taxa. While the closest relatives in Flacourtiaceae are from tropical Asia, the fossil record now indicates that the genus *Populus* had tropical origins in North America during the late Paleocene, about 58 million years ago

(Collinson 1992). These early leaf fossils are very similar to the present day *P. mexicana* in section *Abaso* (Eckenwalder 1996). In the late Eocene, the first Eurasian relatives from other sections appeared, with those of *Turanga* confined to the Old World, and an ancestor of section *Leucoides* invaded temperate habitats. During the Oligocene, precursors of *Tacamahaca* and *Aigeiros* appeared that would not become distinct sections until the Miocene, at which time members of section *Populus* also appeared (Collinson 1992, Eckenwalder 1996).

Table 3.6 Nomenclature of naturally occurring *Populus Hybrids*

Parentage	Hybrid designation	Common name
P. alba × *P. grandidentata*	*P.* × *roulwauiana* Boivin	
P. alba × *P. adenopoda*	*P.* × *tomentosa* Carr..	Chinese white poplar
P. alba × *P. tremula*	*P.* × *canescens* (Ait.) Sm.	grey poplar
P. alba × *P. tremuloides*	*P.* × *heimburgeri* Boivin	
P. angustifolia × *P. deltoides*	*P.* × *acuminata* Rydb. [syn. *P.* × *andrewsii* Sarg.]	Lanceleaf cottonwood, *peuplier à feuilles acuminées*
P. angustifolia × *P. balsamifera*	*P.* × *brayshawii* Boivin	Brayshaw's poplar, *peuplier hybride de Brayshaw*
P. angustifolia × *P. tremuloides*	*P.* × *sennii* Boivin	
P. balsamifera × *P. deltoides*	*P.* × *jackii* Sarg.	Jack's poplar, *peuplier hybride de Jack*
P. balsamifera × *P. tremuloides*	*P.* × *dutillyi* Lepage	
P. deltoides × *P. nigra*	*P.* × *canadensis* Moench cv. Eugenei [syn. *P.* × *euramericana* (Dode) Guinier]	Carolina poplar, *peuplier de Caroline* [syn. Canada poplar, Euramerican poplars]
P. deltoides × *P. tremuloides*	*P.* × *bernardii* Boivin	Bernard poplars
P. deltoides × *P. trichocarpa*)	*P.* × *generosa* Henry [syn. *P.* × *interamericana* Brockh.]	Interamerican poplars
P. fremontii × *P. trichocarpa*	*P.* × *parryi* Sarg.	Parry cottonwood
P. grandidentata × *P. tremuloides*	*P.* × *smithii* Boivin	
P. laurifolia × *P. nigra*	*P.* × *berolinensis* Dippel [syn. *P.* × *rasumowskyana* Schr. and *P.* × *petrowskyana* Schr.]	Berlin poplars, Russian poplars
P. deltoides × *P. balsamifera* × *P. angustifolia* (natural trihybrid)	Unnamed	Unnamed

The evolution of the advanced sections of *Populus* has been characterised by rapid speciation during allopatric cycles, but influenced by widespread introgression, both within and between sections (Eckenwalder 1984b, 1996, Smith and Symata 1990, Kaul 1995). This rapid sequence of events, much conflicting evidence, and the confusion that has characterised the identification of species, has made it difficult to track the recent evolutionary history of poplars in the more advanced sections (Eckenwalder 1996). While there is evidence of evolutionary divergence among the sections, the sections themselves are very widely distributed. Species within the sections are highly related and many are among the most broadly distributed of any tree species.

It is clear that migration of genes to other section members can occur easily throughout a very large portion of the North Temperate zone. Poplars are pioneering species and migrate quickly. Pollen studies have demonstrated that *Populus* species frequently dominate the first forest communities following glaciation (Cwynar 1988, Keenan and Cwynar 1992). In Europe, *P. tremula* is the early pioneering species. *P. nigra* occurs along rivers and in pastures, together with *Salix alba*. Large stands of *P. tremuloides* in North America are thought to have originated soon after retreat of the Pleistocene ice sheet and have been since maintained asexually from root suckers, making them some of the largest and oldest organisms in the world (Barnes 1975, Kemperman and Barnes 1976, Mitton and Grant 1980, Cheliak and Dancik 1982).

4. Reproductive Biology

A. Reproductive development

Poplars are normally dioecious and obligatory outcrossers; however, the occurrence of monoecious inflorescences and perfect flowers has been reported (Lester 1963a, b, Melchior 1967). Reproductive buds in *P. tremuloides* may develop into pistillate, staminate or perfect flowers, initiated at different times (Lester 1963a). *P. lasiocarpa* is a notable exception as it is normally monoecious and self-fertilising (Schreiner 1974). Overall sex ratios of 1:1 have been confirmed for *P. tremuloides* (Einspahr and Winton 1976, Grant and Mitton 1979) and for *P. deltoides* (Farmer 1964b), although an elevational gradient in sex ratio has been observed in *P. tremuloides* in the Rocky Mountains, with females more common at low elevations, while more than 90% are male above 3 200 m (Grant and Mitton 1979).

Reproductive buds are simple (Jackson and Sweet 1972). Their initiation and early development have been described in *P. tremuloides* and *P. deltoides* (Nagaraj 1952, Seitz 1958, Lester 1963a). Floral initiation takes place in buds, located in the axils of leaves on the current-year shoot. These buds are no more than small apices, each with a single bud scale when winter dormancy occurs (Owens and Blake 1985, Kaul 1995). Terminal buds burst in May, and axillary primordia initiate several bud scales during rapid shoot elongation. Reproductive apices are determined around mid-June (Lester 1963a). Pistillate primordia are first to begin development of floral parts by late June, followed by staminate primordia in early July. In *P. deltoides*, staminate flower buds can be readily distinguished from vegetative buds by midsummer, while identification of pistillate flowers requires dissection (Farmer 1976). Floral development continues within buds through September, so that anthers and ovules are well developed before winter dormancy (Owens and Blake 1985, Kaul 1995). For at least some *Populus* species, a chilling requirement must be satisfied before development will resume (Farmer 1964a). Megaspore mother cells differentiate in the spring, and microsporogenesis immediately precedes anthesis (Farmer and Pitcher 1981).

Flowers are borne in catkins (aments) early in the spring, prior to flushing of vegetative buds. When fully developed, both male and female catkins are 10 to 15 cm in length. Female flowers have from two to four, cap- or y-shaped stigmas, while the males have 30 to 80 stamens (Demeritt 1990). Each catkin bears a few dozen, one-celled capsules, each containing 10 to 30 seeds.

Male flowers ripen and shed pollen a few days before females, ensuring that pollen is in the air when the first females are receptive (Farmer and Pitcher 1981). Such pollen-pistil interactions have been largely documented in *Populus nigra* (Villar *et al*. 1987a, Villar *et al*.1993). Variation in flowering date is due to differences among trees and in *P. deltoides* is highly heritable (Farmer 1976). This variation in flowering date extends the pollination period from 2 to 3 weeks. Another study of natural variation in *P. nigra* over 111 sites in France revealed a fair level of diversity and a low overall differentiation, with an important intraregional gene diversity (Legionnet *et al*. 1997).

Pollen germinates within the first few hours after pollination. Fertilisation takes place several days later and is normally complete within two weeks (Farmer and Pitcher 1981). Seed development proceeds rapidly and dispersal occurs in most species by midsummer, before the full growth of the leaves (Schreiner 1974). In North American, the period of seed maturity in sections *Populus* and *Tacamahaca* is determined by temperature sums and is quite uniform within the limits of ecotypic zones (Pauley 1950). On the other hand, seed dispersal in the *Aigeiros* poplars may continue throughout the summer and early fall (Farmer 1966).

B. Mating system and gene flow

Two factors contribute to high gene flow and genetic diversity in poplars. Firstly, most are dioecious, and thus obligatory outcrossers. And secondly, in addition to being wind-pollinated, the long white, silky hairs attached to the short stalks of the seeds promote wind dispersal over great distances (Schreiner 1974), resulting in high rates of migration.

Electrophoretic studies in *P. tremuloides* suggest that gene flow is high, leading to a lack of differentiation among populations for putative neutral allozyme loci. However, the role of non-random mating in these same studies was variable, with no deviations from Hardy-Weinberg equilibrium detected in populations sampled in Minnesota (Lund *et al*. 1992), while an excess of heterozygotes were found in Alberta populations (Cheliak and Dancik 1982), and a deficiency of heterozygotes observed in Ontario populations (Hyun *et al*. 1987).

C. Seed production

Most poplars begin flowering between age 10 and 15 years (Schreiner 1974), although flowering in *P. deltoides* may occur as early as age four (Farmer and Pitcher 1981). *Tacamahaca* and *Aigeiros* poplars produce large annual seed crops. Those in section *Populus* produce some seeds each year, but bumper crops occur at intervals of three to five years. Poplars are prolific seed producers. A typical 12 m *P. deltoides* specimen was estimated to produce almost 28 million seeds in one season, and estimates for *P. tremula* have ranged as high as 54 million seeds. Poplar seeds are very small. Species in section *Populus* can produce 6000 to 8000 seeds per gram, while North American *Leucoides* and *Aigeiros* produce from 300 to 450 seeds per gram (Schreiner 1974).

Typically, the longevity of poplar seeds under natural conditions is quite short – about two to four weeks. Under controlled low-temperature (-18 to 5° C) and stable moisture content (5 to 8%) conditions, storage time has been extended to 140 days for *P. balsamifera* (Hellum 1973), two years for *P. tremuloides* (Fechner *et al*. 1981), and five to six years for *Aigeiros* poplars (Tauer 1979, Muller and Tessier du Cros 1982).

D. Natural regeneration

Poplar seeds germinate or die within a few days after seedfall. Germination is epigeal. A fringe of hairs develops at the base of the hypocotyl, rendering the seedling upright and encouraging the root to grow down into the soil.

A favourable medium such as fine mineral soil is required for germination, together with light and continual moisture (McDonough 1979, Farrar 1995). Such conditions are rare, requiring fresh exposure of mineral soils, as found on shorelines, sandbars and old gravel pits. In North America, regeneration of section *Populus* from seed is confined to newly disturbed areas, whereas the primary mode of reproduction within stands is asexual (Barnes 1966, Schier 1973, Einspahr and Winton 1976).

E. Vegetative reproduction in nature

Except for members of section *Populus*, all poplars sprout vigorously from the stump and root collar. Coppicing occurs occasionally on young aspen (Zsuffa 1975). Reproduction from adventitious shoots on roots (root suckers) is common in many species, although less frequent in those in the *Aigeiros* and *Leucoides* sections.

Clonal groups of *P. tremuloides* in eastern North America are very common, but generally less than 0.1 ha in size, while in areas of Utah, groups as large as 80 ha have been observed (Kemperman and

Barnes 1976). In the semi-arid western United States, some argue that widespread seedling establishment has not occurred since the last glaciation, some 10,000 years ago (Einspahr and Winton 1976, McDonough 1985). Indeed, some biologists feel that western clones could be as old as 1 million years (Barnes 1966, 1975). It has been claimed that a single clone, nicknamed "Pando" (Latin for *I spread*), covers 43 hectares, contains more than 47,000 stems and weighs in excess of 6 million kg, making it the largest known organism (Grant *et al*. 1992, Mitton and Grant 1996)

Studies have also demonstrated that both natural and vegetative propagation occur in nature, for example with *P. nigra* (Legionnet *et al*. 1997).

5. Genetics

A. Cytology

Poplars are normally found in the diploid condition with $2n = 38$ chromosomes (Blackburn and Harrison 1924, Smith 1943). Polyploid individuals are rare and have only been reported in a half-dozen species (Darlington and Wylie 1956). While rare, the first discovery of a triploid forest tree was, in fact, a clone of *P. tremula* (Müntzing 1936). Several other natural triploid clones have since been found in both *P. tremula* and *P. tremuloides*, usually exhibiting larger leaves and exceptional growth (Einspahr *et al*. 1963, Heimburger 1968, Einspahr and Winton 1976).

Some reports suggest the sex determination in poplars is controlled by sex chromosomes (Peto 1938, Smith 1943, van Buijtenen and Einspahr 1959), however, this theory remains controversial. While published reports favour a genetic basis for gender, a linkage analysis of almost 2 500 PCR-based RAPD markers in a segregating family of F1 hybrid *P. trichocarpa* × *P. deltoides* failed to find any markers that were significantly associated with gender (McLetchie *et al*. 1994). The authors suggested that gender might be determined genetically by regions of the genome not sampled by the tested markers or by a complex of loci operating in an additive threshold manner or in an epistatic manner, or that gender is determined environmentally at an early zygote state.

B. Genetic variation

As already mentioned, the genus *Populus* is tremendously varied with species distributed throughout the Northern Hemisphere and the opportunities to generate novel genotypes through hybridisation are enormous. Breeding programs have not hesitated to exploit this genetic variability, although sound quantitative estimates of narrow-sense and broad-sense heritabilities, and covariances among selection criteria would undoubtedly have assisted in making breeding and selection strategies more efficient (Riemenschneider *et al*. 1996). Poplars are ideal species for quantitative genetics studies, as clonal replication can be readily accomplished to describe complex modes of gene action (Foster and Shaw 1988, Mullin and Park 1992, Bradshaw and Foster 1992). It is thus surprising that genetic variation has been studied in detail for only a few species and traits.

The true potential of poplar species can only be determined by genetic studies designed to resolve variation among and within stands. Unfortunately, the concentration of breeders on interspecific hybridisation has left this field largely ignored, and detailed studies of large natural populations are quite recent and only a few species are well documented (Mohrdiek 1983, Farmer 1991).

Population-level variability

Considerable clonal variation among populations may be expressed for growth traits and for *Melampsora* rust resistance, but for other characteristics there is often little geographic differentiation. Overall, data from molecular genetic studies suggest that gene flow through migration has been sufficient

to prevent genetic drift, inbreeding, and other processes that might give rise to geographic variation unrelated to adaptive selection (Farmer 1996).

Significant variation over a 10° latitudinal transect was observed for phenology (Farmer *et al*. 1988a), shoot/root allometric coefficients (Schnekenburger and Farmer 1989), and height growth among four provenances of *P. balsamifera*, with southern sources continuing to grow later in the season (Schnekenburger and Farmer 1989, Farmer 1993). These same populations exhibited very little geographic differentiation for isozyme characters (Farmer *et al*. 1988a), rooting ability (Farmer *et al*. 1989), and date of bud break (Farmer and Reinholt 1986). Another test series including a more restricted sampling of populations over a 3.5° range found significant population differences accounting for about 12% of the variation in two-year height, leaf morphology, sylleptic branching and pest resistance (Riemenschneider *et al*. 1992), with populations grouped into north-western, central, and south-eastern clusters (Riemenschneider and McMahon 1993).

Similarly, in *P. tremuloides*, isozyme and RAPD variation studies have shown little differentiation among populations (*e.g.*, Hyun *et al*. 1987, Lund *et al*. 1992, Yeh *et al*. 1995), whereas considerable variation among populations is well-documented for morphology, growth and wood properties (van Buijtenen *et al*. 1959, Barnes 1969, Einspahr and Winton 1976). Variation among populations generally follows clinal trends, with wood density declining with increasing elevation (Valentine 1962) and from south to north (Einspahr and Benson 1967). A common-garden trial showed that northern and western provenances flushed and ceased growth first, with lower survival when grown in Michigan (Brissette and Barnes 1984), and another showed that better growing clones came from lower Michigan (Reighard and Hanover 1985). There is some evidence of a north-south increase in susceptibility of *P. tremuloides* populations to *Hypoxylon mammatum* (French and Hart 1978). Populations of *P. tremuloides* also vary with respect to ozone sensitivity, with tolerance correlated to maximum daily ozone levels, as well as annual precipitation and minimum temperature (Berrang *et al*. 1991).

Geographic variation is also well documented for *P. deltoides*. A wide range provenance trial established in Nebraska, included sources from Texas in the south, to Minnesota in the north and Pennsylvania in the east, and evaluated bark, stem, crown and leaf morphology, in addition to growth and survival (Ying and Bagley 1976). Clinal patterns of variation from north and west to south and east were observed for most traits. Cuttings from Nebraska, Minnesota and Wisconsin produced significantly higher numbers of roots than those from other sources (Ying and Bagley 1977). A similar clinal trend was found in a study of 40 populations in the southern Great Plains, where NW to SE patterns were observed for two-year height, diameter, branching and *Melampsora* rust resistance (Nelson and Tauer 1987). A study of nine populations in Ontario showed great variation in leaf morphology which was unrelated to latitude or longitude, and was not correlated with the moderate allozyme variation that suggested differences between eastern and western populations (Rajora *et al*. 1991).

A series of studies of *P. trichocarpa* populations in Washington have documented significant variation for leaf, branch and phenology characters (Weber *et al*. 1985), photosynthetic processes (Dunlap *et al*. 1993), survival height growth and biomass production (Heilman and Stettler 1985), volume production, *Melampsora* rust resistance and adaptation to arid sites (Dunlap *et al*. 1994), leaf and crown morphology (Dunlap *et al*. 1995). A sample of 10 populations over a 4.5° latitudinal range showed only weak clinal trends for three-year height and diameter (Rogers *et al*. 1989). Another sample of five riparian populations in Washington showed little difference among populations with respect to flood tolerance of young seedlings and rooted cuttings (Smit 1988).

Individual-level variability

While the amount of genetic variation among populations differs greatly depending on the trait, the variation within populations is moderate to high for virtually all traits. Unfortunately, most genetic testing has focused on clonal materials without any particular family structure, and these studies have concentrated on the species and hybrids of sections *Tacamahaca* and *Aigeiros*. Generally, only estimates of broad-sense heritability (H^2) are available, and genetic structure is rarely partitioned into additive and non-additive components (Riemenschneider *et al*. 1996). In the limited number of studies of seedling populations, narrow-sense heritability (h^2) estimates for growth were similar to estimates of H^2 in *P. deltoides* (Farmer 1970, Ying and Bagley 1976, Nelson and Tauer 1987), and much lower in the case of *P. trichocarpa* (Rogers *et al*. 1989).

A large number of studies of growth and yield characters in *P. deltoides* have produced consistent estimates of H^2 between 0.20 and 0.50, with significant genotype-environment interactions, usually less than half as large as the corresponding genetic main effects (Wilcox and Farmer 1967, Farmer and Wilcox 1968, Mohn and Randall 1971, 1973, Randall and Cooper 1973, Foster 1986). A limited number of clonally replicated trials suggest that much of the genetic variance in yield is non-additive (Foster 1985, Foster and Shaw 1988). Heritability for stem growth in *P. balsamifera* was about $H^2 = 0.50$ (Farmer *et al*. 1988b). In both *P. deltoides* and *P. balsamifera*, C-effects (*in sensu* Lerner 1958) during the first year were often as large as that due to clones (Wilcox and Farmer 1968, Farmer *et al*. 1989), but appear to be less important after field planting (Farmer *et al*. 1988b). Detailed measurements of leaf, branch and phenological characteristics, which are known to affect tree productivity, have been employed to describe ideotypes (Dickmann and Keathley 1996) that may be useful in yield selection of *P. balsamifera* (Riemenschneider *et al*. 1992), *P. trichocarpa* (Riemenschneider *et al*. 1994), and hybrids involving *P. deltoides*, *P. nigra* and *P. simonii* (Wu 1994a, b). Indications of the utility of the ideotype concept for yield selection have been inconsistent.

Heritability of rooting and root characters is typically very high, with H^2 estimates as high as 0.85 to 0.91 in *P. deltoides* (Wilcox and Farmer 1968, Ying and Bagley 1977). Other recent studies also indicate high heritability for rooting in *P. trichocarpa* (Riemenschneider *et al*. 1996).

Much effort has been concentrated on the inheritance of *Melampsora* rust resistance, due to its impact on poplar culture. Early studies of rust resistance in *P. deltoides* gave estimates of h^2 between 0.38 and 0.66, and for H^2 between 0.66 and 0.88 (Jokela 1966). Similarly high estimates for rust resistance or severity have since been reported for *P. deltoides* (Farmer and Wilcox 1968, Thielges and Adams 1975), *P. tremula* and *P. tremuloides* (Gallo *et al*. 1985), *P. balsamifera* (Riemenschneider *et al*. 1992), *P. trichocarpa* and its hybrids (Hsiang *et al*. 1993, Riemenschneider *et al*. 1994), and hybrids among *P. deltoides*, *P. nigra*, and *P. maximowiczii* (Rajora *et al*. 1994).

Molecular genetics

In recent years a great deal of research has been directed towards associating important traits with molecular markers, and in developing corresponding genetic maps (Bradshaw *et al*. 1994, Cervera *et al*. 1997). The emphasis of this work has been directed towards adaptive traits (Bradshaw and Stettler, 1995) and to resistance to diseases (Villar *et al*. 1996), and suggests the role of a few quantitative trait loci (QTL's) that have large effects on these quantitative traits.

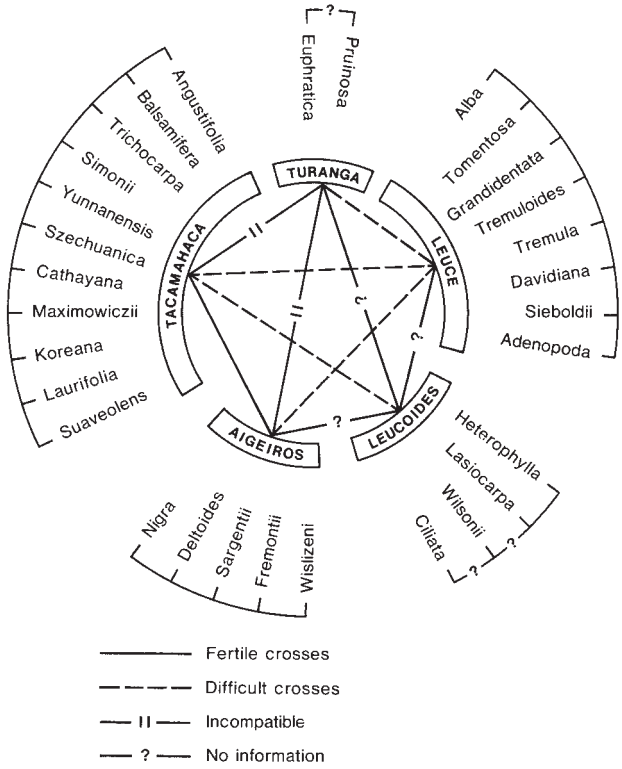
C. Inbreeding depression and genetic load

Given the high rate of gene flow in poplars, one would expect low rates of inbreeding. A study of 200 clones of *P. tremuloides* in Ontario indicated a deficiency of heterozygotes and an average fixation index

of 0.462 (Hyun *et al*. 1987). However, these results might well be due to a sampling phenomenon, Wahlund's effect, rather than inbreeding. In contrast to these observations, populations of *P. tremuloides* in Alberta, where clonal reproduction is more common, showed an excess of heterozygotes (Cheliak and Dancik 1982, Jelinski and Cheliak 1992). While rates of inbreeding in natural populations may be low, genetic load may be expressed as inbreeding depression in pedigrees of hybrid material, giving rise to distortion of expected Mendelian segregation ratios (Bradshaw and Stettler 1994).

6. Crosses

Figure 3.4 Crossability of *Populus* species



Source : Zsuffa, 1975

Extensive crossability studies have been carried out among species in the *Populus*, *Tacamahaca* and *Aigeiros* sections, while few data are available for those in *Turanga* and *Leucoides* (Zsuffa 1975). Interspecific breeding results are summarised in Figure 3.4.

Hybrids between members of the same section are produced easily and are often more vigorous than their parents. Dramatic examples are the hybrids made between *P. tremuloides* and *P. tremula* (Ilstedt and Gullberg 1993). Crossing success between sections is variable. Crosses between *Aigeiros* and *Tacamahaca* are easy, while those between sections *Populus* and *Aigeiros*, and sections *Populus* and *Tacamahaca* are notoriously difficult, resulting normally in dead seed or dwarfed seedlings (Zsuffa 1975). Crosses between sections are sometimes made more easily using interspecific hybrids, rather than pure species, as parents.

The incompatibility of some species is characterised by arrested development of the pollen tube and failure to penetrate the stigma (Melchior and Seitz 1968, Guries and Stettler 1976, Stettler *et al*. 1980, Knox 1984, Villar *et al*. 1987b, Rougier *et al*. 1992, Villar *et al*. 1993). This block can be overcome in some cases by mixing the incompatible pollen with "mentor pollen", *i.e.*, compatible pollen that has been killed by means of gamma radiation (Stettler and Ager 1984, Knox *et al*. 1987, Villar and Gaget-Faurobert 1996), and by treatment of pollen and stigmas with solvents and extracts from compatible pollen (Whitecross and Willing 1975, Willing and Pryor 1976). This technique has made it possible to obtain crosses of section *Populus* with *Aigeiros* and *Tacamahaca* poplars that are otherwise difficult (Stettler 1968, Zufa 1971, Knox *et al*. 1972, Willing and Pryor 1976).

7. Ecology and Associated Species

A. Habitat

Poplars are found in a great variety of forest ecosystems, from boreal to sub-tropical, and from mountainous to riparian. In some environments such as boreal forests and in large river valleys, they form large stands, while in other situations they are found as small stands or groups of trees. Although there is some variation among species, essentially all poplars are very intolerant of shade. They are pioneering species and among the first to invade and re-colonise areas disturbed by harvesting, land clearing and fire. The *Populus* species and their hybrids vary greatly in their adaptability to climate, although all are nutrient demanding and perform best with an abundant and continuous supply of moisture (Heilman *et al*. 1996).

The members of section *Populus* are very broadly distributed and thus found over a tremendous range of climates. In *P. tremuloides*, the southern limit is roughly defined by the 24° C mean July temperature isotherm, while the northern limit corresponds to a mean annual degree-day sum of 700° C, at threshold temperature of 5.6° C (Fowells 1965). Within this range the species occurs where annual precipitation exceeds evapotranspiration. While *P. tremuloides* is found on a variety of soils, ranging from shallow and rocky to deep loamy sands and heavy clays, growth is strongly influenced by drainage and fertility (Perala 1990). Throughout the more restricted range of *P. grandidentata*, moisture is adequate in all seasons, with the least precipitation occurring at the north-west limit on the prairie border in Manitoba with only 510 mm. It is far less adaptable than *P. tremuloides*, developing best on moist, fertile sandy uplands with good aeration (Laidly 1990).

The members of the *Tacamahaca* and *Aigeiros* sections are referred to collectively as the riparian cottonwoods. Species in the *Tacamahaca* section are generally found at higher elevations and latitudes (high river systems of montane and young piedmont valley floodplains) (Braatne *et al*. 1996). In this section, *P. balsamifera* is the most broadly distributed, withstanding climatic extremes similar to that of *P. tremuloides*. It is usually restricted to moist, low-lying ground, and is one of the few boreal species associated with poorly drained clay soils having pH greater than 7.2 (Dix and Swan 1971, Zasada and Phipps 1990). *P. trichocarpa* is most commonly found in the humid coastal forests of the Pacific Northwest, performing best on deep alluvial soils with abundant moisture, nutrients, oxygen and pH 6.0 to 7.0 (Smith 1957, DeBell 1990). *P. angustifolia* has a very limited range where it is a pioneering species on gravel and sand bars near fast-flowing rivers (Brayshaw 1965).

Members of section *Aigeiros* are limited to lower elevations and latitudes (lower river systems of mature piedmont valley flood plains) (Braatne *et al*. 1996). The natural range of *P. deltoides* covers a wide southern range, with frost-free days ranging from less than 100 to more than 200, and rainfall from less than 380 mm in the northwest to more than 1,400 mm in the south. This species performs best on moist, well-drained, fine sandy or silt loams, and on sites that are rarely higher than 6 m above the average level of nearby streams (Cooper and Van Haverbeke 1990).

The only North American member of section *Leucoides*, *P. heterophylla*, is found in warm, humid areas with abundant rainfall. It performs best on deep moist soils of shallow swamps and low-lying areas near tidewater, and occupies sites that are too wet for *P. deltoides* (Johnson 1990).

B. Synecology and associated species

Poplars occur in the early successional stages over a wide range of forest ecosystems, so it is no surprise that ecological associations are tremendously diverse. This is particularly true for species in section *Populus*, as they are not restricted to riparian habitats. In Europe, *P. tremula* is the early pioneer. *P. nigra* occurs along rivers and in pastures, together with *Salix alba, P. tremuloides* is found in pure stands across its range, but also in mixed stands where it is commonly associated with White Spruce (*Picea glauca*), black spruce (*Picea mariana*), balsam fir (*Abies balsamea*), white birch (*Betula papyrifera*), *P. balsamifera*, and jack pine (*Pinus banksiana*). The associations with shrub and herbaceous species are even more numerous and varied (Perala 1990, Farrar 1995). *P. grandidentata* occurs in small pure stands, but more commonly as an associate in poplar stands with either *P. tremuloides* or *P. balsamifera*. It is a minor component of many other forest types and is thus associated with a wide range of shrubs and ground flora (Laidly 1990, Farrar 1995).

In section *Tacamahaca, P. balsamifera* reaches its best development on river flood plains where it occurs as pure stands and is associated with various willows and alders (Viereck *et al*. 1983). However, it also occurs in mixtures with boreal conifers and several hardwood species, and the associated shrub and herbaceous mixtures are numerous (Zasada and Phipps 1990). *P. trichocarpa* generally occurs in mixtures with larger willows, and to a lesser extent with several western conifers (DeBell 1990). Shrub and herbaceous associates are numerous, but better sites are characterised by beaked hazel (*Corylus cornuta*), elder (*Sambucus* spp.), salmonberry (*Rubus spectabilis*), nettles (*Stachys* spp.), swordfern (*Polystichum munitum*) and lady fern (*Athyrium filix-femina*) (Smith 1957).

On riparian sites, *P. deltoides* tends to grow as essentially pure stands or mixed in open stands with other riparian species. In the area where the species performs best, roughleaf dogwood (*Cornus drummondii*) and swamp-privet (*Forestiera acuminata*) are major shrub associates (Cooper and Van Haverbeke 1990).

C. Competition and stand structure

As mentioned earlier, all poplars are shade intolerant, early successional species, and disturbance is often essential in maintaining many poplar ecosystems. The availability of sites suitable for colonisation, particularly following fires, plays a major role in determining seedling establishment (DeByle and Winokur 1985, Jelinski and Cheliak 1992, Kay 1993). Once established, fire can also remove shade-tolerant competitors, allowing vigorous *P. tremuloides* sprouts to emerge from persistent root systems (Bailey *et al*. 1990), with densities in excess of 1 million per hectare (Schier *et al*. 1985). In the absence of disturbance, aspens are regarded as transient, and successional patterns are determined by soil water regime (Roberts and Richardson 1985). Intolerant associates will often out-live aspens, while tolerant hardwoods and conifers will also dominate by virtue of their ability to regenerate under shade.

The riparian cottonwoods are all very flood-tolerant, so that establishment and growth are promoted by the disturbances characteristic of alluvial habitats. In non-alluvial habitats, they take the opportunity to establish on moist agricultural fields, forest clearings and the margins of wetlands, but are eventually dominated by secondary forest species (Braatne *et al*. 1996). Willows and alders may precede the establishment of *P. balsamifera*, which is normally then replaced by White Spruce (Walker and Chapin 1986, Walker *et al*. 1986). *P. deltoides* is a very poor competitor, as it is very intolerant of shade, and

competes well only with willows, owing to its faster growth except on very wet sites (Cooper and Van Haverbeke 1990).

When established as plantations, poplars and their hybrids must generally be established in pure stands. In addition to being highly intolerant of shade, young poplars cannot tolerate competition from grass, weeds or shrubs. Control of vegetation in the first few years is essential, although poplars are highly sensitive to many herbicides used for vegetation control (Demeritt 1990).

D. Ecosystem dynamics

Poplars coexist with a wide range of insects, but most pose a more serious threat only to artificial populations of planted species and their hybrids. In North America the most serious defoliator, particularly in hybrid plantings, is the cottonwood leaf beetle (*Chrysomela scripta*). Other foliage insects include the forest tent caterpillar (*Malacosoma disstria*), the poplar tent maker (*Ichthyura inclusa*), mourning cloak butterfly larvae (*Nymphalis antiopa*), the large aspen tortrix (*Choristoneura conflictana*), a leaf beetle (*Zeugophora scutellaris*), and the aspen blotch miner (*Phyllocnistis populiella*). The cottonwood twig borer (*Gypsonoma haimbachiana*) is particularly destructive, while several other borers may also do damage. Infestations of poplar gall midge (*Prodiplosis morrisi*) and many species aphids are also responsible for damage (Dickmann and Stuart 1983, Demeritt 1990). In China, in Ningzia Autonomous Region, it was reported that 24 million trees were destroyed due to attack by the longicorn, *Anoplophora glabripennis* (Chinese National Report, IPC 1996). Several mechanisms are thought to operate to give hybrids more or less resistance relative to their parental species, but as a rule hybrid populations are centres of insect abundance (Whitham *et al*. 1996).

Other insect pests include:

– Satin moth	*Stilpnoia salicis*
–	*Gluphisia septentrionis*
– Viceroy butterfly larvae	*Basilarchia archippus*
– Gypsy moth	*Lymantria dispar* [European and Asian varieties]
– Mourningcloak butterfly	*Nymphalis antiopa*
– Pandemis leafroller	*Pandemis pyrusana*
– Large aspen tortrix	*Christoneura conflictana*
– Forest tent caterpillar	*Malacosma dissitria*
– Poplar/willow borer	*Chryptorhyuchus lapthi*
– Clear-winger poplar borer	*Panthrene robinae*
– Poplar borer	*Saperda calcerata*
– Bronze poplar borer	*Agrilus grandulatus lirogus*
– Scented willow sawfly	*Nematus salicis odoratus*
– Phratora leaf Beetle	*Phratora californica*
– Flea beetle	*Altica* sp.
– Williamette balley	*Corythucha salicata*
– western willow lace bug	
– Cottonwood twig borer	*Gypsonoma haimbachiana*
– Aphids	

Further information about insect pest species of poplar can be found in Peterson, *et al*. 1996, Hiratsuka, 1987, Furniss and Carolin, 1977, Hepting 1971 and USDA Forest Service 1979. A useful Internet website is “http://ww.cas.psu.edu/docs/CASDEPT/PLANText/poplar.html”.

The fungi associated with *Populus* species are tremendously diverse. More than 250 species are known to be associated with the decay of *P. tremuloides* alone (Lindsey and Gilbertson 1978). Only fungi associated with sections *Populus*, *Aigeiros*, and *Tacamahaca* have been studied to any extent, and "virtually nothing is known" of those associated with other sections (Newcombe 1996).

The five most damaging or potentially damaging diseases of *Aigeiros* and *Tacamahaca* are:

- Melampsora leaf rust (*Melampsora* spp.) which, while causing only moderate levels of mortality in plantations, can cause volume growth reduction of up to 65% (Widin and Schipper 1981). The situation is now becoming serious in Europe as variability in *Melampsora larici-populina* has been reported and interspecific hybrid trees selected for complete resistance to this fungus are now affected by new races of this pathogen (Pinon 1992a,b, Pinon, 1995, Pinon and Frey 1997).

- *Marssonina anthracnose* or leaf spot (*Marssonina* spp.), affecting *Aigeiros* species and some of their intersectional hybrids in particular, and causing an estimated 16% loss from production plantations in Italy (Thielges 1985).

- Bacterial canker (*Xanthomonus populi* Ridé), causing serious damage to non-native *Aigeiros* and *Tacamahaca* species planted in Europe (Thielges 1985).

- Dothiciza canker (*Discosporium populeum*), of minor importance in North America, but causing wide-spread, heavy losses to *P.* × *canadensis* clones in Europe (Waterman 1957, Thielges 1985).

- Septoria leaf spot and canker (*Septoria musiva* Peck), which is generally limited to a leaf spot in native stands but is particularly damaging to hybrids, and has prevented the general use of most *P.* × *canadensis* clones in Canada, the United States and Argentina (Thielges 1985).

Members of the *Populus* section are most likely to be affected by:

- Hypoxylon stem canker [*Hypoxylon mammatum* (Whal.) Miller] is broadly distributed on host species in section *Populus*, but only becomes a disease problem in certain areas (Manion and Griffin 1986, Newcombe 1996). It sometimes causes cankers on *P. trichocarpa* in Europe (Terrasson *et al*. 1988) and on various hybrid clones in North America (Ostry and McNabb 1986).

- White-rotting fungus (*Phellinus tremulae* (Bond.) Bond. & Borisov.) causes serious decay in aspens (Thomas *et al*. 1960), although the mechanism of resistance by members of *Aigeiros* and *Tacamahaca* to decay is unknown (Newcombe 1996).

Various poplar species and hybrids display well-developed adaptations to environmental stress, but in particular to drought, flooding, salinity, cold, and atmospheric pollutants such as ozone (Blake *et al*. 1996, Neuman *et al*. 1996). It is also noted that *P. canescens* shows stability against strong winds.

Many mammals feed on the bark, leaves and roots of *Populus*, notably snowshoe hares (*Lepus americanus*), beaver (*Castor canadensis*), porcupine (*Erethizon dorsatum*), pocket gophers (*Thomomys bottae*), and opossum (*Trichosurus vulpecula*) (Edwards 1978, Bryant 1981, Cantor and Whitham 1989, Basey *et al*. 1990). Ungulates such as deer, moose and elk (*Cervus elaphus*) not only browse on shoots and new sprouts, but also damage bark by chewing and rubbing with their antlers (Romme *et al*. 1995). Cattle and sheep also browse on regeneration and cause root damage to existing trees when allowed to range through stands (Cooper and Van Haverbeke 1990, Perala 1990). While little is known about the response of mammals to patterns of hybridisation in *Populus*, there is tremendous variation in feeding preference

among hybrids and individual clones. It is suspected that this is due to variation in concentrations of phenolic glycosides which are known to be defensive toward mammals (Whitham *et al*. 1996).

Mice and voles can cause severe damage in young plantations (DeBell 1990). Many species of birds thrive in poplar forests, and a few can cause damage through their feeding. The ruffed grouse and the sharp-tailed grouse feed on aspen buds, and the ruffed grouse also feeds on the leaves during the summer months. Red-breasted and yellow-bellied sapsuckers may scar trees with drill holes as they forage for bark insects (Fowells 1965).

The riparian cottonwoods are one of the most productive and sensitive components of riparian ecosystems in western North America. The number of vertebrate species associated with these communities is four times higher than the numbers associated with spruce-fir, lodgepole pine, or Douglas-fir communities, yet human activities result in the loss of over 100 000 ha of riparian habitat each year (Finch and Ruggiero 1993). While riparian ecosystems occur on less than 1% of the western North American landscape, they provide habitat for more bird species than all other vegetation types combined (Knopf *et al*. 1988). Zones of hybridisation are thought to be centres of biodiversity (Whitham *et al*. 1996), representing a refugium for insect species (Whitham 1989), and thus a superior habitat for insectivorous birds (Martinsen and Whitham 1994, Dickson and Whitham 1996).

8. Summary

The wide distribution of *Populus* throughout the Northern Hemisphere represents an important and valuable component of many forest ecosystems and great potential for domestication. Evolution of the genus has been characterised by divergence into various sections, which offer even more opportunities for novel genetic combinations through hybridisation. Gene flow within the range of the individual species is usually very high, with populations distinguished only by their adaptive response to environmental selection pressures.

While initial establishment is by seeds, which may travel long distances to invade newly disturbed areas, the maintenance of populations often relies on poplar's ability to reproduce vegetatively. Plantation culture of poplars has exploited this trait, and most breeding programs are characterised by deployment of selected clones. Clonal variation is high for yield traits, as well as disease resistance and wood quality.

In North America, *P. tremuloides* is the most widely distributed tree species and an important component of many forest types. The riparian cottonwoods, from sections *Aigeiros* and *Tacamahaca*, play an important role in the maintenance of complex riparian communities, in addition to their importance in plantation culture. Although North American poplar communities are still largely intact, small populations on the edge of species' ranges are in need of conservation. Of even greater concern is the erosion of genetic resources, particularly for *P. nigra*, that has resulted from human activity in Europe.

Poplar is ideally suited as a model organism for understanding growth processes in forest trees. It has and will undoubtedly continue to be a target for domestication and forest management in many parts of the world.

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APPENDIX

Genetic Transformation of Poplars

Poplar is the first forest tree species to have been transformed (Fillatti *et al*. 1987). Since that time, a number of different transformation procedures have been optimised on poplar species in many laboratories. These allow for the easy recovery of transgenic poplar plants (Jouanin *et al*. 1993). *Agrobacterium*-mediated transformation appears to be the most widely used vector for DNA transfer in poplar (Leplé *et al*. 1992). The first reports on poplar transformation deal primarily with the introduction of marker genes: the GUS gene or genes conferring selectable traits such as resistance to antibiotics or to herbicides (Brasiliero *et al*. 1992). Since 1990, an increasing number of studies have focused on the expression of genes potentially able to modify agronomic traits, for example: tolerance to insect attack (Robinson *et al*. 1994, Leplé *et al*. 1995, 1998), control of flowering (Weigel and Nilsson, 1995), modification of wood quality through altered lignin content and composition (Baucher *et al*. 1996, Van Doorsselaere *et al*. 1995), improvement to oxidative stress tolerance (Strohm *et al*. 1995). Until recently, most of the results have been obtained using juvenile material grown under controlled conditions, however, an increasing number of field trials are now being set up with transgenic poplars in order to validate results obtained in the greenhouse. Moreover, these field evaluations will answer a number of questions concerning the spatial and temporal stability of transgene expression in mature trees that are subject to a natural changing environment (Pilate *et al*. 1997).

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SECTION 5
SITKA SPRUCE (*PICEA SITCHENSIS* (BONG.) CARR.)

1. General Information

This consensus document addresses the biology of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). Sitka spruce is an ecologically important species of the north temperate coastal rain forest of western North America. It is a valuable species for both pulp and lumber. Its wood offers unique qualities for manufacture of the highest quality sounding boards and tops for many musical instruments. As well, an outstanding strength-to-weight ratio made Sitka spruce strategically important during both World Wars for construction of aircraft (Brazier, 1987). While its natural range is not extensive and the species' economic importance ranks far below that of other western conifers, it is a keystone species in some of the most productive ecosystems of North America, particularly in the Queen Charlotte Islands of British Columbia (Peterson *et al*., 1997). Outside its natural range, Sitka spruce has played an important role in plantation forestry, particularly in Northern Europe (Hermann, 1987). In Great Britain, the species now accounts for almost 70% of the annual conifer planting stock (Malcolm, 1997) and plantations cover over 20% of the forest/woodland area (Cannell and Milne, 1995). Sitka spruce is also a primary plantation species in Brittany, where productivity of stands is similar to that in Britain (Vaudelet, 1982; Serrière-Chadoeuf, 1986; Guyon, 1995).

The general biology of Sitka spruce is described in the context of the species' role in natural forests and its domestication in planted stands. Taxonomic and evolutionary relationships with other *Picea* species are described. Reproductive biology is described with a focus on aspects of mating system, gene flow, seed production and natural stand establishment. The current knowledge of genetic variation within the species is reviewed, highlighting the importance of variation patterns and the potential for improvement by means of recurrent selection breeding strategies. Biological diversity and ecological interactions with higher and lower flora and fauna are discussed. Domestication and operational breeding activities are reviewed. While Sitka spruce reforestation is currently based on seed propagation, vegetative propagation of rooted cuttings is well advanced, and somatic-embryogenesis techniques are available making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

This document was prepared by the lead country, Canada. It is intended for use by regulatory authorities and others who have a responsibility for conducting assessments of transgenic plants proposed for commercialisation, and by those who are actively involved with genetic improvement and intensive management of this species.

2. Taxonomy and Natural Distribution

A. Taxonomy and nomenclature

Sitka spruce (*épinette Sitka* in French Canada, *épicéa Sitka* in France, *Sitkafichte* in Germany) is one of about 40 species of the genus *Picea* A. Dietr. (family Pinaceae) distributed throughout the cooler parts of the North Temperate Zone and higher elevations in the south. It is also one of 7 species native to North America and 5 native to Canada (Farrar, 1995). There is lack of agreement among taxonomists regarding

the subdivision of the genus *Picea* (Schmidt-Vogt, 1977). Most early taxonomists suggested dividing the genus into three sections: Eupicea (or Morinda), Casicta, and Omorika. Mikkola (1969) recommended recognition of only two sections: Abies and Omorika. After extensive crossability studies, Fowler (1983, 1987) has suggested that the section Omorika be further divided into two subsections: Omorikoides and Glaucoides, with Sitka spruce assigned to the latter, together with White Spruce and Engelmann spruce.

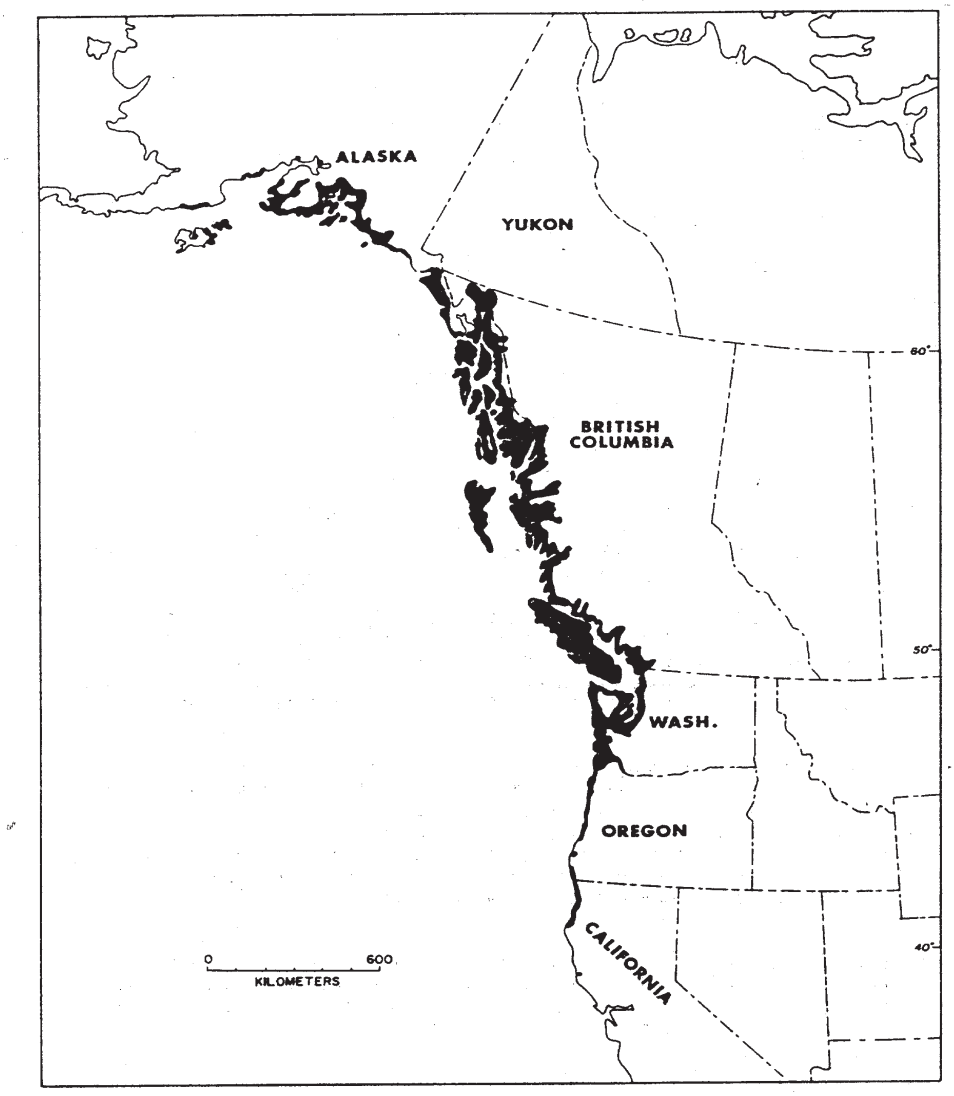
Originally introduced to Great Britain by David Douglas in the early 1800s as *Pinus menziesii*, the species was described soon after by the French botanist Bongard as *Pinus sitchensis*, referring to the origin of his specimens, Sitka Island, Alaska (now known as Baranoff Island) (Peterson *et al*., 1997). Carrière later recognised the species as a member of genus *Picea*, and the species is now well recognised as *Picea sitchensis* (Bong.) Carr. Common names are numerous, including airplane spruce, coast spruce, Menzies spruce, silver spruce, tideland spruce, western spruce, and yellow spruce. A famous rare mutant form found on the Queen Charlottes is known as golden spruce (Peterson *et al*., 1997).

Introgressive hybridisation between Sitka and White Spruce (*Picea glauca* (Moench) Voss) occurs in sympatric areas in north-western British Columbia and Alaska, with the hybrid known as *Picea* × *lutzii* Little (Little, 1953; Daubenmire, 1968; Roche, 1969; Hanover and Wilkinson, 1970; Copes and Beckwith, 1977; Yeh and Arnott, 1986; Woods, 1988). Introgressive hybridisation between white and Englemann spruce (*Picea englemannii* Parry ex Engelm.) is common where the two are sympatric in western Canada, Montana and Wyoming, and the hybrids have given rise to the variety *Picea glauca* var. *albertiana* (S. Brown) Sarg., commonly known as "interior spruce" (Roche, 1969; Roche *et al*., 1969; Daubenmire, 1974). Sitka spruce hybridises with Englemann spruce through controlled crosses (Johnson, 1939; Roche, 1969; Jeffers, 1971; Fowler and Roche, 1977; Kiss, 1989), and there is evidence suggesting that hybrids among Sitka, white and interior spruce also occur naturally (Woods, 1988; Sutton *et al*., 1991a, b, 1994; Coates *et al*., 1994; Grossnickle *et al*., 1996a, b). Several horticultural varieties, most of them dwarf phenotypes, have been recognised (Krüssmann, 1985; Griffiths, 1994).

B Natural distribution

The natural range of Sitka spruce spans a narrow strip on the north Pacific coast of North America, extending for 2 900 km from 61°N latitude in south-central Alaska to 39°N in northern California. Throughout this tremendous north-south range, Sitka spruce is a coastal species, occupying islands of the Alexander Archipelago in Alaska and the Queen Charlotte Islands in British Columbia, and, with the exception of river valleys, they rarely reach more than a few kilometers from the coast along a narrow mainland strip. The southern limit of the species is an isolated population in Mendocino County, California (Harris, 1978). The natural range of Sitka spruce is illustrated in the map given in Figure 3.5.

Figure 3.5 The natural range of Sitka spruce



Source : Harris, 1978

C. Evolution and migrational history

Conifers probably originated around the periphery of the north Pacific basin (Li, 1953). Fossil records indicate that divergence of modern genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin, 1963). Based on comparative immunological studies, Prager *et al.* (1976) have suggested that *Picea* was among the first genera to emerge. Although not supported by fossil evidence, Wright (1955) suggested eastern Asia as the likely origin of *Picea*, based on the abundance of species and particularly the presence of *Picea koyamai* Shirasawa, which he felt was a primitive species. *Picea* is then thought to have migrated to North American in one or more waves of migration via a land bridge between Siberia and Alaska (Wright, 1955).

Phylogenetic relationships within coniferous genera are commonly interpreted from species crossability studies, where it is assumed that the more related are two species, the more easily they can be crossed (Wright, 1955; Critchfield, 1975). The close phylogenetic relationship between the north-western American "white" spruces (Sitka, white, and Engelmann spruce) and the eastern Asiatic *Picea jezoensis* (Sieb et Zucc.) Carr. (Wright, 1955; Roche and Fowler, 1975) supports this theory, at least for the members of the subsection Glaucoides in section Omorika, and suggests that speciation occurred after their arrival in North America.

The fossil record of *Picea* during the Pleistocene era in North America is incomplete, but it is believed that many conifer populations were fragmented and isolated in various refugia during the glacial period (Critchfield, 1984). Sitka spruce probably occupied roughly its present-day range before glaciation, surviving at higher elevations on hills and mountains, and reoccupying lower areas of the long coastal strip in British Columbia and Alaska soon after (Daubenmire, 1968; Page and Hollands, 1987).

3. Reproductive Biology

A. Reproductive development

Sitka spruce is monoecious. Development of the reproductive structures follows a 2-year cycle typical of most conifers in the northern hemisphere, other than *Pinus* species and members of the Cupressaceae family (Owens and Blake, 1985). Bud scales are initiated at the terminal apex and at newly initiated axillary apices within the enlarging vegetative buds, from about mid-April (Owens and Molder, 1976a; Cannell and Bowler, 1978). Apices differentiate as vegetative, pollen cone, or seed cone buds around mid-July, at the cessation of shoot elongation. Pollen cones typically develop from small axillary apices on vigorous distal shoots or terminal apices on less vigorous, proximal shoots. Seed cones usually develop on distal axillary positions on vigorous shoots or from smaller terminal apices on less vigorous shoots (Moir and Fox, 1975a; Owens and Molder, 1976b).

Pollen cone bud development is complete, although meiosis has not occurred before they become dormant at the end of October. Seed cones also do not undergo meiosis prior to becoming dormant in late November (Owens and Molder, 1976b). By the time buds become dormant, all microsporophylls, microsporangia, bracts and functional ovuliferous scales, and leaves have been initiated. The overwintering seed cone, pollen cone, and vegetative buds are small and similar in shape: broadly conical, greenish-brown and covered in a bloom of light grey resin (Moir and Fox, 1975a; Eis and Craigdallie, 1981).

Reproductive and vegetative buds break dormancy at about the same time, in response to photoperiod, while subsequent development is regulated by temperature. Meiosis and subsequent development of pollen occur immediately, followed by maturation of the megagametophyte (Moir and Fox, 1975b; Owens and Molder, 1980). Flushing of reproductive buds precedes that of vegetative buds, and pollen is released over a one-week period, in late-April on Vancouver Island (Owens and Molder, 1980) and by mid-May in Scotland (Moir and Fox, 1975a). The pollen enters receptive seed cones and adheres to the sticky micropylar arms. A week later, a "pollination drop" draws the pollen into the micropyle (Owens and Blake, 1984). Fertilisation occurs 4 to 5 weeks later, and embryo development is completed in mid-August. Without fertilisation, no embryo is formed and the megagametophyte tissue degenerates, leaving a normal-sized, but empty seed (Owens and Molder, 1980).

B. Mating system and gene flow

Sitka spruce is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system (Cottrell and White, 1995). Self pollination occurs to some degree, as the period of pollen release and female receptivity coincide for an individual tree (Owens and Molder, 1980; El-Kassaby and

Reynolds 1990). Female strobili are usually found at the ends of primary branches in the mid- to upper-crown, while males are more prevalent at the ends of secondary branches lower in the crown (Tompsett, 1978; Philipson, 1997), although the effectiveness of this zonation against selfing is questionable (Nienstaedt and Teich, 1972). In the open-grown conditions in a seed orchard, the outcrossing rate was greatly reduced for seeds produced in the lower crown (Chaisurisri *et al*., 1994). The two-step pollination mechanism, whereby pollen is collected in the sticky micropyllar arms over the receptive period, and only then drawn *en masse* by the pollination drop, ensures that pollen from many sources has a chance to fertilise any given ovule (Owens and Blake, 1984; Runions *et al*., 1995).

Gene flow in *Picea* is mediated by small pollen grains, 70-85 μm at their widest point (Eisenhut, 1961), whose bladdery wings make them well-adapted for aerial transport (Di-Giovanni and Kevan, 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright, 1976). Nevertheless, conifer pollen may remain viable for several days and a substantial quantity may travel great distances (Lindgren *et al*., 1995; Lindgren and Lindgren, 1996). Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to 1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan, 1991). While pollen dynamics are not well known in Sitka spruce, a recent study of pollen dispersal dynamics in a black spruce seed orchard indicated that "large amounts" of pollen rose to a height of 300 m above ground level (Di-Giovanni *et al*. 1996). At a steady wind speed of 5 $m \cdot s^{-1}$, the authors calculated that spruce pollen reaching this altitude would drift about 47 km.

C. Seed production

Sitka spruce begins to produce seed at 20 to 25 years of age, with heavy crops occurring at intervals of 3 to 5 years (Malcolm, 1987; Philipson, 1987b). Crop intervals are somewhat longer, 5 to 8 years, in the northern part of the range (Harris, 1969). It is a prolific seed producer and, in a good seed year, an old-growth stand may produce as much as 14.5 kg of seed per hectare (Peterson *et al*., 1997). The seeds themselves are small, and average cleaned seed weight is about 2.2 g/1000 seeds (Safford, 1974).

Initiation and duration of seed dispersal are weather and site dependent. The mature cones open as they lose moisture and the scales flex in dry weather, re-closing during wet periods. Seed dispersal begins in the fall, with over 70% of the seeds dispersed within the first 6 weeks, 90% by February, and the remainder released over the next growing season (Ruth, 1958; Harris, 1969). The seeds are winged and wind-dispersed. The actual distance reached from the source depends on several factors, including height and position of the seed source, local topography and wind conditions (Harris, 1967, 1978). While 80% of the seed usually falls within 30 metres the parent tree, some may travel up to several hundred metres (Mair, 1973).

D. Natural regeneration

Sitka spruce seeds exhibit weak dormancy, and both the rate and total amount of germination can be increased by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Pintaric, 1972; Gordon *et al*., 1976; Gosling, 1988; Chaisurisri *et al*., 1992; Jinks and Jones, 1996). While not always employed in nursery practice, moist-chilling of Sitka spruce seed is often beneficial, particularly when moisture content and temperature are carefully controlled (Gosling and Rigg, 1990; Jones *et al*., 1993; Jones and Gosling, 1994), and stratified seed will tolerate redrying (Jones and Gosling, 1990; Jinks and Jones, 1996; Poulsen, 1996). In the wild, Sitka spruce seeds normally germinate the following spring, as soon as soil surface temperatures are warm enough and provided there is adequate moisture.

Unlike its highly shade tolerant associates western hemlock and western red cedar that can germinate and survive on organic seedbeds, Sitka spruce is more restricted in its seedbed and light requirements and tends to be more disturbance dependent (Taylor, A.H., 1990; Peterson *et al*., 1997). Sitka spruce regenerates naturally on landslides, newly exposed alluvial sites, and openings created by windthrow. Regeneration cutting systems in Sitka spruce must generate sufficient ground exposure and disturbance, by clear cutting, shelterwood or seed-tree methods (Harris and Johnson, 1983; Weetman and Vyse, 1990). Regarding thinning operations in mixed western hemlock, Sitka spruce stands will normally favour regeneration of the hemlock over the spruce, particularly in older stands where thinning intensity is light (Deal and Farr, 1994). Dense natural regeneration has been more commonly observed after harvesting of first-generation planted stands in Britain, with up to several hundreds of thousands per hectare and requiring spacing (Nelson, 1991; Adam and Berg, 1996).

E. Vegetative reproduction in nature

Vegetative reproduction of Sitka spruce is rare under natural conditions or in plantations, although layering can occur on moist sites (Cooper, 1931; Roche and Fowler, 1975). Rooting is most likely to occur when lower branches of open-grown trees come in contact with the ground and become covered by soil or organic materials on the edges of bogs or near the timber line (Harris, 1978).

4. Crosses

Potential crosses with Sitka spruce are summarised in Table 3.7. Natural hybrids between Sitka and White Spruce were first collected in North America by H.J. Lutz on the Kenai Peninsula, Alaska, and given the name *Picea* × *lutzii* by E.L. Little (1953). Before this, white-Sitka spruce hybrids had been observed in Denmark as a result of natural crossing between adjacent plantations (Thaarup, 1945; Bornebusch, 1946). Populations resulting from introgressive hybridisation have since been documented in the Skeena, Nass, and Bulkley river valleys in British Columbia where the two species are sympatric (Daubenmire, 1968; Roche, 1969). The hybrid has frequently been made artificially with parents from outside the sympatric area (Fowler, 1987), often in the hope of imparting the resistance of White Spruce to the white pine weevil. The degree of cold hardiness of the hybrid is related to the proportion of White Spruce germplasm (Ying and Morgenstern, 1982), and growth performance of the hybrid depends greatly on the origin of the parents (Sheppard and Cannell, 1985). Though *Picea breweriana* and *Picea sitchensis* can cross successfully, *P. breweriana* has a very small range that is rarely, if ever, sympatric with *P. sitchensis* given that the populations are separated by elevation. The category of "easily crossed, probably occurring naturally" indicates species that readily cross with *P. sitchensis* if grown together in artificial plantations, but are not naturally sympatric.

Table 3.7 Species cross compatibility with Sitka spruce

Species	Origin	References
Commonly occurring in sympatric range		
P. glauca (Moench) Voss.= *Picea* × *lutzii* Little	Canada, Northeast USA	Daubenmire, 1968; Roche, 1969; Fowler,1987; Woods, 1988
Easily crossed, probably occurring naturally		
P. englemannii Parry ex Engelm.	Canada, Western USA	Johnson, L.P.V. 1939; Roche, 1969; Jeffers, 1971; Fowler and Roche, 1977; Kiss, 1989
P. breweriana Wats.	Northwest USA	Langner 1952
P. mariana (Mill.) B.S.P.	Canada, Northern USA	Fowler, 1983
Successful crosses		
P. jezoensis (Sieb. & Zucc.) Carr.	Japan	Wright, 1955; Roulund, 1969
P. omorika (Pancic) Purkyne	Western Serbia, Eastern Bosnia	Johnson, 1939; Langner, 1959; Roulund, 1971; Geburek and Krusche, 1985
Possible crossability		
P. abies (L.) Karst (=*P. excelsa* (Lam.) Link)	Northern, Central, Eastern Europe	Langner, 1952
P. likiangensis (Franch.) Pritz.	China	Roche and Fowler, 1975
P. pungens Engelm.	Western USA	Roche and Fowler, 1975
P. wilsonii Mast.	China	Roulund, 1969

5. Genetics

A. Cytology

Sitka spruce vegetative cells normally have $2n = 24$ chromosomes (Burley, 1965b; Fox, 1987), although some trees exhibit a small 13th pair (Moir and Fox, 1972; Kean *et al*., 1982). These supernumerary or B-chromosomes seem to be restricted to provenances in the southern half of the species range (Moir and Fox, 1977), but have not been associated with any detectable effect on growth (Moir and Fox, 1976).

B. Genetic variation

Population-level variability

Before 1970, information on population variation of Sitka spruce was only available from small studies with limited sampling. Even in these first limited trials, there was strong evidence of clinal variation for many traits, associated with latitude, elevation, and distance from the coast (Burley, 1965a; Roche and Fowler, 1975). In 1969/70, an extensive sampling of seed sources from across the entire range was organised by the International Union of Forest Research Organizations (IUFRO). Ten of these sources were widely planted in field tests in North America and many European countries (O'Driscoll, 1978).

Clinal variation patterns are expressed for phenological traits such as cessation of growth (Lines and Mitchell, 1966; Pollard *et al*., 1975; Kraus and Lines, 1976), and is greater among provenances than within

(Falkenhagen, 1977; Deleporte, 1984). Southern coastal provenances produce up to 100% more height growth than northern inland sources (Cannell, 1974; Cannell and Willett, 1975; Cannell and Willet, 1976). While southern sources grow faster, they are more susceptible to frost damage, particularly in the nursery (Magnesen, 1986; Lines, 1987b; McKay, 1994). Provenance trials in the former Federal Republic of Germany showed a north-south trend in growth, with latitude accounting for over 80% of the among-provenance variation (Kleinschmit, 1984). Results of a 19 year provenance trial in Ireland demonstrated that the most productive provenances of Sitka Spruce for the mild, coastal conditions in Ireland originated from southern Washington and northern Oregon (Thompson and Pfeifer, 1995).

Ecotypic variation related to bioclimatic and physiographic factors has been demonstrated among provenances for seed and cone traits (Falkenhagen, 1978; Falkenhagen and Nash, 1978). Even for growth traits that normally exhibit clinal variation patterns, substantial variation may be present at the microgeographic level, attributable to such local site factors as slope and aspect (Campbell *et al*., 1989). Variation in biochemical composition appears to be clinal for sources from Alaska to north Washington, while more southerly sources show no geographic trends, perhaps reflecting the post-glacial recolonisation of northern parts of the range (Forrest, 1975b, 1980; Wellendorf and Kaufmann, 1977).

Population differences have also been demonstrated for susceptibility to insect attack. Provenances from Kitwamga (inland Skeena River) and Big Qualicum (SE Vancouver Island) suffer less damage from the white pine weevil (Alfaro and Ying, 1990; Tomlin and Borden, 1994; Ying and Ebata, 1994). Density of green spruce aphids attacking a provenance test in northern Ireland was related to latitude of seed origin, with southern provenances especially susceptible (Day, 1984). Lignified stone cell masses in spruce bark are considered an important physical defence against insects and fungi, and there is a clinal increase in bark lignin with increasing latitude of provenance origin (Wainhouse and Ashburner, 1996).

In contrast to many other characters, geographic variation at polymorphic allozyme loci appears to be weak. In a study of the 10 IUFRO provenances, only 8% of the diversity at polymorphic loci was due to differences among populations, whereas 92% resided within populations (Yeh and El-Kassaby, 1980).

Individual-level variability

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within-populations as the source of genetic gains. For productivity traits, on average, 40% of the genetic variation for Sitka spruce is at the provenance level, while 60% is within provenance (Fletcher, 1992).

Estimates of narrow-sense heritabilities for height growth during the first six years was around 25% for open-pollinated progeny of randomly selected trees in a single population (Samuel and Johnstone, 1979), while another study estimated heritability at over 40% for height after eight years (Birot and Christophe, 1983). Other quantitative estimates of additive genetic variation in growth traits have also varied widely (Falkenhagen, 1977; Yeh and Rasmussen, 1985; Samuel, 1991), but narrow-sense heritability is normally more than sufficient for individual tree selection and breeding to be effective, particularly when provenance and family performance are combined in a multiple-trait selection index (Christophe and Birot, 1983). Height superiority of individual trees is not necessarily associated with production of "free growth", although progeny of plus-trees have been noted to improve their height rankings over the first six years on better sites by production of free growth (Cannell and Johnstone, 1978).

Perhaps the most precise estimates of genetic variance for Sitka spruce have been reported for a 7-tree diallel cross, planted at two test sites in Scotland and Wales (Samuel *et al*., 1972; Samuel, 1991). In this experiment, genetic variation for height growth, although under some additive genetic control, was predominantly controlled by non-additive effects, whereas diameter was only controlled by additive

effects. Practically all the variation in monoterpene composition for these crosses was attributable to additive genetic effects, and reflected the differences in parental means in additive combination with little significant deviation due to specific combining ability or reciprocal effects (Forrest and Samuel, 1988).

The ease of vegetative propagation of Sitka spruce offers opportunities to capture additional gains earlier in the breeding cycle. Clonal selection has been demonstrated to be highly effective in Sitka spruce, for characters such as planting stock phenology, frost resistance (Nielsen and Roulund, 1996), root-growth potential (Deans *et al*., 1992), early height and diameter growth, and branching habit (Cahalan, 1981) . Clonal testing has also demonstrated that it is possible to select clones that combine good growth with high wood density (Costa e Silva *et al*., 1994).

A provenance test in British Columbia demonstrated significant difference in susceptibility to white pine weevil, both among provenances and among families within provenances (Alfaro and Ying, 1990; Ying, 1991). Unfortunately, a study of variation within a resistant provenance found that taller families were more likely to be attacked (Alfaro *et al*., 1993).

Variation in biochemical composition for trees within provenances is high (Forrest, 1975a, b; Wellendorf and Kaufmann, 1977), a trend that is also found for variation at polymorphic enzyme loci (Yeh and El-Kassaby, 1980). While southern provenances are generally less resistant to frost, there is sufficient variation within populations to select frost resistant, fast-growing individuals (Nicoll *et al*,. 1996).

Genotype-environment interactions are of concern to tree breeders who generally seek broad adaptability within bred material. A seedling test of provenances grown under controlled environments found that provenances near the centre of the range were more broadly adapted (Mergen *et al*., 1974). A test of families originating from the latitudinal range of Sitka spruce and planted at eight locations in Britain found highly significant family-site interaction for six-year height, but found that above-average families could be selected that were broadly adapted (Johnstone and Samuel, 1978). A progeny test established at 3 locations in Denmark demonstrated genetic control of growth, stem form, wood density (pilodyn penetration), frost resistance and resistance to aphids, in addition to substantial genotype–environment interaction for growth characters (Jensen *et al*., 1996). Another clonal test in Denmark found that 15% of the clones contributed over 50% of the GE interaction variance (Nielsen and Roulund, 1996).

C. Inbreeding depression and genetic load

Self pollination in Sitka spruce has severe effects on seed set, early growth, and survival (Samuel *et al*., 1972). Among those that survive, strong inbreeding depression continues with selfed individuals only 68% as tall as outcrossed trees at 15 years of age (Samuel, 1991). Inbreeding depression is also exhibited by progeny originating from seed collections in small stands (Phillips, 1984).

D. Breeding programs

Breeding strategies for Sitka spruce generally utilise a system of progeny testing and recurrent selection for generation advancement, combined with clonal seed orchards for production of improved seed. Flowering of Sitka spruce grafts can be stimulated by means of various cultural treatments, particularly those involving gibberellin $A_{4/7}$, and this has facilitated the turnover of breeding cycles (Philipson, 1985a, b, 1987a, 1992; Philipson *et al*., 1990; Ross, 1991; Owens *et al*., 1992). While most seed orchards currently in production were established by grafting cuttings from plus-trees, and their placement in cultivated field environments, some programs have also experimented with the management of containerised Sitka spruce orchards, with the possibility of vegetative multiplication of small quantities of seed by rooted cuttings (John and Mason, 1987; Philipson and Fletcher, 1990).

Breeding programs have been established in all the areas where Sitka spruce is an important plantation species. In British Columbia, where planting of Sitka spruce is severely limited due to risk of damage by the white pine weevil, the breeding plan emphasises weevil resistance. Currently, more than 250 open-pollinated families and 300 clones are included in weevil-resistance screening trials (King, 1994; King *et al*,. 1998). The intensive breeding program in Britain began in the early 70's (Fletcher and Faulkner, 1972), and is by far the most ambitious. From an initial 2 800 plus-tree selections, the breeding population now consists of 200 tested parents, subdivided into sub-populations targeted for different geographic areas (Faulkner, 1987; Fletcher, 1992; Malcolm, 1997). Breeding programs are also carried out in Denmark (Roulund, 1990) and in northern France (Deleporte and Roman-Amat, 1986), where Sitka spruce is an important component of plantation forestry operations.

E. Conservation of genetic resources

Domestication of a key species such as Sitka spruce can influence diversity of genetic resources: (1) indirectly, by the method of seed collection, extraction, and storage, and by nursery and plantation culture; and (2) directly, by intentional selection to increase the frequency of genes for desirable traits (Chaisurisri and El-Kassaby, 1994; Morgenstern, 1996). The inadvertent loss of genes by natural processes and human activity can have negative consequences on the adaptability of populations and the potential for future gains from breeding. The need for gene conservation for a species can be assessed by evaluating (1) its current status of protection, (2) its frequency of occurrence, (3) the extent of its botanical range, (4) ease of natural regeneration, and (5) its representation in genetic testing and breeding programs (Lester, 1996). Using this approach, Yanchuk and Lester (1996) ranked the need of Sitka spruce for gene conservation as higher than some of its associates (Douglas-fir, western red cedar and western hemlock), but lower than others (mountain hemlock, amabilis fir, yellow-cedar and western white pine).

In the case of Sitka spruce, *in situ* conservation of genetic resources is practised by protection of ecological reserves, special areas, and parks (Pollard, 1995), and integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of isolated, small populations (Yanchuk, 1995). *Ex situ* conservation, by cryopreservation of germplasm, by off-site maintenance of populations in arboreta and clone banks, and by multi-population breeding strategies (Eriksson *et al*., 1993; Namkoong, 1995), has been practised to a much lesser extent, although many provenances and families are now represented in field tests and seed bank collections (Edwards and El-Kassaby, 1993). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller, 1992; Yanchuk and Lester, 1996).

6. Ecology and associated species

A. Habitat

In its natural range, Sitka spruce is primarily a coastal species, but may extend well inland where optimum environmental conditions occur (Harris, 1990). It is primarily a low elevation species and is uncommon at elevations above 500 m. It is, however, much less restricted by edaphic factors than by climate and physiography (Roche and Haddock, 1987).

Climate

The natural range of Sitka spruce is restricted to an area of maritime climate with abundant moisture throughout the year, relatively mild winters and cool summers. Annual precipitation is high throughout the range, but somewhat greater in the north where summer precipitation as light drizzle and fog are frequent. Summer temperatures in these coastal habitats lack the extremes of more continental areas and, while

moderated by the ocean current of the north Pacific, decrease northward. Although winters are mild, accumulated heat input varies with latitude and probably accounts for much of the variation in productivity, soil development, and associated species (Harris, 1978).

Soils and site type

While Sitka spruce grows on soil derived from a variety of parent materials, their best development is on deep, moist, well-aerated soils. Growth is poor on swampy sites. Sitka spruce commonly occupies alluvial soils along streams, coarse-textured soils, or soils with a thick accumulation of organic matter. In Alaska, it is a pioneering species on immature soils exposed by glacial retreat or uplift from the sea. Soils in the natural range are acidic, with pH values varying from 4.0 to 5.7 (Harris, 1978).

Sitka spruce is relatively nutrient demanding, particularly at young ages prior to crown closure (Miller and Miller, 1987). It is most productive on nitrogen rich soils, but also requires relatively large amounts of calcium, magnesium and phosphorus (Krajina, 1969). Nitrogen and phosphorus have been found to be limiting on sites in both British Columbia and Britain, and applications of fertilisers may be necessary for successful plantation establishment (McIntosh, 1981, 1983; Miller and Miller, 1987; Taylor, C.M.A., 1990; Taylor and Tabbush, 1990; Prescott and Weetman, 1994).

B. Synecology and associated species

Sitka spruce occurs most commonly in mixed stands, usually associated with western hemlock (*Tsuga heterophylla*) (Harris and Johnson, 1983). Red alder (*Alnus rubra*) and black cottonwood (*Populus trichocarpa*) are associated throughout the range. Other associates vary with latitude: Douglas-fir (*Pseudotsuga menziesii*), Port-Orford-cedar (*Chamaecyparis lawsoniana*), western white pine (*Pinus monticola*), redwood (*Sequoia sempervirens*), and bigleaf maple (*Acer macrophyllum*) are limited to the south; shore pine (*Pinus contorta* var. *contorta*) and western red cedar (*Thjua plicata*) extend into south-east Alaska; while yellow cedar (*Chamaecyparis nootkatensis*), mountain hemlock (*Tsuga mertensiana*), subalpine fir (*Abies lasiocarpa*), and Sitka alder (*Alnus sinuata*) are limited to northern sites and higher elevations in the south (Harris, 1978). In Alaska and the Skeena, Nass, and Bulkley river valleys of British Columbia, Sitka spruce is associated with White Spruce (*Picea glauca*), and hybrid populations are found (Daubenmire, 1968; Roche, 1969). Pure stands of Sitka spruce are common on tidewater areas that receive quantities of salt spray, and in early succession situations following disturbance. Sitka spruce is an aggressive pioneer and, by itself or together with intolerant associates such as alder or cottonwood, will invade landslides, dunes, uplifted beaches and glaciated terrain.

Due to the latitudinal spread of the Sitka spruce range and the variation in precipitation and exposure, the species is a component of several ecological associations, characterised by available moisture and nutrient regimes. On the mid- to upper-slopes of the Queen Charlotte Islands, the western hemlock – Sitka spruce forest is typically associated with Alaska blueberry (*Vaccinium alaskaense*), red huckleberry (*V. parifolium*), ovalleaf huckleberry (*V. ovalifolium*), and several mosses (*Rhytidiadelphus loreus*, *Hylocomium splendens*, and *Mnium glabrescens*). On exposed coastal locations, pure stands of salt-tolerant Sitka spruce are associated with reed grass (*Calamagrostis nutkaensis*), salal (*Gaultheria shallon*) and finger moss (*Stokesiella oregana*). Sheltered alluvial sites find Sitka spruce and its associates western hemlock and red alder on grass meadows with *Trisetum cernum*, *Gymnocarpium dryopteris*, *Hylocomium splendens* and *Leucolepis menziesii* (Roche and Haddock, 1987; Hanley and Hoel, 1996; Hanley and Brady, 1997).

Inland valleys and eastern slopes of coastal mountains tend to be drier. It is on these areas that Sitka spruce is most likely to be sympatric with White Spruce and hybridisation can occur. Within this drier zone, valley bottoms and mountain slopes typically support mixed conifer forests with ground vegetation

dominated by Alaska blueberry, ovalleaf huckleberry, red huckleberry, and rustyleaf menziesii (*Menziesia ferruginea*). Devil's club (*Oplopanax horridus*) appears within this zone on fluvial sites of the Skeena and Nass Rivers, and skunk cabbage (*Lysichiton americanum*) and salmonberry (*Rubus spectabilis*) are found on the driest sites in flat areas (Roche and Haddock, 1987; Harris, 1990).

C. Competition and stand structure

While Sitka spruce is rated as tolerant to shade, it is less tolerant than its usual associate, western hemlock (Daniel *et al*., 1979; Minore, 1979; Kobe and Coates, 1997), so that the general successional tendency is toward a western hemlock climax type, although few climax stands proceed to pure hemlock. As Sitka spruce is physically large, long-lived, and able to invade small openings resulting from windthrow, it is commonly maintained as a stand component, even under climax conditions. In south-east Alaska, mixed stands of hemlock and spruce are regarded as the climax stand type, with Sitka spruce regenerating on mineral soil mixtures exposed by windthrow and other disturbance, and hemlock seeding in on organic substrates (Harris, 1990; Deal *et al*., 1991; Peterson *et al*., 1997).

Sitka spruce is one of few conifer species that produce epicormic shoots along the stem. These shoots may originate from either dormant or adventitious buds (Stone and Stone, 1943) in response to light intensity (Isaac, 1940; Herman, 1964). Increasing exposure of stems to sunlight by thinning of stands will stimulate epicormic branching and affect the future quality of the trees (Farr and Harris, 1971).

D. Ecosystem dynamics

Many abiotic factors interact with Sitka spruce in natural and planted forests, and some may cause significant damage. Windthrow is probably the most serious damaging agent, particularly in plantations of Sitka spruce that are established in Great Britain where shallow rooting on unfavourable soils and exposure to strong winds results is risk of instability (Miller, 1986; Coutts and Philipson, 1987; Mason and Quine, 1995; Malcolm, 1997). Elsewhere in Europe, planted Sitka spruce has suffered significant wind damage, but has proven more wind-firm than other conifers such as *Picea abies*, *Abies* spp. and *Pinus sylvestris* in France (de Champs *et al*,. 1983; Touzet, 1983), Denmark (Neckelmann, 1981) and Norway (Lohmander and Helles, 1987). In North America, Sitka spruce is considered less wind-resistant than *Pseudotsuga menziesii* and *Thuja plicata*, but more-so than *Tsuga heterophylla* and *Abies amabilis* (Minore, 1979).

While Sitka spruce is among the least fire-resistant species in coastal forests, wild fires are not a major cause of damage within the native range (Minore, 1979; Agee, 1990). On the other hand, Sitka spruce regeneration benefits rather more from slash burning than several of its conifer associates (Hawkes *et al*., 1990; Otchere-Boateng and Herring, 1990). Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils (Shaw *et al*,. 1987; Goulet, 1995). Autumn frost is a particular problem in Great Britain, where faster-growing southern provenances are particularly susceptible (Redfern and Cannell, 1982; Lines 1987b; Nicoll *et al*,. 1996).

In the following table, other species interactions with Sitka Spruce are shown.

Table 3.8 Species Interactions with Sitka Spruce

Insects	
Common name	**Agent**
White pine weevil [Johnson, 1965; McMullen, 1976; Alfaro, 1982; Martineau, 1984; de Groot, 1985; Wallace and Sullivan, 1985; Hulme, 1986, 1987; McMullen *et al*,. 1987; Alfaro, 1989a,b; Hulme and Harris, 1989; McLean, 1989; Alfaro and Omule, 1990; Alfaro and Ying, 1990; Warkentin *et al*., 1992; Fraser and Heppner, 1993; Alfaro, 1994; Fraser and Szeto, 1994; Hulme, 1994; Sahota *et al*., 1994; Spittlehouse *et al*., 1994; Tomlin and Borden, 1994; Alfaro *et al*., 1995; Hulme, 1995; Alfaro, 1996a,b; de Groot and Zylstra, 1996; Tomlin *et al*., 1996; and, Tomlin and Borden, 1997a,b:]	*Pissodes strobi;* The most serious economic insect pest of Sitka spruce in North America. Larvae tunnel down the inner bark of the shoot, killing the leaders. Not yet a pest where Sitka spruce is planted in Europe.
Green spruce aphid [Powell and Parry, 1976; Carter, 1977; Dixon, 1977; Koot, 1983; Evans, 1987; Nichols, 1987; Carter, 1989; Sutherland *et al*., 1989; Finck *et al*., 1990; Seaby and Mowat, 1993; Straw, 1995; Schwenke, 1972]	*Elatobium abietinum;* a sap sucking insect, can result in mortality at high infestations levels
Bark beetles (Scolytidae) Spruce beetle Great spruce bark beetle [Bejer-Petersen, 1976; Lemperiere and Bailley, 1986; Fielding *et al*., 1991; Kirkeby-Thomsen, 1992; Rose *et al*,. 1994, and Reynolds and Holsten, 1996]	*Dendroctonus rufipennis;* feeds and breeds in galleries between bark and wood. *D. micans* (ditto)
Fungi	
Disease	**Agent**
Annosus root rot [Pratt, 1979a, b; and Morrison *et al*., 1986]	*Heterobasidoion annosum;* causes butt-rot and can lower yield and quality
Armillaria root rot [Boullard and Gaudray, 1975; Redfern, 1978; and Morrison, 1981]	*Armillaria mellea* complex; may kill younger trees
Laminated root rot [Nelson and Sturrock, 1993; and Thies and Sturrock, 1995]	*Phellinus weirii;* butt decay that may kill younger trees
Rhizinia root rot [Phillips and Young, 1976; Gregory and Redfern, 1987; and Callan, 1993]	*Rhizinia undulata;* can affect young seedlings and pole sized trees

For other rust fungi, stem decay, nursery moulds and diseases of seed and cones see: [Gregory and Redfern, 1987; Sutherland *et al*., 1987, 1989 and Sutherland and Hunt, 1990]	
Animals	
Common name	**Species name**
Sitka spruce stands provide cover for many species of fish (salmon and trout species), mammals and birds [Hartman and Brown, 1988; and Staines *et al*., 1987]	
Black-tailed deer [Sullivan *et al*,. 1990] Red deer Sika deer Roe deer Fallow deer [Welch *et al*., 1987, 1991, 1992; Hannan and Whelan, 1989 and de Jong *et al*., 1995]	*Odocoileus hemionus columbianus;* heavy numbers may cause browsing damage *Cervus elaphus* *C. nippon* *Capreolus caprelous* *Dama dama* All may cause damage by bark stripping and browsing
Porcupine [Sullivan *et al*., 1986]	*Erethizon dorsatum;* may feed cause slight damage
Red squirrel [Syme, 1985]	*Tamiasciurus hudsonicus;* damages shoots in removing cones
Seed-eating birds	Many bird species commonly eat quantities of seed, as well as insects associated with Sitka spruce.

E. Symbiotic Relationships - Mycorrhizae

Relatively little research has been done on mycorrhizas of Sitka spruce, although results from forest trials show that inoculation with selected mycorrhizal fungi can give significant early growth effects (Walker, 1987). For example, seedlings inoculated with E-strain fungi, the dominant mycorrhizal fungi of nurseries, were smaller than those inoculated with either *Thelephora terrestris* or *Laccaria laccata* (Thomas and Jackson, 1983). The dominant mycorrhizal fungus in the nursery, the 'E-strain', decreases in frequency with age after planting out. Some mycorrhizal types are found at all forest sites in Britain: of these types, *Thelephora terrestris* has been found on all age classes of Sitka spruce. Other mycorrhizal species recorded on Sitka spruce include *Amanita rubescens, Laccaria amethystea, Lactarius hepaticus, L. tabidus, L. turpis* and *Russula ochroleuca* (Thomas *et al*., 1983). The successions of fruit bodies of mycorrhizal fungi under differently aged British plantations of Sitka spruce were determined to be *Laccaria/Paxillus-Inocybe-Cortinarius-Lactarius* (Dighton *et al*., 1986).

Eighty-four potentially mycorrhizal macrofungi have been recorded with Sitka spruce in Scotland. They derive primarily from the native flora of birch and pine and many are fungi with a wide host range. Specific mycorrhizal associates do not occur. The saprotrophic macrofungi are species that are common in a range of vegetation types (Alexander and Watling, 1987). In nurseries in the Irish Republic, *Piceirhiza horti-inflata* was the most frequent mycorrhizal association during the first year of growth but appeared to be replaced by *Hebeloma* sp. and *Amphinema byssoides* in 2-year-old seedlings. There was a greater diversity of mycorrhizas on container-grown seedlings, which included *Thelephora terrestris*, *Hebeloma* sp. and *Piceirhiza guttata* (Grogan *et al*., 1994). Sitka spruce trees in a plantation established in Normandy in 1956 were affected by *Armillaria mellea* root rot and it is suggested that the formation of mycorrhizae on *P. sitchensis* in France (where the tree is an exotic) is in some way incomplete and affords inadequate protection against *A. mellea* (Gaudray, 1973).

The ability of six ectomycorrhizal fungi (*Thelephora terrestris, Hebeloma crustuliniforme* strains Siv and 81a, *Paxillus involutus, Laccaria laccata* and *Lactarius rufus*) to form mycorrhizas on plantlets of Sitka spruce derived from somatic embryos was investigated by Sasa and Krogstrup (1991). Mycorrhizal synthesis was achieved only on the oldest plantlets during the third week after inoculation. The rate and development of mycorrhizal formation varied according to the fungal species, with infection by *T. terrestris* the highest (92% of the total number of root tips), and *Lactarius rufus* failing to form any mycorrhizas.

7. Domestication

In 1930, Sitka spruce seedlings were among those planted in British Columbia's first reforestation project in the Fraser River Valley, near Vancouver (Young, 1989). While previously planted at a level of about 10 million seedlings per year in British Columbia, Sitka spruce has been all but eliminated from reforestation programs in North America, due to damage from the white pine weevil (King *et al*., 1998). Today, less than 2 million seedlings are planted each year, primarily on cool, coastal areas of the Queen Charlotte Islands. Meanwhile, Sitka spruce is the backbone of plantation forestry in Great Britain, accounting for about 70% of the seedlings planted (Malcolm, 1997), and is a commonly planted species in other European countries such as France and Denmark (Hermann, 1987). In spite of the good growth potential of Sitka spruce in the former Federal Republic of Germany, planting has been drastically reduced due to frost damage, drought and storm damage, and foraging by deer (Kleinschmit, 1978).

A. Deployment of reforestation materials

While Sitka spruce planting stock has traditionally been produced in bare root nurseries as 2+0 seedlings or 1+1 transplants, an increasing proportion is now produced in containerised growing systems, particularly in North America (Daniels and Simpson, 1990; Van Eerden and Gates, 1990; Aldhous and Mason, 1994). A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently.

Sitka spruce planting stock can also be produced by means of vegetative propagation. Practical propagation systems have been developed (Kleinschmidt, 1992). Juvenile cuttings are easily rooted to produce planting stock as "stecklings" (Mason, 1984, 1992; John and Mason, 1987; Mason and Keenleyside, 1987) whose performance after planting is comparable to that of conventional transplant stock (Roulund, 1978; Roulund and Bergstedt, 1982; Baldwin and Mason, 1986; Mason *et al*., 1989). While steckling planting stock has been actively promoted in Great Britain, higher nursery production costs have kept annual production to a few million (Mason, 1991, 1992; Mason and Sharpe, 1992). Despite the higher production costs for steckling stock, the ability to bulk-up (vegetatively multiply) scarce seed sources and tested crosses between selected individuals is expected to more than compensate by realising potential genetic gains earlier (Gill, 1983; Mason and Gill, 1986; Mason and Harper, 1987; Lee, 1992).

Techniques for the initiation and regeneration of somatic embryos are available (Krogstrup *et al*., 1988), and embryogenic lines can be successfully regenerated after cryostorage (Find *et al*., 1993; Kristensen *et al*., 1994), making it possible to maintain genotypes in a completely juvenile condition during clonal testing. Sitka spruce plantlets derived from tissue culture propagation systems are also being automated further through the application of bioreactor technology (Moorhouse *et al*., 1996).

Sitka spruce has been established by direct seeding on an experimental basis, where it has been shown that the seedlings produce a much smaller amount of adventitious roots, compared with bare root transplants (Coutts *et al*., 1990). However, direct seeding has not been used operationally as a regeneration

technique for Sitka spruce. Its use in British Columbia is considered a poor option, due to the very slow growth of germinants, which predisposes them to drought and competition (Mitchell *et al,*. 1990).

B. Provenance transfer

Within the native range, plantations established on sites with a strong maritime climate will be faster growing if seeds are transferred from more southerly latitudes. Conversely, transfers of seed from coastal origins to planting sites further inland involve higher risk (Lester *et al*., 1990; Ying, 1990).

Sitka spruce seedlots from British Columbia have also been certified under the OECD scheme for sale in Europe (Pollard and Portlock, 1990; Portlock, 1996). In Britain, southern provenances (below 47°N latitude) grow fastest, but are susceptible to spring and autumn frosts (Lines, 1987a, b), although clonal testing has demonstrated substantial variation in frost hardiness within provenances and potential for selecting southern genotypes with low risk of frost damage (Nicoll *et al*., 1996). Material from the Queen Charlotte Islands is generally recommended over much of Britain, although origins further south in Washington are better for south-west England, Wales, and parts of west Scotland (Fletcher, 1992). In the north of Germany, provenances from Washington are recommended, while fast-growing sources from Oregon are deemed to be too susceptible to frost damage (Stratmann and Tegeler, 1987). Provenances have been recommended for use in France, where plots have been established at four locations to demonstrate seed source differences (Bastien and Lemoine, 1986; François, 1986; Steinmetz, 1986). In Denmark, naturalised seed sources of the second and subsequent generations have grown faster and shown better adaptation than trees from seed imported directly from North America (Nielsen, 1994). In Germany, provenances from British Columbia (Canada) are also recommended.

8. Summary

Sitka spruce is an economically important species of the north temperate coastal rain forest of western North America. While not as commercially important as other conifers within its native range, it is a keystone species in some of the most productive ecosystems in North America. Sitka spruce is now widely planted in North Europe, where it forms the backbone of plantation forestry and is of enormous economic value in some regions. It is closely related to the other North American "white" spruces, *Picea glauca* and *P. englemannii*. As an outcrossing, wind-pollinated species and prolific seed producer, it can transfer its genes rapidly to neighbouring populations and to other related spruces.

Sitka spruce exhibits clinal variation for many growth traits, associated with latitude, elevation, and distance from the coast. Population differences are also demonstrated for resistance to insect attack. While there is great variation among populations, more than half of the genetic variation in many growth traits is found among individuals within populations. Heritabilities for growth and quality traits are sufficiently high to expect substantial genetic gain from conventional recurrent-selection breeding programs. The species is readily propagated by rooted cuttings, offering potential to capture non-additive genetic variance and to accelerate the pace of genetic improvement. Statistically significant genotype-environment interactions have been observed, but broadly adapted individuals are rather common.

The distribution of Sitka spruce is limited to an area of maritime climate with abundant moisture. It may occur as pure stands, particularly on exposed coastal sites, but more commonly occurs in mixtures with western hemlock. While Sitka spruce is tolerant to shade and may occur in climax forest types, it is dependent on disturbance for regeneration and can be an aggressive pioneer in earlier stages of succession. The white pine weevil is by far the most serious threat to stands in North America, killing the leader and seriously affecting growth and merchantability. While the weevil does not affect planted stands in Europe, the green spruce aphid, various species of deer, and windthrow can cause significant damage.

Sitka spruce is well suited to artificial regeneration. While constituting a minor component of the reforestation effort within its native range, Sitka spruce plantation programs are well developed in some parts of Europe. Genetically improved materials from local seed orchards now constitute a significant portion of deployed planting stock. While most Sitka spruce reforestation is currently based on seed propagation, vegetative propagation techniques for cuttings and regeneration of somatic embryos are well advanced, making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

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SECTION 6
STONE FRUITS (*PRUNUS SPP.*)

1. Introduction

A. General Background

The genus of ***Prunus*** *sensu latu* comprises more domesticated (also cultivated) species of temperate fruits than the other genera in the family of *Rosaceae* (*Malus, Pyrus, Sorbus, Cydonia, Rubus, Fragaria*). One of the obvious reasons for the abundant domestication might have been the coincidence between the location of the centre of variability of *Prunus* and the site of human evolution and/or of the first ancient high civilisations of human history.

Improvement of fruit trees through traditional breeding methods is a long-term effort because of their lengthy generation time. Thus, new approaches are researched to attain the envisaged breeding goals in a reasonable time frame. Genetic transformation is potentially useful, because specific genetic changes can be made. In the last few years successful examples of resistance breeding against viruses from different plant virus families have been reported, using the coat protein-mediated cross protection approach (Beachy *et al*., 1990). However, only very few fruit trees have been among these experiments due to the difficulties in transformation protocols.

"Cross protection" was originally described as the phenomenon of protection of a plant against the invasion of a severe disease-causing virus due to prior inoculation of the plant with an attenuated virus strain (McKinney, 1929). Hamilton postulated in 1980 that the expression of sequences from the viral genome, if expressed in transgenic plants, could possibly cause a protection against viruses. In fact by the expression of the viral coat protein gene in transgenic plants, similar effects could be obtained, and it was therefore distinguished as coat protein mediated protection (Beachy *et al*., 1990).

This still continues to be a difficult task among fruit trees, as can be seen from the limited number of successful reports of regeneration in woody species (McGranahan *et al*., 1988; Laimer da Câmara Machado *et al*., 1989; James and Dandekar, 1991; Oliveira and Pais, 1992; Mante *et al*., 1991) and in *Prunus* species, in particular plum (Scorza *et al*., 1994; Ravelonandro *et al*., 1997) peach (Hammerschlag *et al*., 1989), apricot and cherry rootstocks (Laimer da Câmara Machado *et al*., 1992; da Câmara Machado *et al*., 1995a,b).

B. Topics for this case study

One subject of the present study is to assess tools intended to cope with the new plagues, such as the currently incurable Plum Pox Virus (PPV) disease, which harass the growth of *Prunus* species and endanger the mere existence of the most precious, highly esteemed ancient, as well as, new cultivars.

Attempts at developing GMOs have been initiated by several research teams around the world and on such crops as plums (Scorza *et al*., 1994) and apricots (Laimer da Câmara Machado *et al*., 1992; da Câmara Machado *et al*., 1995c). Similar research is currently sponsored in Hungary, especially, to save the traditional plum variety, Besztercei. This precious local variety found fame in the last century in Hungarian

and later Bosnian dried fruit, jam, distilled beverages, etc. The rapid decline of plum production ensued, as surmised, with the release of viruses triggered by the developing trade of nursery grown graftings. The plum growing regions of the former Yugoslavia were hit most severely. As a result, the original site of the variety Besztercei was assigned to Romania after the First World War. Traditional breeding to find or to introduce resistance to PPV started about the middle of the 20th century at Cacak (former Yugoslavia). In parallel, surveys of varieties resistant or at least less affected by PPV were undertaken, but, little hope exists of finding a solution (Cociu *et al*., 1997; Hartmann, 1988).

In this study, an attempt is also made to provide background information for science-based decision making, in case such GMOs should be released into the environment. As well, this study focusses the assessment on risks and develops strategies for avoiding or counteracting said risks.

2. General Description and Use as a Crop

The genus *Prunus is* comprised of approximately 400 species of trees and shrubs. Many species and cultivars are grown for their edible fruits, while others are planted for their ornamental value. Approximately 25 species are native to the US, of which 18 reach tree size (Maynard *et al*., 1991). Like *P. avium* L. in Europe, black cherry (*P.serotina* Ehrh.) is the only member of this genus with commercial importance as a timber species in the US (Fowells, 1965). It is a high-value hardwood timber species, prized for cabinetry, furniture and veneer (Panshin and De Zeeuw, 1970).

Stone fruits (Table 3.9) are appreciated world-wide either for fresh consumption, or in the processing industry: drying, distillation, canning, production of jams, syrups and fruit juices, etc. (Druart and Gruselle, 1986). As well, they are used for their timber and their value as ornamental crops (Moore and Ballington, 1991).

Table 3.9 Stone fruit production (1000 metric tonnes) in the world from 1989 through 1999

Crop	1989-91	1997	1999
Peaches and nectarines	9317	11286	12044
Plums	6270	7845	7346
Apricots	2226	2375	2720
Almonds	1288	1554	1632

Source : FAO Production Yearbook, 1999

Plum species are found native throughout the Northern Hemisphere but mostly in the temperate zone. The earliest writings about plums date back some 2,000 years (Gautier, 1977). Plums may have been the first species among all the fruits to attract human interest. It is more remarkable that the earliest cultivation of *P. domestica* began somewhere between Eastern Europe and the Caucasian mountains, whereas *P. salicina* and *P. simonii* were brought into cultivation in Asia.

It is remarkable that other cultivated temperate fruits of the *Prunus* genus, apricot and peach, reached Europe even before the Roman empire. The Latin names of the crops refer to Armenia and Persia, respectively, indicating the path of trade in ancient times. The centre of origin of those species is rather diffuse, but much more in the East, *i.e.* in Central and East Asia. Both species "grew up" as important crops in modern Europe. Some of the reasons might be their abundance and associated wealth as well as, a whole year round offer of subtropical fruits competing with the short season temperate fruits.

The peach is one of the most varied of all fruit species, falling between trees and shrubs of fruit. There are several types of them in the canopy, vegetative and generative characteristics, namely fruit, stone and

seed traits. All commercial cultivars belong to *P. persica* L. Batsch, and are primarily grown in temperate zones between latitudes 30° and 45° N and S, and in the tropics and subtropics at higher elevations (Hammerschlag, 1986).

A close relative of the peach, the almond, represents an entirely different food quality. Its cultivation in generally dry, if not marginal habitats, is as extensive as an almost semi-domesticated fruit, less subject to phytosanitary problems. Interspecific hybrids of *P. amygdalus* and *P. persica* are well known in fruit growing as an important rootstock for peach production, *e.g.*, GF 677.

A somewhat detached subgenus of the genus *Prunus* includes the cherry and sour cherry which are, equally, ancient cultivated fruits, one of them being diploid, the other, tetraploid.

P. avium is primarily a European species, which occurs abundantly in wild form on the forest slopes of Southern, Central and Western Europe. Pomologically, according to fruit firmness, cherry cultivars are divided into the Heart cherry group, with mainly early ripening cultivars that have a soft flesh, and the Bigarreau group. The Bigarreau group includes late cultivars with firm flesh, such as Lambert, Stella, Bing, Van, Windsor, Schmidt, Hedelfingen, Napoleon, and Gold that have dark red, black, yellowish or light-coloured fruits. The major portion of the harvest is processed into solid, liquid or frozen products, and part is kept for direct consumption in the fresh state (Ivanicka and Pretová, 1986).

Sour cherry is widely naturalised and its distribution area covers almost all European countries and SW Asia. However, it is cultivated in many other parts of the world, mainly in North America. Sour cherry production is about one-third that of sweet cherry (FAO Yearbook, 1975, data not included in later editions). More recent data (in thousands tons) indicate that the most important producers are the USSR with 450 (Kramer, 1985) and the USA with 119 (Westwood, 1978). Other countries with great productions are: Germany (91), Former Yugoslavia (47), and Hungary (41) (Christensen, 1985; Kramer, 1985). Although the most important cultivars used are Schattenmorelle and Montmorency, the list of cultivars reaches a great number. Thus, in the USSR it runs up to 80 sour cherry varieties listed in various district catalogs (Kramer, 1985). The use of Stockton Morello in North America as a cherry rootstock is very minor (Tukey, 1964). The predominant root stocks in North America are mazzard (*Prunus avium*) or *P. mahaleb*.

The predominance of one or two major apricot cultivars in each production area is partly responsible for large fluctuations in yield and makes this crop species vulnerable to adverse environmental conditions, diseases and pests (Mehlenbacher *et al*., 1991). Moreover, the major cultivars of the main apricot producing countries (Spain, Italy, the United States, Greece, France, Morocco, Hungary, Romania, South Africa, Bulgaria, Australia, Algeria) belong to the European group, which by their origin are known to have a very narrow genetic background (Kostina, 1969).

Apricot production is rapidly changing in Europe. Spain, the main producer keeps its production constant, while France is increasing production and Italy and Greece are decreasing their production levels.

Especially drastic is the situation in Greece, where the annual production of 100,000 tonnes about 10 years ago has decreased to 30-50,000 tonnes, mainly due to damage caused by late frosts and the Sharka virus.

The same holds true for Hungary, where at the beginning of the Seventies 60-130,000 tons were produced on an area of 13-14,000 ha, while in the early 90s the orchard area decreased to 2,500 – 3,000 ha and production dropped to 20-40,000 tons/a (Pedryc, Budapest, pers. comm).

3. Taxonomic Situation

A. Taxonomy

In the past different approaches were chosen to present the phylogeny of the subfamily of *Prunoideae* belonging to the family of *Rosaceae*. There were two main contrasting conceptions, *i.e.* all stone fruits belong to the genus *Prunus*, or the genus *Prunus* contains only plums and prunes. Here the classification is presented according to Strasburger *et al*. (1991).

The seven subgenera in *Prunus* are determined basically by how the leaves are rolled up in the bud, whether the flowers are organised in cymes or in racemes and finally by morphological characteristics of the generative organs, *i.e.* the size and colour of flowers, fruit, stone and seed traits.

- AMYGDALUS (almonds): **P. amygdalus**, P. bucharica, P. fenzliana, P. kuramica, P. nana, P. orientalis, P. webbii
- PERSICA (peaches): P. davidiana, P. ferganensis, P. kansuensis, P. mira, **P. persica**
- ARMENIACA (apricots): P. ansu, **P. armeniaca**, P. brigantiaca, P. x dasycarpa, P. holosericea, P. mandshurica, P. mume, P. sibirica
- *PRUNUS* (plums and prunes): P. cerasifera, P. divaricata, **P. domestica**, P. insititia, P. italica, P. spinosa, P. syriaca, **P. salicina**, P. simonii, P. ussuriensis, P. americana, P. angustifolia, P. hortulana, P. maritima, P. mexicana, P. munsoniana, P. nigra, P. rivularis, P. subcordata
- CERASUS (sweet and sour cherries): **P. avium, P. cerasus**, P. fruticosa, P. japonica, P. maackii, P. mahaleb, P. pseudocerasus, P. pumila, P. serrulata, P. tomentosa
- PADUS (bird cherries) P. padus, P. serotina
- LAUROCERASUS (bay-cherries)

This study will focus on essential data about the species in bold (*P. amygdalus, P. persica, P. armeniaca, P. domestica, P. avium, P. cerasus* and *P. salicina),* since they are the most widely grown species with horticultural interest. However, interactions with wild or escaped relatives will also be considered.

B. Number of chromosomes

The phenomenon of polyploidy is a widespread occurrence and of great importance in the evolution of new species or forms. For example, many genera of flowering plants contain a series of species characterised by varying degrees of ploidy. Polyploidy is important, too, from a practical point of view, since plants with this character are often very vigorous, and may be more resistant to frost and the attacks of parasitic fungi. Moreover, changes of flower structure and self-fertility according to the number of chromosomes have been observed. In *Prunus*, the basic number in vegetative cells is eight chromosomes. Polyploidy, due to interspecific hybridisation, took place during the phylogeny of the genus and is responsible for self-sterility and intersterility. The C-value is the DNA amount in the unreplicated haploid nucleus (pg/cell). The DNA amount in the unreplicated haploid or gametic nucleus of an organismis referred to as its C-value (Swift, 1950), irrespective of the ploidy level of the taxon. C-value equals genome size in diploid species, but always exceeds genome size in polyploid species. Nuclear DNA C-value and genome size are important biodiversity characters with fundamental biological significance and many uses (Bennett and Leitch, 1995).

The following different number of chromosomes and degrees of ploidy have been reported:

Genus	Species	Chromosome number	Reference
Amygdalus	*P. amygdalus*	2n=16 (diploid)	Darlington *et al*., 1945
	P. bucharica	2n=16 (diploid)	
	P. fenzliana	2n=16 (diploid)	Darlington *et al*., 1945
	P. kuramica	2n=16 (diploid)	
	P. orientalis	2n=16 (diploid)	
	P. tenella	2n=16 (diploid)	Darlington *et al*., 1945

The cultivated almond was designated *Amygdalus communis* L. by Linnaeus in 1753. Miller (Webb, 1967) first used the name *Prunus* in 1768 in designating the cultivated 'sweet' almond as *Prunus dulcis*, describing it apparently as a 'botanical variety'. The species was later named *Prunus amygdalus* by Batsch (1801), the species name meaning 'Greek nut.' Archangeli (1882) later used the name *Prunus communis* for almond. Schneider (1904) and Rehder (1924) accepted *Prunus amygdalus* Batsch as the scientific name for almond and by which the species had been known in American botanical and horticultural literature for many years. In 1964, a discrepancy in name priority was determined to exist by the General Committee of Botanical Nomenclature of the International Botanical Congress (Punt, 1964). As a result, the name *Prunus dulcis* (Miller) D.A.Webb was proposed for the cultivated sweet almond (Webb, 1967). *Prunus amygdalus* Batsch (1801) and *Prunus communis* L. Archangeli (1882) are listed as synonyms. A flowering almond species appreciated as an ornamental is *Prunus triloba*.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
Persica	*P. davidiana*		2n=16 (diploid)	Missouri Botanical Garden, 1990
	P. ferganensis		2n=16 (diploid)	Missouri Botanical Garden, 1991
	P. kansuensis		2n=16 (diploid)	Missouri Botanical Garden, 1990
	P. mira		2n=16 (diploid)	
	P. persica	0.6	2n=16 (diploid)	Darlington *et al*., 1945 Bennett and Leitch, 1995

It has been suggested by Watkins (1979) that almond and peach, which are both regular diploids (2n = 16) originated from the same primitive species but evolved separately following the mountain development of the Central Asian massif. Almonds evolved in the arid steppes, deserts and mountainous areas to the west, south and southwest, whereas the peach evolved eastward towards China in a more humid environment and at lower elevations.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
Armeniaca	*P. ansu*		2n=16 (diploid)	
	P. armeniaca	0.6	2n=16 (diploid)	Darlington *et al*., 1945 Bennett and Leitch, 1995
	P. brigantiaca		2n=16 (diploid)	
	P. mandshurica		2n=16 (diploid)	
	P. x dasycarpa		2n=16 (diploid)	
	P. holosericea		2n=16 (diploid)	
	P. mume		2n=16 (diploid), 24	Darlington *et al*., 1945
	P. sibirica		2n=16 (diploid)	

All apricot species are regular diploids with eight pairs of chromosomes (2n=16). No difficulties have been reported in intercrossing *P. armeniaca, P. siberica, P. mandshurica* and *P. mume*, although not all combinations have been attempted.

P. x dasycarpa Ehrh., the black or purple apricot, is a naturally occurring hybrid of *P. cerasifera* Ehrh. and *P. armeniaca* and is found as isolated trees, where the distribution of the two species overlaps (Mehlenbacher *et al*., 1991). *P. x dasycarpa* has been backcrossed to both *P. cerasifera* and *P. armeniaca*; crosses to the plum parent are generally easier.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
Prunus	*P. americana*		2n=16 (diploid)	Darlington *et al*., 1945
	P. angustifolia		2n=16 (diploid)	Janick and Moore, 1975
	P. cerasifera		2n=16 (diploid), 24	Janick and Moore, 1975
	P. domestica	1.8	2n=48 (hexaploid)	Darlington *et al*., 1945 Bennett and Leitch, 1995
	P. hortulana		2n=16 (diploid)	Janick and Moore, 1975
	P. insititia		2n=48 (hexaploid), 24	Darlington, 1945, Tischler, 1950
	P. italica		2n=48 (hexaploid)	
	P. maritima		2n=16 (diploid)	Darlington *et al*., 1945
	P. mexicana		2n=16 (diploid)	Janick and Moore, 1975
	P. munsoniana		2n=16 (diploid)	Janick and Moore, 1975
	P. nigra		2n=16 (diploid)	Darlington *et al*., 1945
	P. rivularis		2n=16 (diploid)	Janick and Moore, 1975
	P. salicina		2n=16 (diploid)	Darlington *et al*., 1945
	P. simonii		2n=16 (diploid)	Janick and Moore, 1975
	P. spinosa		2n=32(tetraploid), and natural hybrids with 16, 24, 40, 48	Darlington *et al*., 1945, Janick and Moore, 1975
	P. subcordata		2n=16 (diploid)	Janick and Moore, 1975
	P. salicina		2n=16 (diploid)	Darlington *et al*., 1945, Jannick and Moore, 1975
	P. syriaca		2n=16 (diploid)	
	P. ussuriensis		2n=16 (diploid)	

The most important commercial species of plums are generally classified in two groups, the European plums (*Prunus domestica* L.) and related forms with hexaploid chromosome number (2n=6x=48) and the Japanes plums (*Prunus salicina*) and their hybrids with diploid chromosome number.

P. domestica is believed to have arisen as a natural alloploid between *Prunus cerasifera* (diploid) and *P. spinosa* (tetraploid) (Crane and Lawrence, 1952).

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
Cerasus	*Prunus avium*	0.7	2n=16 (diploid), 24, 32	Darlington *et al*., 1945 Bennett and Leitch, 1995
	P. besseyi		2n=16 (diploid)	
	P. cerasus	1.2	2n=32 (tetraploid)	Missouri Botanical Garden, 1985 Bennett and Leitch, 1995
	P. fruticosa		2n=32 (tetraploid)	
	P. mahaleb		2n=16 (diploid)	Darlington *et al*., 1945
	P. pumila		2n=16 (diploid)	Darlington *et al*., 1945
	P. serrulata		2n=16 (diploid), 24	Darlington *et al*., 1945
	P. subhirtella	0.6	2n=16 (diploid)	Darlington *et al*., 1945 Bennett and Leitch, 1995
	P. tomentosa		2n=16 (diploid)	Darlington *et al*., 1945

Among cherries, the sweet (*P. avium*) and sour (*P. cerasus*) cherry, flowering ornamental cherry species, and a few others used as rootstocks for cherries are important. The earliest description of the "keration" comes from Theophrastus about 300 B.C. *P. fruticosa*, the ground cherry, is considered the probable parent of both *P. avium* and *P. cerasus*, sweet and sour cherry respectively (Fogle, 1975).

The chromosome number of *P. cerasus* is 32 (Crane and Lawrence, 1952). As 8 is the base number of the genus *Prunus* and following De Candolle´s hypothesis (Coutanceau 1953) it seems that sour cherry is a tetraploid originating from an unreduced *P. avium* (2n = 16) gametophyte, thus by chromosome doubling.

Genus	Species	DNA amount 2C (pg)	Chromosome number	Reference
Padus	*P. padus*		2n=16 (diploid)	Tischler, 1950
	P. serotina	1.0	2n=32 (tetraploid)	Kumar and Subramanian, 1987 Bennett and Leitch, 1995

C. Molecular markers for the identification of genotypes

Since morphoplogical markers sometimes are prone to equivocal interpretations and generally time consuming, the search for biochemical and molecular markers was initiated in the genus *Prunus*. The development of DNA markers like RFLPs or RAPDs is very recent in fruit trees (Eldredge *et al*., 1992).

Initially, isoenzyme markers in *Prunus* crops like peach (Messeguer *et al*., 1987; Monet and Gribault, 1991), almond (Cerezo *et al*., 1989; Arús *et al*., 1994a) and cherry (Santi and Lemoine, 1990; Boskovic and Tobutt, 1994) were developed.

In addition to the isozyme markers, RAPD, RFLP and AFLP (Arús *et al*., 1994a,b) which are used to clearly distinguish among the different stone fruit cultivars available on the international fruit market, additional markers, such as SSRs have recently been developed for peach (Cipriani *et al*., 1999) and apricot (da Camara Machado et. al., submitted).

4. Centres of Origin/ Diversity

A. Geographic origin and natural distribution of *P. amygdalus*

Populations of almond have been described to occur in two areas: (a) the south west slopes and deep gorges of Kopet-Dagh Mountains of Turkmenistan (800-1700m), in an area, which is dry and snowless and (b) in Uzbekistan on the western slopes of the Tian Shan Mountains at similar elevations.

The almond *Prunus dulcis* (Miller) D.A Webb has been grown in cultivation for its edible seed since ancient times. From its centre of origin in Central Asia, it was disseminated to all ancient civilisation in Asia (2000 BC), Europe (350 B.C.), and North Africa (600-700 A.D.) (Vavilov, 1930; deCandolle, 1964). Almonds were initially introduced into California during the Spanish Mission Period, but significant plantings were not made until after the settlement of California following the Gold Rush (Wickson, 1910; Wood, 1925; Taylor and Philip, 1925). During the same period (1850-1900), almonds were introduced into West Australia (Quinn, 1928), South Africa, and parts of South America (particularly Chile and Argentina) in regions with the same climate as California.

Almond production is concentrated in three regions of the World: Asia, Mediterranean area (of Europe and Africa) and California (Kester and Horel, 1980).

The different species have different geographic distributions:

- P. *amygdalus* Batsch. syn. *P. communis* Arcang. is native in the south west slopes and deep gorges of the Kopet-Dagh and western slopes of the Tien-Shan Mountains
- *P. bucharica* (Korsh.) Fedtsch. is native in the steppes of Central Asia
- P. fenzliana Fritsch. is distributed in the Caucasian Mountains, near Ararat and Armenia
- *P. kuramica* Korchinsky is feraly native in slopes of the Hindukush Mountains
- *P. orientalis* Mill. is distributed in South-central Asia
- *P. webbii* (Spach) Vieh., a European almond species, which is found in the Balkans
- *P. tenella* is a European almond species with strong sprouting ability

B. Geographic origin and natural distribution of *P. persica*

Peaches are native to China and its culture dates back at least 4000 years (Wang, 1985). Wolda peaches are known as “Maotao” (hairy peach) or “Yitao” (wild peach) currently exist in remote areas of China, where they are used as seedling rootstock for improved cultivars (Li, 1984). The Chinese recognise three groups of peaches (Li, 1984; Wang, 1985). The Southern group of peach is grown along the Yangtze River in the provinces of Jiangsu, Zhejiang, Jiangxi, Hubei, Hunan and Sichuan. The Northern group of peach is found along the Yellow River in Shandong, Hebei, Henan, Shanxi, Shaanxi, and Gansu provinces, and a third group is found in the arid northwest of China. Peaches spread west from China following the trade routes through Persia. In Egypt, peaches were used in offerings to the “God of Tranquillity” about 1400 B.C. (Roach, 1985). According to Plinius, the peach was planted in Greece by 332 B.C. and was mentioned by Virgil (70-19 B.C.) in Roman literature. Along the path of distribution through Europe, adapted populations of local peaches can be found. Among these are the “vineyard” peaches of France, Romania, and the former Yugoslavia (Parnia *et al*., 1988) towards the Hungarian Great Plain. The peach was common in England by the 14th century (Bunyard, 1938). Peaches were brought to North and South America by the early Spanish explorers through St. Augustine, Florida and to settlements in Mexico by 1600.

Five species are considered as peaches:

- *P. davidiana* (Carr.) Franch. is native of North China
- *P. ferganensis* (Kost. et Rjyb.) Kov. et Kost., which is found in Western China
- *P. kansuensis* Rehd., which is native in North-western China
- *P. mira Koehne*, which is found in the Himalayan mountains and along the Yellow and Yangtze Rivers
- *P. persica* (L.) Batsch. which contains most of the cultivated peaches and nectarines (convarietas *leavis)*, be it freestone (provar. *glabra*) or clingstone (provar. *nudicarpa*).

C. Geographic origin and natural distribution of *P. armeniaca*

Prunus armeniaca L., the cultivated apricot, is believed to have originated in the mountains of Northern and North-eastern China in the same area as the Great Wall and overlapping the southern branch of the distribution of *P.sibirica* L. (Mehlenbacher *et al*., 1991). The apricot was brought via Armenia and Asia Minor into Italy over 2000 years ago, to England in the 13th century and to North America only by 1720 (Westwood, 1978).

Wild apricots also occur in the Tien Shan Mountains in the Xinijang autonomous region (Wang, 1985) and Dzhungar and Zailing Mountains in Soviet Central Asia. This is believed to be the secondary centre of origin (Zeven and de Wet, 1982). The area of distribution of the cultivated apricot is much larger and includes areas where seedling orchards are common such as Central Asia, Afghanistan, Kashmir, Iran, Turkey, and Trans-Caucasia (Kostina, 1936; Mehlenbacher *et al*., 1991). All of these areas are valuable sources of germplasm.

Apricot production is severely restricted by ecological conditions. The gene pool of apricot contains only few species and varieties, which range in areas of adaptation from the cold winters of Siberia to the subtropical climate of North Africa and from California, the deserts of Central Asia and the humid areas of Japan and Eastern China. However, commercial production areas are still very limited (Mehlenbacher *et al*., 1991; Faust - Surányi and Nyujtó, 1998).

In China, the Yu's order (2200 B.C.) refers to apricot growing and there are also documents from the 7th century (Löschnig and Passecker, 1954; Nyujtó and Surányi, 1981; Faust *et al*., 1998). Kostina (1969) presented an excellent eco-geographical grouping for apricot cultivars and species. There are Central Asian, Irano-Caucasian, European, Northern Chinese, Tibetan, North-eastern Chinese, Eastern Chinese and Dshungar-Zailij groups (Faust *et al*., 1998; Mehlenbacher *et al*., 1991). Basic species are identified, as follows:

- *P. ansu* Maxim. is distributed in Eastern China, South Korea and Japan
- *P. armeniaca* L. is native of Northern and North-eastern China
- P. *brigantiaca* Vill. (Alpine apricot) is distributed in the region of the Alps, in South-eastern France
- *P. mandshurica* (Maxim.) Koehne is native in north east of China
- P. x dasycarpa, a hybrid between P. cerasifera and P. armeniaca Ehrh.
- *P. holosericea* (Batal) Kost. (Tibetian apricot), which is native of Tibetian Mountains
- *P. mume* (Sieb.) Sieb. et Zucc., which is native in south of China

- *P. sibirica* L., which is distributed along Baikal Lake, Mandshuria, North Korea

D. Geographic origin and natural distribution of *P. domestica* and *P. salicina*

Plum species are found native throughout the Northern Hemisphere but mostly in the temperate zone. The earliest writings about plums date back some 2000 years (Cullinan, 1937) and De Candolle assumes that plums have been known for 2000-4000 years (Banegal, 1954).

Prunus domestica seems to have originated in Southern Europe or Western Asia around the Caucasus Mountains and the Caspian sea (Cullinan, 1937). However, it is also widespread in the Balkans and Mediterranean countries.

Prunus salicina originated in China and was introduced into Japan 200-400 years ago. In China, it has been cultivated since ancient times where it is thought to occur in the wild in the Tsunglin range in Shensi and Kansu. Recently, it reached Europe by way of California and Italy.

Plums are a diverse group of plants with many botanical species, that have been cultivated for the last 3000 years. The most important species of *Prunus* are generally classified into three groups, the European, the Asian and the American plums. Plums may have been the first species among all the fruits to attract human interest. Six of the most important species of plums, *P. domestica, P. italica, P. syriaca, P. salicina, P. simonii and P. americana* are not known in the wild and presumably were selected and cultivated very early by humans. It is more remarkable that the earliest cultivation of *P. domestica* began somewhere between Eastern Europe and the Caucasian mountains, whereas *P. salicina* and *P. simonii* were brought into cultivation in Asia.

Regarding the Krieche/Haferpflaume (*P. insistitia var. juliana*), repeatedly described as “wild”, Körber-Grohne (1996) mentions, that this is not the case in SW-Germany. As a welcome fruiting shrub, it has served as a shrubby hedge (Hag) around farm gardens or as a division between fruit orchards. It is not present in hedges or open fields nor is it found in woods or wood margins, as is the case with the crab apple. The oldest subfossil fruitstones have been found in Neolithic settlements in Germany and Switzerland (Ehrenstein, Robenhausen). The Krieche/Haferpflaume (*P. insistitia var. juliana*) is a typical example of the continuity of domestication from the Neolithic until the present, which has been attributed to the propagation by grafting from Roman times onwards, or in the case of plum cultivars through root suckers.

The classification of plums is divided into geographic groups:

European group	*P. spinosa L.*	Europe, Asia Minor and North Africa
	P. cerasifera Ehrh. (and *P. divaricata* Ledeb.)	With some eco-geographical subspecies in Balkan, Asia Minor, Caucasian region and Central Asia
	P. insititia L.	In Central Europe, Balkan, Western Asia
	P. domestica L.	Native in Western Asia
	P. italica (Borkh.) em. Kárpáti	Hybrid between *P. domestica* and *P. insititia* with convarietas (*pomariarum, claudiana, ovoidea and mamillaris)*
	P. syriaca (Borkh.) em. Kárpáti	Hybrid between *P. cerasifera* and *P. domestica*
Asian group	*P. salicina*	Native in the Basin of Yangtze River
	P. simonii	No wild form, only cultivated
	P. ussuriensis	Along Ussuri River
American group	*P. subcordata*	Native of California and Oregon
	P. mexicana	South-western Kentucky to Western Tennessee to Oklahoma and Mexico
	P. rivularis	Native in Texas
	P. maritima	From Brunswick to Virginia
	P. americana	From Massachusetts to Georgia to near the Golf of Mexico and to the west
	P. nigra	From New Brunswick to Northern Ohio
	P. angustifolia	From Delaware to Florida and Texas
	P. hortulana	Native in Central Kentucky and Tennessee, to Iowa and Oklahoma
	P. munsoniana	From Kentucky to Kansas and Texas

E. Geographic origin and natural distribution of *P. avium* and *P. cerasus*

Watkins (1976) suggests that the first diploid *Prunus* species arose in central Asia, and that species in the section Cerasus which includes sweet, sour and ground cherry, were early derivatives of this ancestral *Prunus*. The Cerasus cherries developed to the west of the central Asian subgenus Cerasus' centre of origin while most other Cerasus species evolved to the east.

Hedrick (1915) described the geographic range of wild sweet cherry as all of mainland Europe well into the Southern U.S.S.R. and as far east as Northern India, with the greatest prevalence between the Casparian sea and the Black sea. In contrast, the ground cherry centre of origin is Western and Central Asia (Watkins, 1976).

It is reported that sweet cherries (*Prunus avium*) were brought in 74 B.C by the Roman General Lucullus from Cerasunt on the Black Sea to Rome, and from there spread to Germany and Britain.

Sweet cherries (mazzard) have been grown from Southern Russia, north of the Caucasian mountains to the north of France for a long time. *Prunus fruticosa* Pall., the ground cherry, has a wider area of distribution, which overlaps with the centre of wild cherry, thus giving new hybrids as sour cherries. The sour cherry is native in the Carpathian Basin. Domestication and cultivation has resulted in some ecotypes of sweet and sour, or ground cherries in the various areas of Europe, and partly in Asia. Several sweet and sour types are adapted to harsh winter conditions (Iezzoni *et al*., 1990; Faust and Surányi, 1997).

Although cultivated cherries are ubiquitous in the temperate zone, there has been little effort to take them further south into subtropical regions. There are those cultivars which require low-chilling among the cherry genotypes, although the existing high quality cherry cultivars all have high chilling requirements. Commercial production of sweet cherries is limited by rain fall during the ripening period, which causes cracking of Bigarreau-type sweet cherries and subsequent brown-rot infection, which destroys the value of the fruit.

Sour cherries are not subject to cracking, nevertheless, they are also better adapted to cool climates. Most of the world cherry production is in Europe, its native home.

Duke cherries are hybrids between sweet and sour cherry varieties.

Wild and cultivated species are useful in breeding and cultivation:

P. avium L.	native of Eurasia
P. cerasus L.	a hybrid between *P. avium* and *P. fruticosa*
P. fruticosa Pall.	grows in Southeast Asia and Central and Western Europe
P. mahaleb L.	grows in Southern and Central Europe and Asia Minor
P. pseudocerasus L.	originated in the North China
P. tomentosa Thunb.	native in the Chinese provinces and Eastern Tibet

Furthermore, there exist American and Asian ecotypes:

- *P. capuli L.* Mexican cherry
- P. japonica Thunb.
- P. maackii Rupr.
- P. pumila L.
- P. serrulata Lindl.

5. Reproduction Biology of the Genus *Prunus*

A. Sexual reproduction

This genus is exceptional in the *Rosaceae* family - representing the evolutionally most advanced taxon - with a pistil reduced to one carpel only. In the pistil, there are regularly two ovules but in the majority of cases only one grows to seed. The rate of two (or even more) seeds per stone are sometimes typical for particular varieties in Pomaceous pistils but are rare in *Prunus.* The androeceum, with its three whorls of stamina with regular anthers does not show much difference from the average of the family. Seeds of pollinated fruits with embryos issued from zygotes as a product of fused sexual cells (gametes) are the main form of reproduction, even though interspecific hybrids with generative sterility and many cultivars are able to produce tillers. There is no indication of apogamy or parthenogenesis, neither of parthenocarpy, except in some varieties with signs of aborted embryos as a result of selection for extra early ripening. In that case, however, *in vitro* rescue of excised embryos may secure the survival of the offspring. The genetic dynamics of the species are secured by a high rate of outbreeding conditioned by a polyallelic system of autoincompatibility (2.4.2). The seeds are tightly closed in the stone, *i.e.*, the lignified endocarp of the fruit. For germination, however, the seeds need to be stratified, *i.e.*, exposed to temperatures below 10°C for some weeks or months, practically over the winter to start germination, moreover, excised seedlings from ripe seeds also need some "chilling" in order to develop normally, *i.e.* to produce elongated shoots axes. The same holds true for the excised, underdeveloped embryos rescued for breeding purposes.

In almond, self-incompatibility is controlled by different alleles of a gametophytic self incompatibility gene (Kester and Asay, 1975; Crossa-Raynaud and Grasselly, 1985; Socias i Company and Felipe, 1988). Self-fertility genes have been found in almond and related species. Recently, 17 additional self-fertile genotypes have been identified in wild almond populations in Italy (Reina *et al.*, 1985).

Prunus persica is self-fertile. However, pollen sterility in *P. persica* could be useful for interspecific hybridisation.

In apricot, most Central Asian cultivars are self-incompatible, while most European cultivars are self-compatible (Mehlenbacher *et al.*, 1991).

Most sweet cherry cultivars presently being grown are self-incompatible. Self-incompatibility in sweet cherry was first identified by East and Mangelsdorf (1925) and later determined to be of the monofactorial gametophytic type with multiple allelic S locus (Lewis, 1948).

Self-fertility in sweet cherry has been obtained through X-ray radiation applied to flower buds at the pollen mother cell stage (Lewis, 1948; Lewis and Crowe, 1954) and by spontaneously occurring mutations (Lewis, 1951).

B. The mating system of most cultivated *Prunus* species

The mating system of most cultivated *Prunus* species is determined by the clearly defined system of auto-incompatibility, which is inherently combined with inter-incompatibility. However, the high number (20-40) of known alleles of the single (Sx) locus with gametophytic determination allows little chance of meeting incompatible mates amongst the varieties cultivated. Those incompatible combinations are registered according to experimental proofs accumulated during the last, nearly seventy years since the phenomenon has been detected. Auto-incompatibility is expressed at different degrees between total, intermediate and scarcely identified cases. The apparent lack of such a mechanism is found in peach, although male sterility occurs at a relatively high frequency, *e.g.*, the cultivar J.H. Hale, and in some plums, *e.g.*, the cvs Tuleu gras, Pitestan and Carpentin (Silbereisen *et al.*, 1996). The evolutionary role of male sterility is highly analogous to that of incompatibility. As a general tendency of the domestication process, the increased rate of self-fertility is derived from the selection pressure for high and regular yields in crops grown for their generative organs. This can be seen in the *Prunus* species as many, mainly recently developed, varieties are self-fertile. In modern times, self-fertility has been consciously favoured. Another condition of self-fertility is polyploidy appearing on the margin of the spreading species like the European plum and sour cherry as amphiploids of interspecific hybrids in which activity of the S alleles is impaired. No doubt that some of those clones are also tillering spontaneously. Conditions of cultivation, introduction to new habitats included, are similar to the marginal areas of a species where rare mutations enjoy better chances to survive. All those reasons enhance the chances of self-fertile genotypes. Some exceptions in European plum and sour cherry prove that auto-incompatibility did not disappear entirely with amphiploidy, so a conscious effort in the research for high productivity gave rise to new, self-fertile sour cherry cultivars.

Search for radiation-induced mutants in cherry populations has spotted the Sf allele, which proved to be dominant in relation to the rest of the sterility alleles. This phenomena is employed in breeding programmes in order to produce new self-fertile cherry cultivars. The first self-fertile sweet cherry cultivar was released from a breeding program in 1968 and named Stella (Lapins, 1971). It was the result of a cross between Lambert and JI 2420. The cross was made in 1956. JI 2420 came from the John Innes Institute in the UK and was the result of a cross between Emperor Francis and irradiated pollen from Napoleon. At the moment all self-fertile cultivars have Stella in its pedigree somewhere. The John Innes Institute had a couple of other selections that were self-fertile but they have not yet made it into a named cultivar.

Bees play a major role as pollinating agents, as the pollen of *Prunus* species cannot be carried by wind and even self-pollination requires the mechanical intervention of insects.

C. Natural vegetative multiplication

Natural vegetative multiplication is rather exceptional in the genus of *Prunus*, but all subgenera have members which build up extended colonies by tillering, and some successful cultivars have been maintained by tillers since ancient times, *e.g.*, European (hexaploid) plums in the NE-Hungary (in the riverside of the Tisza river) and there are clones of semi-wild cultigens, blackthorn and sour cherry. Most of the existing cultivars are, however, multiplied by grafting and trading, except, some old varieties, such as the sour cherry variety Cigánymeggy (Hungary) and Oblacinska (Former Yugoslavia). In the group of almonds the only tillering wild species, *P. tenella*, is eligible as a potentially dwarfing stock.

More attempts have been invested into the vegetative propagation of *Prunus* rootstocks. As very few are inclined to develop tillers spontaneously, the tools of micropropagation *in vitro* gained particularly high interest, for example, in the rapid multiplication of GF 677. In fact, millions of plants are produced world-wide by *in vitro* techniques (Rosati and de Paoli, 1992). The production of self-rooted plantlets by green cuttings under a mist curtain seems to be less favoured mainly because of the questionable value of the self-rooted trees and/or the low efficiency of the technique. Grafting techniques, on the other hand, became routine several centuries ago. In addition, some stocks are propagated with cuttings.

6. Crossability

A. Interspecific and intergeneric hybrids

Interspecific (and intergeneric) relations in *Prunus* are not clear, perhaps due to the widespread presence of auto-incompatibility and the relative fertility of interspecific hybrids. The prolonged time period for flowering in the *Prunus* species and even within varieties of one species, substantially influences the possibility of mutual pollination between different cultivars, as well as different species. This is due to the different phases in flowering during the blooming season of the cultivated *Prunus* species.

The physiological or ecological diversity of the species caused sufficient isolation of their individual habitat. Consequently, they were scarcely sympatric in their natural environment, whereas, some *Prunus* species were grown, regularly, in home gardens if not in larger orchards. Interspecific barriers did not develop during natural evolution, but by the appearance of cultigenous hybrids, which triggered their development from the first steps of domestication up to the limits set by taxonomic divergence. Although the cherries are perhaps the most distant from the rest of the species, we find bridging species between plums and cherries as documented for *P. salicina*. Less difficult seems to be the gene flow between plum-apricot-peach and almond as documented by the list of successful interspecific crosses (Table 3.10).

Table 3.10 Interspecific hybrids with *Prunus persica*

P. amygdalus	x *P. persica*
	x *(P. amygdalus x P. davidiana)*
P. armeniaca	x *P. davidiana*
	x *P. persica*
P. besseyi	x *P. persica*
P. cerasus	x *P. persica*
P. hortulana	x *P. persica*
P. nana	x *P. persica*
P. persica	x *P. amygdalus*
	x *P. davidiana*
	x P. *cerasifera* var. divaricata
	x cherry (sps?)
	x *P. kansuensis*
	x *P. mira*
	x *P. nana*
	x *P. besseyi*
	x *P. salicina*
	x *P. spinosa*
P. salicina	x *P. persica*
P. spinosa	x *P. persica*
P. tenella	x *P. davidiana*
	x *P. persica*

Source : after Janick and Moore, 1975

All the species mentioned have been intercrossed with various degrees of difficulty, and grafted on each other within reasonable limits, which is proof of their genetic and physiological affinities. The use of rootstocks enlarges, dramatically, the possibilities of occupying ecological niches previously inaccessible with species on their own roots.

Hybridisation readily takes place between *Prunus amygdalus* and *Prunus persica* (Kester and Asay, 1975, 1988). Naturally interspecific hybrid rootstocks, 'GF 667', are common, where the two species are grown together.

North-American species and their interspecific hybrids, created between 1907 and 1965, represent a distinct group of cultivated *Prunus* species, the "cherry plums" (different from the species *P. cerasifera*, the cherry plum in the traditional sense). They are derived essentially from *P. besseyi* and *P. pumila*, with the western and the eastern sand cherry as a common parent (Janick and Moore, 1975) (Table 3.11).

Table 3.11 Hybrids of the sand cherries (*P. besseyi* and *P. pumila*) with other species

Sand Cherry	x *P. americana*
Sand Cherry	x *P. salicina*
Sand Cherry	x *P. salicina*
Sand Cherry	x *P. simoni*
Sand Cherry	x *P. armeniaca*
Sand Cherry	x *P. persica*

Source : Janick and Moore, 1975)

It is remarkable that peach (*P. persica*) is one of the most flexible species of *Prunus* regarding its use in interspecific crosses for breeding purposes. The documented products of these breeding efforts are divided into two distinct groups: Hybrids mostly fertile (A) and mostly sterile (B). A complicated polyhybrid background is surmised in most cases from taxa *P. davidiana (d), ferganensis (f), kansuensis (k), mira (m) and persica* (p) according to Scorza and Okie (1990) and Janick and Moore (1996) (Table 3.12). In group (A), we may consider the hybrid products as potentially new fruits, *i.e.* distinct commodities, as it happened with the sand cherry derivatives in the Midwest of the United States.

Table 3.12 *Prunus* species reported as hybrids between peach and peach species

Species		Hybrid	Common name	Origin
P. amygdalus	(A)	d.m.p.	almond	SW Asia
P. davidiana	(A)	k.p.	mountain peach, shan tao	N China
P. ferganensis	(A)	p.	xinjiang tao	NE China, S Russia
P. kansuensis	(A)	d.p.	wild peach, kansu tao	NW China
P. mira	(A)	p.	Tibetan peach, xizang tao, smooth-pit	W China-Himalayas
P. persica	(A)	d.f.k.m.	peach, maotao	China
P. americana	(B)	p.	American plum	USA
P. armeniaca	(B)	d.p.	Apricot	Asia
P. besseyi	(B)	d.p.	western sand cherry	N USA, Canada
P. brigantina	(B)	p.	Briancon apricot	France
P. cerasifera	(B)	d.p.	myrabolan plum	W Asia
P. cerasus	(B)	p.	sour cherry	W Asia, SE Europe
P. domestica	(B)	p.	European plum	W Asia, Europe
P. hortulana	(B)	p.	wild plum	C USA
P. japonica	(B)	p.	Chinese bush cherry, Korean b.c.	China
Species		**Hybrid**	**Common name**	**Origin**
P. nigra	(B)	p.	Canadian plum	N USA, Canada
P. pumila	(B)	p.	eastern sand cherry	N USA
P. salicina	(B)	f.p.	Japanese plum	China
P. simmonii	(B)	p.	Simon's plum	N China
P. spinosa	(B)	p.	sloe or blackthorn	Europe, W Asia, N Africa
P. tenella = nana	(B)	d.p.	Siberian almond	SE Europe, W Asia
P. tomentosa	(B)	p.	Chinese bush cherry, Manchu cherry	N&W China, Japan
P. virginiana	(B)	p.	choke cherry	N USA, Canada

(A) Closely related to peach producing fertile hybrids
(B) Hybrids mostly sterile. The codes of species used as parent in the ancestry:
P. davidiana (d), *ferganensis* (f), *kansuensis* (k), *mira* (m) *and persica* (p)

The only valid example of commercially recommended interspecific pollinations is between sweet and sour cherry (Nyéki and Soltész, 1996). As parthenocarpy, understood as seedless fruit, does not exist in *Prunus*, it seems obvious that seed abortion might have little chance in fruit production, however, some extra early ripening cherry and peach varieties are used to develop unviable seeds. It was proved that the excision of the embryos before the fruit have ripened facilitates the rescue of plantlets under *in vitro* conditions. Thus an efficacious technique has been developed for use by breeders in combining genes of those extra early varieties, *e.g.*, Bailey and Hough.

Because of the ease of natural hybridisation of *P. fruticosa* with *P. cerasus* and *P. avium*, some *P. fruticosa* rootstocks under testing may be interspecific hybrids.

Prunus tomentosa has been hybridised with cherry (Fisher and Schmidt, 1938; Noznikov, 1951). *Prunus salicina* hybridises easily with *P. simonii*, *P. armeniaca*, and American plum species.

No difficulties have been reported in intercrossing *P. armeniaca*, *P.sibirica*, *P. mandshurica* and *P. mume*, although not all combinations have been attempted. *P.*x *dasycarpa* has been backcrossed to both *P. cerasifera* and *P. armeniaca*; crosses to the plum parent are generally easier.

Results to date indicate that crosses between true apricot species (*P. armeniaca*, *P. mandshurica*, *P. sibirica* and *P. mume*) are successful when made in either direction and resulting hybrids are viable and fertile.

A large number of crosses between various plum and apricot species have been reported. Listed in order of flowering date, they are *P. salicina* Lindl., *P.*X *dasycarpa, P. cerasifera* Ehrh., *P. domestica* L., *P. besseyi* Bailey, and *P. maritima* Marsh. The initial cross is generally more successful when plums are used as the female parent. *P.cerasifera* x *P. armeniaca* produced hybrids resembling the natural interspecific hybrid species *P.X dasycarpa.*

Hybrids of the Asian plum species *P. salicina* with *P. armeniaca* have also been generated with little difficulty. Fertility of the hybrids varies; pollen fertility is generally quite low.

Several authors also report successful hybridisation of the hexaploid plum *P. domestica* with apricot. Resulting hybrids are tetraploid.

The beach plum, *P. maritima* has also been hybridised successfully with common apricot. More distant hybrids of apricots with peach and almond have been reported. These crosses are quite difficult to make and the resulting hybrids are often weak and sterile. The incorporation of genes from *P. persica* could conceivably greatly expand the areas in which apricots could be grown.

B. Introgression into wild relatives

The introgression between cultivated and wild species is scarcely documented. There is no doubt concerning the physical possibility. Escapes of cultivated varieties are frequently found in woods, pastures, abandoned orchards, ruderal, suburban, and marginal areas. Intercrosses with really wild populations have very little chance, as blackthorn, hedge cherry and dwarf almond (*P. tenella*) are extremely different in morphology, as well as in adaptation, *i.e.* eventual hybrids could only survive in a much protected environment. Cherries may have more chances as far as introgression into the wild populations is concerned. It is worthwhile to consider the escapes of varieties and species introduced as rootstocks to nurseries and grown out from the roots and stumps of destroyed grafts in abandoned orchards. That is how a high diversity of cherry plums have been naturalised recently. As a result, the cherry plum has become much more tolerant than the European plum and apricot to the destructive effect of Plum Pox Virus (PPV). Escaped rootstock varieties and spontaneous hybrids of ancient, as well as, recently introduced varieties are a general phenomena found in neglected orchards, and escapes of no immediate relation to fruits growing in the area are found. For example, *P. serotina, P. mahaleb, P. padus,* bitter almonds.

In Central Europe, the possibility of introgression is much more limited to the Near East, Caucasus, Iran, Central Asia and the Chinese subcontinent, where a huge wealth of intermediate and semi-cultivated forms reside.

7. Domestication of *Prunus* sp.

A. Breeding of *Prunus*

One of the obvious reasons for the abundant domestication of the *Prunus* species might have been the coincidence between the centre of variability of *Prunus* and the site of human evolution and/or of the first ancient high civilisations of human history. The easily fossilised stone of the fruit proved that fruit of

considerable size existed long before the appearance of man. Plums "offered themselves" to man to be domesticated. According to ecological arguments, it is highly probable that the today despised species, *e.g.*, blackthorn (*P.spinosa*) followed men as a "secondary crop" which is reflected in its occurrence as a witness of the ancient Neolithic culture on the outskirts of the villages, roadsides and pastures as hedges. The ancestors of that tetraploid species are unknown. It survived adversities of severe pasturage but benefited from deforestation during the spreading of primitive agriculture in the Near East and Europe, where its more vigorous relative, the favoured fruit tree, the diploid cherry *plum (P. cerasifera)* presented a permanent temptation for crossbreeding. The appearance of the European plum (*P. domestica* and *insititia* included) was not an unique and endemic event in the history of the Eurasian region. The hexaploid, (amphiploid) species has been reproduced at several instances by purposeful breeding, according to the model of bread wheat, triticale, tobacco, oilseed rape, garden strawberry, and other cultivated species, first perhaps by Rybin, a disciple of Vavilov in the 1930s. Since ancient and medieval times, the European plum made an important carrier, first owing to its ability of producing tillers as its alleged ancestor, the blackthorn also did, and secondly, because it became naturalised in mesophytic marginal cultivated areas and some river flats (*e.g.*, of Felsö-Tisza). As the most important fruit and almost staple food, it served the well being of poor people for centuries. However, later on, it became one of the first horticultural products to be exported and became of this, was seen as a symbol of wealth (as the greater number of plum trees on the manor of a member of the gentry, the more wealthy he was). The distilled drink achieved its fame as the national drink in S-E Europe, replacing gin. Recently, the fate of the European plum has been severely impaired, by the Plum Pox Virus, which was identified during the first part of the 20th century.

In the rich choice of plums, a host of species originating from East Asia and North America are diploid. These species have been inter-crossed with the cherry plum with considerable success. As a result, in the last few decades there has been an impetus of the so-called Japanese Plums. The first documented attempts are due to Luther Burbank, a Californian breeder at the end of the 19th century. Those interspecific hybrids, however, well represented in the list of cultivars, only represent a small fragment of the huge gene reserves of the Northern Hemisphere. As a source of precious genes, especially for resistance and special qualities lacking from the traditional European plum, these varieties are to be kept in mind.

B. Conservation of *Prunus* genetic resources

The International Plant Genetic Resources Institute (IPGRI), formerly known as the International Board for Plant Genetic Resources (IBPGR) has elaborated a descriptor list for plum, peach, cherry and apricot (IBPGR 1984a,b,c, 1995) and developed recommendations for the safe movement of germplasm of stone fruits (Diekmann and Putter, 1996).

The European Information Platform on Crop Genetic Resources has been established under the umbrella of the European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR) to facilitate access to information about genetic resources conserved in genebanks throughout the Region. The European *Prunus* database has been maintained by Dr. Anne Zanetto at the "Institut National de la Recherche Agronomique" (INRA) in Bordeaux, France, under the initiative of the European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR) since 1994. The database includes, in 26 European countries, the collections of all *Prunus* species, cultivated stone fruit and their related species (even the wild ones). The database is comprised of 19 passport data from the IPGRI/FAO Multicrop descriptors list, 13 descriptors common for all the different species and 3 to 7 specific descriptors depending on the species of the accession. These descriptors are mainly morphological. The possibility of including more agronomic or physiological descriptors is under consideration by the ECP/GR *Prunus* Working Group. The database has been supported for three years by the European Union in the "European Programme on the conservation, characterisation, collection and utilisation of genetic resources in

agriculture", under the title "International Network on *Prunus* genetic resources" (GENRES61). In addition, there are gene banks in China and Japan.

C. Synecology

In Europe, some wild, native and escape species of *P. nana, P. avium, P. fruticosa, P. mahaleb* and *P. spinosa* can be found in natural and cultivated forests or ruderals.

D. Interaction with pathogens

Rosaceae, in general, and *Prunus* species, in particular, are prone, in varying degrees, to infections by a range of pathogens, *e.g.* fungi such as *Monilia laxa*, *Taphrina deformans*, bacteria, such as *Pseudomonas,* and *Xanthomonas*, viruses, such as PPV, PNRSV and PDV and phytoplasmas such as European Stone Fruit Yellows (ESFY) (http://www.boku.ac.at/pbiotech/phytopath).

The main problems associated with apricot growing include: die-back or apoplexy, sensitivity to viruses, frost damage in winter and spring, fungi cankers and alternate bearing.

In recent years, a viral pathogen became the major threat to stone fruit cultivations in large areas of Central and Southern Europe and other Mediterranean countries. This pathogen was responsible for considerable economic losses and reduction of production areas. The Plum Pox Virus (PPV), causal agent of Sharka disease and a member of the potyvirus family, was classified by US and EC plant quarantine agencies as the most important pathogen in apricots, plums and peaches (the only plant pathogen for which an APHIS plan exists (Scorza, 1991). Sharka infection data from Spain, Greece, France and Italy clearly demonstrates the economic consequences of this threat (COST 88 Plum Pox Virus Workshop -Potyvirus Group, Valencia June 1993), as apricot cultures are increasingly being replaced. Apricot appears to be the most sensitive stone fruit towards infection with PPV. Apricot production was practically erased in the late 70s in some valleys of Northern Italy, *e.g.*, Vintschgau (Eynard *et al*., 1991), and is seriously threatened in some Austrian valleys, *e.g.* Wachau (Pieber, pers. comm). After its appearance in the South American Continent (Herrera *et al*., 1997) in 1999, it has been confirmed for the first time in the US (http://aphis.usda. gov/lpa/press/1999/10/plumpox.txt). This prompted Canada to close the entry of *Prunus* material from the US (http://www.cfia-acia.agr.ca/english/corpaffr/ newsrelease/19991122e.shtml).

Considering the severity of the disease, the difficulty to control its spread, and the lack of resistant cultivars, the necessity of resistant cultivars is evident and a straight-forward strategy is required. In fact there are no species resistant to Plum Pox Virus among the species sexually compatible to crop cultivars. This means it is not possible to obtain resistant cultivars by conventional hybridisation.

The information concerning the interrelation of *Prunus* species gives an opportunity of considering their pathosystems. Most pathogens and pests have developed along evolutionary pathways in parallel with the domestication and evolution of new species and hybrids. Because of geographical continuity, most species coming from the East carried their parasites freely. One of the best examples is the green peach aphid, *Myzus persicae*, which proved its fidelity to the peach tree in spite of being polyphagous; neither related species substituted the peach as a primary host. It is remarkable that even though the peach was one of the last oriental species to arrive in Europe, the green peach aphid became one of the most efficient vectors of viral diseases in Northern Europe. On the contrary, a pathogen *Taphrina deformans* did not harm either of the relatives of peach. Although, another adapted disease, *Polystigma rubrum* kept to the European plum (and blackthorn) as host, in spite of the permanent sympatric presence of relatives of the *Prunus* genus. Different susceptibilities to other diseases and pests indicates the existence of genetic resistance in spite of the possibly, small genetic divergence, *e.g.* the reduced proliferation of aphids on apricots in relation to peach and plum.

The increasing threat of stone fruit production in Mediterranean countries by phytoplasma diseases has been recognised in recent years. Although severe decline of European stone fruits was reported as early as 1924 on apricots in France and in 1933 on Japanese plums, in Italy it was only in 1973 that their phytoplasma aetiology was discovered. At that time, phytoplasmas were called mycoplasma-like organisms (MLOs). As different *Prunus* species were affected, different disease names were given: apricot chlorotic leaf roll (ACLR) on apricots, plum leptonecrosis (PLN) on Japanese plums, peach yellows, peach rosette and peach vein clearing (PVC) on peach and several other decline diseases on European plum, almond and flowering cherry. *Prunus* rootstocks are also severely affected by similar disorders. Common symptoms are yellowing and leaf roll in summer, off-season growth in winter, die-back and a more or less rapid decline. Up to now these diseases have been restricted to the southern half of Europe with their northern border in Germany. In the past few decades they have been of increasing economic importance, *e.g.* ACLR and PLN are especially devastating for apricots and Japanese plums. Molecular analysis of the pathogen revealed that only one type of phytoplasma, the European stone fruit yellows (ESFY) phytoplasma, is associated with all these diseases (Jarausch *et al*., 2000). ESFY phytoplasmas are genetically different from phytoplasmas infecting *Prunus* species in North America. ESFY phytoplasmas are classified as quarantine organisms by European legislation (Laimer da Câmara Machado *et al*., 2001; Heinrich *et al*., 2001). Upon experimental inoculation, apricot, peach and Japanese plum are the most susceptible stone fruits whereas European plum and almond are more tolerant and cherries appear to be resistant (Jarausch *et al*., 2000).

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SECTION 7
WHITE SPRUCE (*PICEA GLAUCA* (MOENCH) VOSS)

1. Forestry Practices

White Spruce is the most commonly planted tree species in Canada, accounting for more than one-third of all reforestation throughout the (Kuhnke 1989). Active tree breeding and orchard programmes exist in every Canadian province, a distinction not shared by any other species (Fowler and Morgenstern 1990; Lavereau 1995). Breeding programmes in the United States are active, with productive White Spruce seed orchards in New England, New York and the Lake States (Carter and Simpson 1985; Carter 1988; Stine *et al.* 1995).). White Spruce is the dominant reforestation species in the upper Lake States (Rauscher 1987). In Germany, *Picea glauca* has had limited use as a species with potential for reclaiming badly polluted industrial areas (Weiss 1986). Elsewhere in the world, White Spruce is of much less importance. With the exception of limited ornamental use, it is not generally planted outside its North American range.

A. Deployment of reforestation materials

White Spruce has a long history as a preferred species for reforestation across its range. In the early years, most planting stock were produced as seedlings or transplants in bareroot nurseries (Stiell 1976). Following developments in nursery technology, most planting stock for White Spruce are now produced from seed in containerised systems, in soil-less growing media. A variety of containers are used and stock is raised in both heated and unheated greenhouse structures. Cultural techniques have become highly sophisticated, ensuring that high-quality planting stock can be produced reliably and efficiently (Landis *et al.* 1989, 1990a, b, 1992, 1995).

White Spruce planting stock can also be produced by means of vegetative propagation. The simplest approach is to bulk-up (vegetatively multiply) tested crosses between selected individuals. White Spruce cuttings taken from the seedlings can then be rooted to produce stecklings for deployment (Russell and Ferguson 1990).

Techniques for the initiation and regeneration of somatic embryos have been available for White Spruce for about 10 years (*e.g.*, Hakman and Fowke 1987; Lu and Thorpe 1987; Hakman and von Arnold 1988). In fact, work on *Picea* glauca has been responsible for many *in vitro* technologies now used with coniferous plants. Since then, technical progress has been rapid, and the production of White Spruce planting stock is also now possible by means of somatic embryogenesis. In addition to *P. glauca* somatic embryogenesis has also been achieved in *P. engelmanni* and in *P. glauca engelmannii* complex (Wilson and Thorpe, 1995). Work by Lulsdorf *et al.* (1993) also describes the development of encapsulation of somatic embryos. While embling production systems have not yet achieved operational status in White Spruce, nursery and field testing has demonstrated that performance of emblings is comparable to that of seedling stock (Grossnickle and Major 1994a, b; Grossnickle *et al.* 1994). Embryogenic lines can be successfully regenerated after cryostorage (Cyr *et al.* 1994; Park *et al.* 1994), making it possible to maintain genotypes in a completely juvenile condition during clonal

testing. White Spruce embling propagation systems are also being automated further through the application of bioreactor technology to produce "synthetic" seeds (Attree *et al.* 1994).

The advancement of *in vitro* propagation of *P. glauca* has played an important role in the more recent success in genetic transformation of the species. *P. glauca* was the second coniferous species to be stably transformed (Ellis *et al.* 1993). The number of stably transformed coniferous species remains rather low (five to ten species).

The use of direct seeding as a regeneration technique for White Spruce has fluctuated, and results have been erratic (Waldron 1974; Stiell 1976). Its operational use has been largely restricted to Alberta, where direct seeding of White Spruce is often performed as a species mixture with *Pinus contorta* (Kuhnke 1989).

B. Provenance transfer

A long history of provenance tests has demonstrated the general wisdom of using local White Spruce seed sources in the absence of tested alternatives. While some movement of genetic material from point of collection to site of establishment is inevitable, these transfers are normally controlled either a set of transfer rules, regulating distance of movement, or seed zones, where it is assumed that adaptation of populations has been shaped by climate and other ecological factors (Morgenstern 1996). Both are appropriate for a species like White Spruce, where genetic variation is predominantly clinal. Seed zones have been most commonly used throughout the range of White Spruce, where they are normally based on ecological classification schemes (*e.g.*, Fowler and MacGillivray 1967; Konishi 1979). With provenance test data in hand, White Spruce seed zones may be revised to recognise the amplitude of genetic variation and stability over regions (Govindaraju 1990).

While local seed sources are generally recommended for White Spruce, some provenance transfers have been demonstrated to be particularly promising. Provenances from the Ottawa Valley region have continued to perform better than local sources in several field experiments, to the point that breeding programmes based on this material have been recommended in the Lake States (Nienstaedt and Kang 1983), New Brunswick (Fowler 1986) and Quebec (Beaulieu 1996).

C. Breeding programmes

With experimental evidence that substantial genetic variation was to be found within populations, selection of plus-trees has been a common starting point for most improvement programmes. The actual improvement realised through plus-tree selection systems may vary considerably, depending on the techniques used and the stand situations, all of which affect selection intensity, genetic variance and heritability for traits of interest (Morgenstern and Mullin 1988; Cornelius 1994). In White Spruce it is particularly important that sampling of the founder population by selection be balanced, and that breeding strategies maintain this balance to avoid rapid loss of genetic diversity in the breeding population (Nienstaedt and Kang 1987).

Breeding programmes are now well established throughout the range where White Spruce is planted. Regional breeding strategies have been prepared which generally utilise a system of progeny testing and recurrent selection for generation advancement, combined with clonal seed orchards for production of improved seed and usually involving multi-agency co-operation (*e.g.*, Carter and Simpson 1985; Fowler 1986; Dojack 1991; Lamontagne 1992; Stine *et al.* 1995). Flowering of young White Spruce grafts can be stimulated by means of various cultural treatments, particularly those involving gibberellin $A_{4/7}$, and this has facilitated the turnover of breeding cycles (Greenwood *et al.* 1991; Daoust *et al.* 1995).

Most seed orchards currently in production were established by grafting cuttings from plus-trees, and establishment in cultivated field environments. Early data indicated that such orchards would average over 1 million viable seeds per hectare by the time they entered their productive period (Nienstaedt and Jeffers 1970; McPherson *et al.* 1982). Many of these first-generation orchards are now in production, and some regional nursery requirements are now met completely by orchard seed. Some programmes have also experimented with the management of containerised White Spruce orchards. While container orchards can be conveniently managed to maximise genetic value and to promote flower production, the yield has seldom been more than 10-15 filled seed per cone and requires further development of cultural protocols (Webber and Stoehr 1995).

D. Conservation of genetic resources

Domestication of a key species such as White Spruce can influence diversity of genetic resources (1) indirectly, through the method of seed collection, extraction and storage, and through nursery and plantation culture; and (2) directly, through intentional selection to increase the frequency of genes for desirable traits (Morgenstern 1996). The inadvertent loss of genes through natural processes and human activity can have negative consequences for the adaptability of populations and the potential for future gains from breeding.

Throughout most of the range of White Spruce, *in situ* conservation of genetic resources is practised by protecting of ecological reserves, special areas and parks (Pollard 1995), and is integrated with domestication activities that control the movement of seed, active management of existing stands to maintain biological diversity, and protection of small isolated populations (VanBorrendam 1984; Dhir and Barnhardt 1995; Villeneuve 1995; Yanchuk 1995). As outcrossing rates in White Spruce stands can be lower than those of other conifers, inbreeding depression related to population size is a concern for *in situ* conservation efforts. Studies have been initiated to develop guidelines on minimum viable population size (Mosseler *et al.* 1995).

Ex situ conservation, through cryopreservation of germplasm, off-site maintenance of populations in arboreta and clone banks, and multi-population breeding strategies (Eriksson *et al.* 1993; Namkoong 1995), has been practised to a much lesser extent, although many White Spruce provenances and families are now represented in field tests and seed bank collections (Plourde *et al.* 1995). Such "active" forms of gene management must be accelerated in preparation for response to rapid environmental and climate changes (Ledig and Kitzmiller 1992).

2. Taxonomy

White Spruce (*épinette blanche* in French Canada) is one of about 40 species of the genus *Picea* A. Dietr. (family Pinaceae) distributed throughout the cooler parts of the North Temperate Zone and higher elevations in the south, and one of seven species native to North America and five native to Canada (Farrar 1995). Its scientific name is now well recognised as *Picea glauca* (Moench) Voss, although it has also been referred to in the literature under an array of botanical synonyms including *Picea canadensis* B.S.P. and *Picea alba* Link. (Sutton, 1970; Krüssmann, 1985). Its colloquial synonyms are even more numerous. They include cat spruce, skunk spruce and Canadian spruce in English, and *épinette à bière*, *épinette des champs* and *sapinette blanche* in French (Sutton 1970).

A variety is generally recognised as Porsild spruce (*Picea glauca* var. *porsildii* Raup) in northern Alberta, the Yukon and Alaska (Farrar 1995). Introgressive hybridisation between white and Englemann spruce (*Picea englemannii* Parry ex Engelm.) is common where the two are sympatric in western Canada, Montana and Wyoming, and the hybrids have given rise to a variety known as *Picea glauca* var. *albertiana* (S. Brown) Sarg. (Roche 1969; Roche *et al.* 1969; Daubenmire 1974).

Introgressive hybridisation between white and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) also occurs in sympatric areas in northwestern British Columbia and Alaska, with the hybrid known as *Picea* × *lutzii* Little (Roche 1969; Copes and Beckwith 1977; Yeh and Arnott 1986). A rare natural hybrid between white and black spruce (*Picea mariana* (Mill.) B.S.P.), known as "Rosendahl" spruce, has been recognised in the southern part of the range (Little and Pauley 1958; Riemenschneider and Mohn 1975) and has been reported as occurring commonly in northwestern Canada (Larsen 1965; Roche 1969), but its F1 hybrid status has been questioned (Parker and McLachlan 1978). Many named horticultural varieties are recognised (Krüssmann 1985; Griffiths 1994).

There is lack of agreement among taxonomists regarding the subdivision of the genus *Picea* (Schmidt-Vogt 1977). Most early taxonomists suggested dividing the genus into three sections: Eupicea (or Morinda), Casicta and Omorika. Mikkola (1969) recommended recognition of only two sections: Abies and Omorika. After extensive crossability studies, Fowler (1983, 1987a) has suggested that the section Omorika be further divided into two subsections, Omorikoides and Glaucoides, with White Spruce assigned to the latter together with Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Engelmann spruce (*Picea engelmanni* Parry).

3. Centres of Origin/ Diversity

A. Natural distribution

The natural range of White Spruce extends from the Atlantic to within 100 km of the Pacific Ocean, and from the northern tree limit across North America south into northern New England, New York and the Lake States (Sutton 1970). Over this tremendous range it is found at elevations ranging from sea level to 1 520 m (Nienstaedt and Zasada 1990). Alone or with black spruce and tamarack (*Larix laricina* (Du Roi) K. Koch), White Spruce forms the northern limit of tree-form growth. Outlier populations have been reported as far south as the Black Hills in Wyoming and South Dakota (Sutton 1970).

Several range maps have been prepared for White Spruce, but that drawn by E.L. Little, Jr. and presented in Fowells (1965) has formed the basis for maps found in current reference publications (Nienstaedt and Zasada 1990; Farrar 1995). Little's map is shown in Figure 3.6.

B. Evolution and migrational history

Fossil records indicate that divergence of genera in Pinaceae occurred some 135 million years ago during the late Jurassic or early Cretaceous period (Florin 1963). Based on comparative immunological studies, Prager *et al.* (1976) have suggested that *Picea* was among the first genera to emerge.

Although not supported by fossil evidence, Wright (1955) suggested eastern Asia as the likely origin of *Picea*, based on the abundance of species and particularly the presence of *Picea koyamai* Shirasawa, which he felt is a primitive species. *Picea* is then thought to have migrated to North America in one or more waves via a land bridge between Siberia and Alaska (Wright 1955). Critchfield (1984) cites fossil evidence that the White Spruce extended in a broad, shifting pattern across much of North America by the Late Pleistocene.

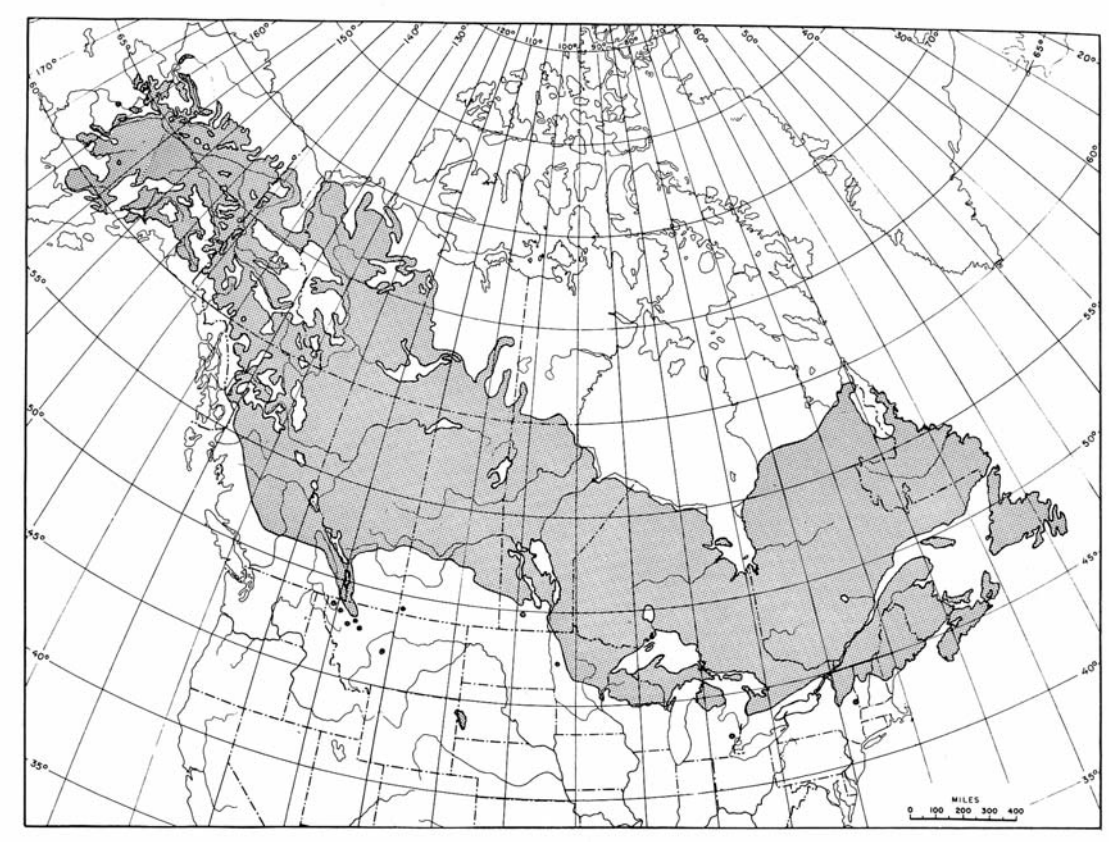
Phylogenetic relationships within coniferous genera are commonly interpreted from species crossability studies, where it is assumed that the more related are two species, the more easily they can be crossed (Wright 1955; Critchfield 1973). The close phylogenetic relationship between the northwestern American "white" spruces (white, Sitka and Engelmann spruce) and the eastern Asiatic

P. jezoensis (Sieb et Zucc.) Carr. (Wright 1955; Roche and Fowler 1975) supports this theory, at least for the members of subsection Glaucoides in section Omorika.

Fossilised cones of an extinct species, *Picea banksii*, found on Banks Island in Arctic Canada can only be distinguished from those of White Spruce on the basis of mean size. These provide evidence that White Spruce, or a close ancestor such as *P. banksii,* was the link between North America and Asia, rather than *P. jezoensis* (Hills and Ogilvie 1970).

Radiocarbon evidence suggests that White Spruce was likely found at least 280 km further north during the Climatic and Little Climatic Optima, 3 500 and 900 years ago (Sutton 1970). During the Pleistocene glaciation, a main eastern refugium extended further south into the Great Plains and perhaps as far as Lee County, Texas (Potzger and Tharp 1943; Graham and Heimsch 1960), and into North Carolina (Frey 1951). Meanwhile, western refugia are considered to have existed in the Yukon-Alaska and the lower eastern slopes of the Rockies, joined by a "fluctuating corridor" through Alberta (Nienstaedt and Teich 1972). It is considered that these east and west populations then followed the retreat of the ice sheet, meeting in the Great Lakes region (Halliday and Brown 1943; Löve 1959).

Figure 3.6 The natural range of White Spruce



Source : Fowells, 1965

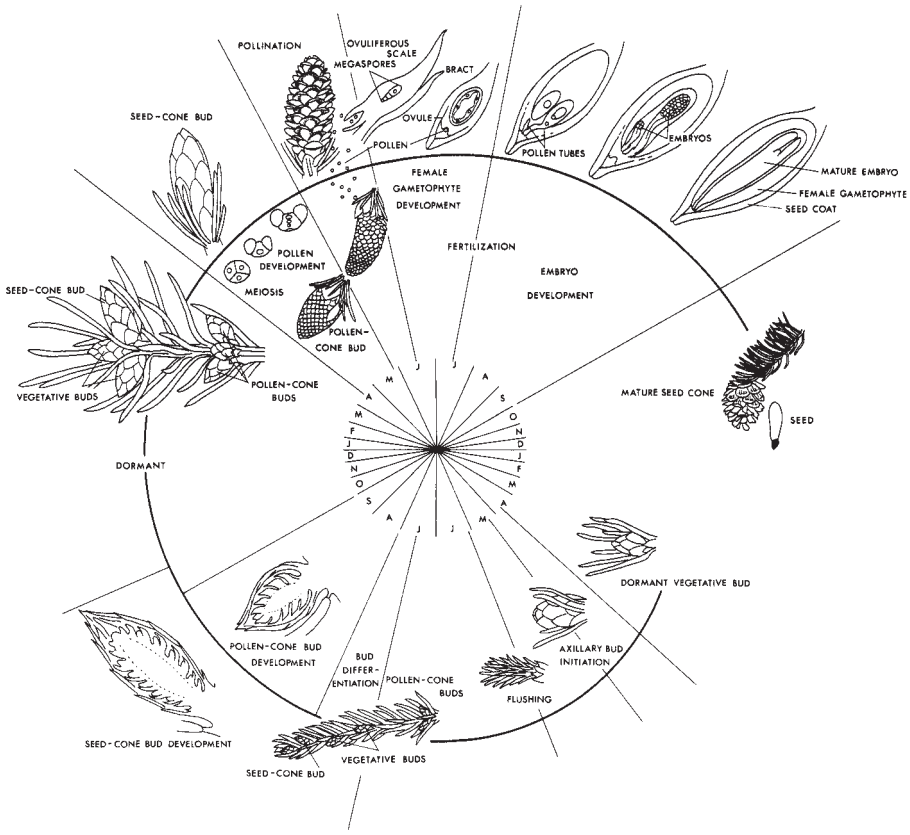
4. Reproductive Biology

A. Reproductive development

White Spruce is monoecious. Development of the reproductive structures follows a two-year cycle typical of most conifers in the northern hemisphere (Figure 3.7), other than *Pinus* species and members of the Cupressaceae family (Owens and Blake 1985). Bud scales are initiated at the terminal apex, and at newly initiated axillary apices within the enlarging vegetative buds, from about late April (Owens *et al*. 1977). Apices differentiate as vegetative, pollen cone or seed cone buds around mid-July, at the cessation of shoot elongation. The proportion of apices differentiating as reproductive buds may be increased by hot, dry weather at the time of differentiation, particularly if preceding cone crops have been poor (Owens and Blake 1985; Nienstaedt and Zasada 1990).

Pollen cone bud development is complete by early October, when they become dormant. Completion of seed cone and vegetative bud development follows shortly thereafter. By the time buds become dormant, all microsporophylls, microsporangia, bracts and functional ovuliferous scales, and leaves have been initiated. Megaspore mother cells are present in the dormant seed cone buds, although meiosis has not begun (Owens and Molder 1977, 1984). Overwintering vegetative buds are small and dome-shaped. Reproductive buds, usually terminal or subterminal, may be identified by their larger size and ovate to obovate shape. Distinguishing between male and female buds may be difficult without dissection, but males are generally found in the middle to lower crown (Eis 1967a; Eis and Inkster 1972).

Figure 3.7 The reproductive cycle of White Spruce



Source : Owens and Molder, 1984

Reproductive and vegetative buds break dormancy at about the same time, in response to photoperiod, while subsequent development is regulated by temperature. Meiosis and subsequent development of pollen occur immediately, followed by maturation of the megagametophyte. The developmental morphology of reproductive structures was well-documented with colour photographs by Ho (1991). Flushing of reproductive buds precedes that of vegetative buds, and pollen is released over a one-week period. The pollen enters receptive seed cones and adheres to the sticky micropylar arms. When the cones close, a "pollination drop" draws the pollen into the ovule (Owens and Molder 1979; Ho 1984; Runions *et al.* 1995). Transport of the pollen to the micropyle may be facilitated by rainwater (Runions and Owens 1996). Fertilisation occurs three to four weeks later, and embryo development is completed in early to late August (Owens and Molder 1979; Zasada 1988). Female gametophytes may abort at many stages of development, but most commonly at or shortly after meiosis (Owens and Molder 1984). Without fertilisation, no embryo is formed and the megagametophyte tissue degenerates, leaving a normal-sized but empty seed (Owens and Molder 1979).

B. Mating system and gene flow

White Spruce is a wind-pollinated, monoecious species, and outcrossing is by far the most prevalent mating system. Self-pollination occurs to some degree, as the period of pollen release and female receptivity coincide for an individual tree (Nienstaedt 1958). Female strobili are concentrated in the top quarter of the crown, while males are more prevalent in the mid to lower crown, but the effectiveness of this zonation against selfing is questionable (Nienstaedt and Teich 1972). The two-step pollination mechanism, whereby pollen is collected in the sticky micropyllar arms over the receptive period, and only then drawn en masse by the pollination drop, ensures that pollen from many sources has a chance to fertilise any given ovule (Runions *et al.* 1995). Under controlled pollination, where large quantities of pollen are applied at one time, the micropylar arms become fully occupied and it is more likely that the first-on become first-in (Franklin 1974; Ho 1985). The nucellus itself can accommodate two or three pollen grains, and there is potential of some postzygotic selection to reduce the number of selfed embryos (Fowler 1987b). The incidence of selfing also varies greatly as a function of population size and structure. Selfing rates in a White Spruce seed orchard varied from 0 to 22% (Denti and Schoen 1988). However, the presence of several ramets of the same clone might generate a pollen cloud quite unlike that in a natural stand. Although outcrossing pollen has no advantage over self-pollen prior to fertilisation and self-fertilisation, can occur, most selfed embryos fail to develop, likely as a result of homozygous lethal recessive genes (Mergen *et al.* 1965; Nienstaedt and Teich 1972).

Natural inbreeding among related trees is common in small populations. In two such stands in New Brunswick, the average inbreeding coefficient was estimated as $F = 0.145$ (Coles and Fowler 1976; Park *et al.* 1984). An electrophoretic study of six stands near the limit of the range in subarctic Quebec displayed deficiencies of heterozygotes at 60% of the loci analysed and suggested rapid accumulation of inbreeding. Differences among these isolated populations were large ($F_{st} = 0.113$), suggesting that genetic drift might be important (Tremblay and Simon 1989). Substantial deviations from random mating and high rates of inbreeding were also observed within stands in Newfoundland (Innes and Ringius 1990) and in a seed production area in Alberta (King *et al.* 1984). Another electrophoretic study of trees throughout a 19 ha stand in eastern Ontario demonstrated that, while selfing was not a major component of the mating system and there was an excess of heterozygotes in both the parental and filial populations, other forms of non-random mating among relatives and restricted transmission distances of effective pollen gametes were important (Cheliak *et al.* 1985).

Gene flow in *Picea* is mediated by small pollen grains, 70-85 nm at their widest point (Eisenhut 1961), whose bladdery wings make them well-adapted for aerial transport (Di-Giovanni and Kevan 1991). Various studies of pollen dispersal in conifers indicate that over 90% of the pollen comes to rest less than 100 m from the source (Wright 1976). Nevertheless, a substantial quantity may travel great distances; Gregory (1973) cites reports that pollen of *Pinus* and *Picea* may travel as far as 600 to 1 000 km, and several authors have concluded that isolation distances of less than 1 km often have little impact on contamination rates in conifer seed orchards (see review by Di-Giovanni and Kevan 1991). A recent study of pollen dispersal dynamics in a black spruce seed orchard indicated that "large amounts" of pollen rose to a height of 300 m above ground level (Di-Giovanni *et al.* 1996). At a steady wind speed of 5 m s^{-1}, the authors calculated that spruce pollen reaching this altitude would drift about 47 km. Another study examined pollen contamination within a White Spruce seed orchard that a forest fire had isolated from native stands by as much as 3 km upwind (Caron *et al.* 1994; Mercier *et al.* 1994). In a heavy pollen year, contamination levels within this well-isolated orchard were estimated at 93%.

C. Seed production

Cones and seeds may be produced on White Spruce as young as four years (Sutton 1970), but most trees do not produce seed until 10 to 15 years. Significant seed production normally occurs on trees that are at least 30 years of age (Nienstaedt and Teich 1972). Periodicity of seed production and crop size are extremely variable. Good cone crops are borne irregularly, but on average every four years (Stiell 1976). In a "heavy" crop year, Waldron (1965) estimated that a mature stand in Manitoba produced 13.8 million seeds/ha, but that only 59% were sound. In a "moderate" seed year, the stand produced 2.5 million seeds/ha. Maximum seed production over a 13-year period in a stand in Alaska was 40 million seeds/ha, and in three years exceeded 10 million seeds/ha (Nienstaedt and Zasada 1990).

Initiation and duration of seed dispersal are weather and site dependent (Zasada 1988). The mature cones open as they lose moisture and the scales flex in dry weather, reclosing during wet periods. Seed dispersal begins in mid- to late-August, with most seeds released in September (Crossley 1953; Waldron 1965; Dobbs 1976). The interval between seed ripening and beginning of dispersal can be less than two weeks, which creates problems in determining the best time to collect cones (Smith 1983; Mercier and Langlois 1992). At some northern sites, seed dispersal may begin before seeds are fully mature (Mercier 1994). In practice, cone collection can begin a couple of weeks earlier if seeds are allowed to "artificially ripen" by storing the cones under cool, moist conditions (Winston and Haddon 1981; Caron *et al.* 1990, 1993).

The seeds are winged and wind-dispersed. The actual distance reached from the source varies from site to site (Dobbs 1976), but in one study less than 5% of the seeds were dispersed more than 100 m from the source (Zasada and Lovig 1983). The seeds themselves are small, and average cleaned seed weight is about 2.0 g/1 000 seeds (Safford 1974).

D. Natural regeneration

White Spruce seeds exhibit varying degrees of dormancy that may be broken by exposure to low temperatures under moist conditions, *i.e.*, cold stratification (Wang 1974). Dormancy results from inhibition of embryo development, induced by the seed coat and/or megagametophyte tissue (Downie and Bewley 1996). Seed dormancy may vary greatly among stands, individual trees and crop year (Hellum 1968; Wang 1976; Caron *et al.* 1990). In the wild, White Spruce seeds normally germinate the following spring, as soon as soil surface temperatures are warm enough and provided there is adequate moisture (Nienstaedt and Zasada 1990).

Natural regeneration of White Spruce can be difficult to predict and is not easily established under most harvesting systems. As a shade-tolerant species, White Spruce is able to regenerate under mature stands of spruce and early successional species, but advance regeneration stocking is often poor (Jablanczy 1967, 1979; Krasny *et al.* 1984; Walker *et al.* 1986; Nienstaedt and Zasada 1990). Freshly disturbed areas with exposed mineral soils offer the best conditions for germination and establishment (Eis 1967b; Lees 1970; Dobbs 1976; Zasada *et al.* 1978). Thick organic layers are common under mature stands, but such surfaces restrict germination success and shallow root penetration leads to mortality if the canopy is opened suddenly (Jablanczy 1967). Where advance regeneration is established on thick moss, survival after logging is often poor and seedlings are soon replaced by other more aggressive species. Under the more open canopies of stands growing on alluvium sites, the sudden increase of competing vegetation after harvesting prevents seedling establishment and causes severe mortality to advance regeneration (Eis 1981). Advance regeneration that does establish under closed canopies will not survive suppression as long as in other more tolerant

species such as balsam fir (Jablanczy 1967, 1979). Allelopathic effects of *Cladonia* lichens may inhibit the establishment of regeneration (Fisher 1979).

E. Vegetative reproduction in nature

While vegetative reproduction is rare over much of the range of White Spruce, layering is common at the northern limit where regeneration from seed is limited because of climatic conditions (Nienstaedt and Zasada 1990; Fayle and Scott 1995). Rooting occurs when lower branches of open-grown trees come in contact with the ground and are covered by soil or organic materials. Populations in these arctic areas likely originated from seed at a time when climatic conditions were warmer, and vegetative propagation is now the only possible means of regeneration (Elliott 1979).

5. Genetics

A. Cytology

Vegetative cells are normally diploid, with $2n = 24$ chromosomes (Mehra and Khoshoo 1956; Santamour 1960). Aneuploidy and polyploidy are very rare (De Torok and White 1960); about 1 in 13 000 seedlings have been observed, mostly tetraploid, and most of them stunted (Winton 1964).

B. Genetic variation

Population-level variability

White Spruce was an early candidate for provenance research, and evidence of clinal variation for height growth related to latitude and elevation of origin appeared as early as 1950 (Morgenstern 1996). North-south adaptive variation has also been observed for such characters as cold hardiness of buds, foliage and stems (Simpson 1994), optimal and threshold germination temperature (Fraser 1971), germination rate (Roche 1969), seed quality (Khalil 1986), juvenile growth (Dunsworth and Dancik 1983; Khalil 1986), date of bud flush (Blum 1988), and various other seedling morphological and phenological traits (Nienstaedt and Teich 1972).

There is evidence of east-west variation patterns in such taxonomic characteristics as needle colour, number of stomata and branch pubescence (Nienstaedt and Teich 1972), cortical monoterpenes (Wilkinson *et al*. 1971), DNA content (Miksche 1968) and cpDNA allele frequencies (Furnier and Stine 1995). These data are consistent with the two-refugia theory of White Spruce remigration, following the Pleistocene glaciation, with a major division at about 95°W, with latitudinal clines within each division (Nienstaedt and Teich 1972).

While White Spruce generally exhibits clinal variation for adaptive traits, edaphic ecotypes have been identified in eastern Ontario that produce superior height growth on granitic and limestone sites (Teich and Holst 1974; Murray and Skeates 1985). White Spruce populations from moist-warm habitats of the sub-boreal spruce biogeoclimatic zone in the interior of British Columbia have displayed greater resistance to white pine weevil (*Pissodes strobi*) attack (Alfaro *et al.* 1996). In a 20-year-old trial in the badly polluted industrial Erzgebirge region of East Germany, there was great variation in performance of 16 tested White Spruce provenances. The best of these (from Sundridge, Ontario) was superior to the best of 17 tested provenances of Norway spruce (Weiss *et al.* 1988).

Local provenances are generally well-adapted and grow well, but it is not uncommon for provenances from more southerly locations to exhibit better growth (Nienstaedt and Teich 1972). Some particular sources have demonstrated superior performance over a wide range of sites. A

provenance from Birch Island, British Columbia, has proven exceptional and in coastal nurseries will match the growth of Sitka spruce (Nienstaedt and Zasada 1990). Provenances from the Ottawa Valley have performed well at many locations from the Lakes States through to Newfoundland (Nienstaedt 1969; Corriveau and Boudoux 1971; Teich *et al.* 1975; Fowler and Coles 1977; Radsliff *et al.* 1983; Khalil 1985). Although these sources grew well in Newfoundland, survival was sometimes poor (Hall 1986). In Nova Scotia, Ottawa Valley sources were surpassed in height growth by provenances from Prince Edward Island (Bailey 1987). In a range-wide provenance test in Alberta, the 10 best provenances included sources from Saskatchewan, Manitoba, Ontario and Quebec, and had 15% greater height and only slightly lower survival after 15 years than did the local seed sources (Hansen *et al.* 1995).

When provenances of diverse geographic origin are tested, population differences may explain 10 to 15% of the phenotypic variation in wood relative density (Stellrecht *et al.* 1974; Beaulieu and Corriveau 1985; Corriveau *et al.* 1987). However, population differences within a smaller geographic area can be negligible, even while family differences within stands can account for 16% of the variation in relative density of outer wood (Corriveau *et al.* 1991).

In contrast to many other characters, geographic variation at polymorphic allozyme loci appears to be weak. A 19-year-old test in Minnesota of 22 provenances from across the range of White Spruce demonstrated that while 48.0% and 54.1% of the variation in height at ages nine and 19, respectively, was due to differences among populations, an average of only 3.8% of the allozyme variation was due to population differences (Furnier *et al.* 1991). The variance among enzyme systems at 13 loci in four populations in Alaska, on an altitudinal gradient from 120 to 750 m, was such that only 2% of the variance was among populations, while 97% of the genetic diversity was within-stand, suggesting that the allozyme systems studied were selectively neutral (Alden and Loopstra 1987).

Individual-level variability

While variation among provenances is important in determining the risks and benefits of transferring seed sources, genetic improvement from mass selection relies primarily on variation within populations as the source of genetic gains. The partitioning of genetic variance among and within populations is greatly influenced by the range of adaptive variation sampled by the tested provenances. A wide-range sample of provenances tested in Wisconsin estimated population variance to be two to three times the family-within-population variance for height at nine and 15 years (Nienstaedt and Riemenschneider 1985). Another sample drawn from across Quebec and Ontario indicated that population variance was as large as that of families-within-populations (Li *et al.* 1993). Field trials using hierarchal sampling over a limited area of southeastern Ontario showed high within-stand variation for height growth and phenology, while variation among stands was low (Dhir 1976; Pollard and Ying 1979a, b).

The oldest White Spruce progeny tests were established on four sites at the Petawawa Forest Experiment Station in 1958 (Holst and Teich 1969). Narrow-sense heritabilities for this material were reported at age eight to 11 years in the range of 0.15 to 0.35 for height, and at three of the four sites was similar at age 22 (Ying and Morgenstern 1979). A similar progeny test in Minnesota produced heritability estimates for height of 0.27 at age nine, increasing to 0.35 at age 12 (Mohn *et al.* 1976), and another in Wisconsin produced estimates of 0.16 and 0.25 at ages nine and 15, respectively (Nienstaedt and Riemenschneider 1985).

Heritability estimates for diameter have typically been lower: from 0.05 to 0.10 at age 22 in the Ontario test (Ying and Morgenstern 1979), and 0.14 in the Minnesota test (Merrill and Mohn 1985).

The Petawawa trial was revisited by Magnussen (1993), who found that heritability estimates were much higher when only the "crop" trees were considered, as would be the case during selection in older stands. Stem analysis of almost 300 trees from 18 open-pollinated families at 36 years of age showed strong heritability of height growth, in the range of 0.3 to 0.6. Heritability for volume was also moderately strong, peaking at about 0.3 at age 20 and declining rapidly thereafter.

White Spruce in the western part of the range is frequently deformed by the white pine weevil (*Pissodes strobi*). Resistance to this pest varies substantially among individuals within a population, and the genetic basis has been demonstrated (Kiss and Yanchuk 1991; Alfaro *et al.* 1996).

While significant variation in wood specific gravity exists among populations, there are few correlations with environmental gradients or growth, and most of the variability exists among individuals within stands (Beaulieu and Corriveau 1985). Corriveau *et al*. (1991) studied the variation of wood quality characters in 19-year-old open-pollinated progenies from eight populations of White Spruce in the Upper Ottawa Valley. Their results indicated that the relative density of outer wood in White Spruce is under strong genetic control, with 16% of the variation explained by family differences and narrow-sense heritability estimated to be 0.63. An open-pollinated family test in British Columbia produced a similarly high estimate of heritability for wood specific gravity, 0.47, at age 15 (Yanchuk and Kiss 1993).

Substantial genetic variation has also been demonstrated in the initiation, maturation and germination of somatic White Spruce embryos from zygotic embryonic tissue (Park *et al.* 1993, 1994). Of particular concern to clonal selection programmes, a substantial portion of the genetic variance in the response to cultural treatments and the maturation and germination of somatic embryos was due to non-additive genetic variance.

C. Inbreeding depression and genetic load

Strong inbreeding depression has been reported in White Spruce (Mergen *et al.* 1965; Fowler and Park 1983; Park *et al.* 1984), and height growth losses as great as 33% have been reported (Ying 1978). Compared with other conifers, the number of lethal equivalents per zygote, 12.6, is high, and selfing has severe effects on seed set, early growth and survival (Fowler and Park 1983). Selection likely acts to remove selfed and highly inbred individuals early in the life-cycle, prior to the age of reproduction (Furnier *et al.* 1991). Nevertheless, natural inbreeding among related trees is common in small populations. In two such stands in New Brunswick, the average inbreeding coefficient was estimated as $F = 0.145$ (Coles and Fowler 1976; Park *et al.* 1984).

6. Crosses

Potential crosses with White Spruce are summarised in Table 3.13 (modified from Nienstaedt and Teich 1972). Introgressive hybridisation between white and Englemann spruce is widespread where the species are sympatric over large areas of British Columbia and Alberta (Nienstaedt and Teich 1972). In these areas breeding programmes simply treat the hybrid complex as a single species, "interior spruce".

Table 3.13 Species cross compatibility with White Spruce

Species	References
Commonly occurring in sympatric range	
P. englemannii Parry ex Engelm. = *P. glauca* var. *albertiana* (S. Brown) Sarg.	Roche 1969; Daubenmire 1974
P. sitchensis (Bong.) Carr. = *Picea* × *lutzii* Little	Roche 1969; Fowler 1987a
Successful crosses; hybridity verified	
P. jezoensis var *hondoensis* (Mayr.) Rehder	Wright 1955
P. koyamai Shirasawa	Wright 1955
P. omorika (Pancic) Purkyne	Jeffers 1971; Gordon 1980
P. pungens Engelm.	Hanover and Wilkinson 1969; Bongarten and Hanover 1982; Gordon 1980
P. schrenkiana Fisch. & Mey.	Fowler 1966; Gordon 1980
Limited crossibility; hybridity verified	
P. likiangensis (Franch.) Pritz.	Jeffers 1971; Gordon 1986
P. maximowiczii Reg.	Jeffers 1971
P. mexicana Martinez	Gordon 1980
P. mariana (Mill.) B.S.P.	Gordon 1986; Little and Pauley 1958; Parker and McLachlan 1978
P. smithiana Boiss.	Mergen *et al.* 1965; Nienstaedt and Fowler 1982
Possible crossibility; hybrids not verified	
P. abies (L.) Karst.	Jeffers 1971
P. asperata Mast.	Mergen *et al.* 1965
P. chihuahuana Martinez	Gordon 1980
P. glehnii (Fr. Schmidt) Mast.	Anonymous 1962
P. montigena Mast.	Jeffers 1971
P. orientalis (L.) Link	Mergen *et al.* 1965
P. retroflexa Mast.	Jeffers 1971
P. rubens Sarg.	Gordon 1980; Bongarten and Hanover 1982

Source : Modified from Nienstaedt and Teich, 1972

7. Ecology and Associated Species

Much of the information in this section has been derived from the excellent chapter on the silvics of White Spruce by Hans Nienstaedt and John Zasada, in USDA Forest Service Agricultural Handbook 654 (Nienstaedt and Zasada 1990). Other citations are given as appropriate when specific information is attributable to other sources.

A. Habitat

Having repopulated a tremendous area following glaciation, White Spruce can grow under a great variety of conditions, including extreme climates and soils, and is regarded as a "plastic" species. It is tolerant of shade, but recovers well after release from suppression and exposure to more light (Farrar 1995). Although it is a climax species in succession, it not only succeeded in establishing itself soon after glaciation, but also demonstrated an ability to invade abandoned farmland throughout eastern Canada, occupying about 200 000 ha of old fields in Nova Scotia alone (Drinkwater 1957).

Climate

The northern limit of the White Spruce is likely determined by a number of climatic, biotic and abiotic factors. What is clear is that climatic extremes in this area are significant. Mean daily

temperatures for January throughout much of the species range in Alaska, the Yukon and the Northwest Territories are in the vicinity of -29°C, whereas those is July reach only 13°C. Moisture is also limited in this area, with mean annual precipitation of only 250 mm. While photoperiod north of the Arctic Circle is 24 hours at the summer solstice, the length of the growing season at the northern limit is only about 60 days and may be as short as 20 days.

The southern limit of White Spruce's dominance as a species in forest stands roughly follows the 18°C July isotherm, except in the Prairie Provinces where it swings somewhat north. Maximum summer temperatures as high as 43°C have been recorded within the range in Manitoba, and mean annual precipitation can be as high as 1 270 mm in Nova Scotia and Newfoundland. Low mean annual precipitation in the range of 380 to 510 mm combines with mean high temperatures in July of over 24°C to produce the most severe conditions along the southern edge of the range in the Prairie Provinces.

Soils and site type

A wide range of soils and site conditions support White Spruce, although the diversity of sites becomes more limited in northern areas with increasing severity of climate (Sutton 1970). Within its range, it is found on soils of glacial, lacustrine, marine and alluvial origin derived from geologically diverse substrata, including granites, gneisses, sedimentaries, slates, schists, shales and conglomerates.

Podzolic soils are most common, but White Spruce also grows on brunisolic, luvisolic, gleysolic and regisolic soils. It can also be found as a minor species on sand flats and other coarse-textured soils, on shallow mesic organic soils in Saskatchewan, and on organic soils with black spruce in the central Yukon.

While White Spruce can occupy extremely harsh site conditions, it is generally regarded as more demanding than other associated conifers, requiring higher moisture and fertility to achieve best development on moderately well-drained soils. Optimum pH values are probably in the range of 4.7 to 7.0 (Sutton 1970; Stiell 1976), but White Spruce stands are found on strongly acidic soils at pH 4.0, as well as alkaline soils as high as pH 8.0. Ecotypic variation has been observed in White Spruce, with some ecotypes adapted to limestone sites (Teich and Holst 1974; Murray and Skeates 1985). White Spruce stand development itself can have an impact on organic layers and on properties of the mineral soil. Brand *et al.* (1986) found that soil pH decreased by 1.2 units in plantations established on abandoned farmland in Ontario.

B. Synecology and associated species

Distributed over such a wide range, it is no surprise that White Spruce is an important component of several different forest types. In the eastern part of its range, it occurs in pure stands on abandoned fields in New England and the Maritime Provinces (Drinkwater 1957; Sutton 1970) and in moist boreal regions in the north. It more commonly occurs as a major stand component in association with black spruce (*Picea mariana*), red spruce (*Picea rubens*), balsam fir (*Abies balsamea*), white birch (*Betula papyrifera*) and trembling aspen (*Populus tremuloides*), and to a lesser extent with yellow birch (*Betula alleghaniensis*) and sugar maple (*Acer saccharum*). When White Spruce occurs in communities with intolerant species such as trembling aspen, white birch or red pine (*Pinus resinosa*), its greater shade tolerance leads to its assuming increasing importance as succession progresses. In northern Quebec, White Spruce is associated with lichen (*Cladonia*), feathermosses (*e.g.*, *Hylocomium splendens*, *Pleurozium schreberi*, *Ptilium cristacastrensis*, and *Dicranum* spp.), dwarf birch (*Betula nana*) and many ericaceous plants.

Pure stands are more common in the western part of the range. Associated species in such stands in Alaska include white birch, trembling aspen, black spruce and balsam poplar (*Populus balsamifera*), whereas in western Canada the pure White Spruce type is associated with subalpine fir (*Abies lasiocarpa*), balsam fir, Douglas fir (*Pseudotsuga menziesii*), jack pine (*Pinus banksiana*) and lodgepole pine (*P. contorta*). In northwestern Canada and Alaska, closed White Spruce stands occur in communities with willows (*Salix* spp.) and buffalo berry (*Shepherdia* spp.), combined either with northern goldenrod (*Solidago multiradiata*) and crowberry (*Empetrum* spp.), or with huckleberry (*Gaylussacia* spp.), dewberry (*Rubus* spp.) and peavine (*Lathyrus* spp.).

In low elevations of western Canada and throughout interior Alaska, White Spruce is found in mixed-wood stands with trembling aspen. Common understorey shrubs found under such canopies in Alaska include green alder (*Alnus crispa*), willows, common bearberry (*Arctostaphylos uva-ursi*), highbush cranberry (*Viburnum edule*) and mountain cranberry (*Vaccinium vitis-idaea*). In the Prairie Provinces, the White Spruce-aspen type is associated with common snowberry (*Symphoricarpos albus*), red osier dogwood (*Cornus stolonifera*), western serviceberry (*Amelanchier alnifolia*) and western chokecherry (*Prunus virginiana* var. *demissa*).

Mixed White Spruce-paper birch stands are also common in western Canada and parts of Alaska. In this stand type, the understory vegetation usually includes willows, green alder, highbush cranberry, prickly rose (*Rosa acicularis*), mountain cranberry, bunchberry (*Cornus canadensis*) and Labrador-tea (*Ledum groenlandicum*).

Both the White Spruce-aspen and White Spruce-white birch stand types are successional stages leading to the pure White Spruce type or, in alpine treeline communities, the black spruce-White Spruce type. The latter occurs as open stands that, depending on moisture availability, may also support resin birch (*Betula glandulosa*), alders, willows, feathermosses and *Cladonia* lichens, together with Labrador-tea, bog blueberry (*Vaccinium uliginosum*), mountain cranberry and black crowberry (*Empetrum nigrum*).

Where White Spruce occurs as an important component of the boreal spruce-fir forest, green alder is the most commonly associated tall shrub, with willows important in western areas, and mountain maple (*Acer spicatum*), showy mountain ash (*Sorbus decora*) and American mountain ash (*S. americana*) important in the east. Common medium to low shrubs are highbush cranberry, red currant (*Ribes triste*), prickly rose and raspberry (*Rubus idaeus*). Ground vegetation commonly includes fireweed (*Epilobium angustifolium*), one-sided wintergreen (*Pyrola secunda*), one-flowered wintergreen (*Moneses uniflora*), northern twinflower (*Linnaea borealis*), naked bishop's cap (*Mitella nuda*), bunchberry, dwarf rattlesnake plantain (*Goodyera repens*), stiff club moss (*Lycopodium annotinum*) and horsetail (*Equisetum* spp.) (la Roi 1967). Many bryophytes occur in these boreal spruce-fir stands. The most common mosses are *Pleurozium schreberi*, *Hylocomium splendens*, *Ptilium cristacastrensis*, *Dicranum fuscescens* and *Drepanocladus uncinatus*. Common liverworts are *Ptilidium pulcherrimum*, *P. ciliare*, *Lophozia* spp. and *Blepharostoma trichophyllum*. Common lichens include *Peltigera apthosa*, *P. canina*, *Cladonia rangiferina*, *C. sylvatica*, *C. alpestris*, *C. gracilis* and *Cetraria islandica* (la Roi and Stringer 1976).

C. Competition and stand structure

White Spruce can exist in various stand types and various stages of succession. Under shade, it is classified as intermediate to tolerant (Nienstaedt and Zasada 1990; Farrar 1995). It will compete with, but not necessarily outperform, other shade-tolerant conifers such as hemlock, black and red spruce, balsam fir, sugar maple and beech. In association with less tolerant early-successional species such as aspen, white birch and lodgepole pine, it may remain a suppressed, understorey component, becoming

more prominent at later successional stages. White Spruce competes poorly against the dense growth of perennials, bracken fern and understorey shrubs (Fowells 1965).

While White Spruce can form pure stands, particularly in the northwestern part of its range and in the New England States and Maritime Provinces, these stands are not always self-sustaining climax types. Where White Spruce pioneers to form even-aged stands on old fields in the Maritimes, advance regeneration is often outnumbered and outperformed by balsam fir seedlings that become a larger component after release (Jablanczy 1979). In mixtures, particularly with less tolerant species, the response to release by disturbance or cutting can be much more successful (Crossley 1976; Berry 1982; Nienstaedt and Zasada 1990).

White Spruce can be a component of multi-aged stands, either as pure stands or mixed with other tolerant late-successional conifers and hardwoods. Older age classes in such stands can be as high as 200 to 250 years in Alberta (Day 1972; Nienstaedt and Zasada 1990). As establishment is facilitated by disturbance, the age distribution in such stands is not continuous, but rather grouped according to periods of successful establishment.

D. Ecosystem dynamics

Many abiotic factors interact with White Spruce in forest ecosystems; some pose a direct threat to the species or cause significant damage. Wild fires can eliminate seed supply and leave a seedbed that is more conducive to the establishment of other species such as lodgepole pine, intolerant hardwoods and even black spruce. Stands established on flood plains may benefit from deposit of seedbed materials or suffer from disturbance to young regeneration. Frost heaving can cause severe damage, particularly to container seedlings planted on finer-textured soils. Root form and depth of White Spruce can vary greatly depending on site conditions (Strong and la Roi 1983), and shallow-rooted stands may be prone to windthrow. Periodic storms may cause considerable damage from hail, ice and snow (Dobbs and McMinn 1973; Gill 1974; Sampson and Wurtz 1994). Late-spring frosts can cause significant damage to flushing vegetative and reproductive buds.

While a great number of insects are a natural component of White Spruce forest types, few are responsible for large losses. Of these, the eastern spruce budworm (*Choristoneura fumiferana*) is the most destructive. Massive epidemics of this defoliator occur periodically, resulting in heavy mortality and loss of growth, particularly where White Spruce is associated with balsam fir (Rose *et al.* 1994). Several other defoliators cause damage or weaken trees on a smaller scale, including the yellow-headed spruce sawfly (*Pikonema alaskensis*), European spruce sawfly (*Diprion hercyniae*), needleminers, needleworms, loopers, tussock moths and the spruce harlequin. Other groups of insects attack buds and shoots of White Spruce, including gall-forming adelgids (*Adelges* spp.), spruce bud moths (*Zeiraphera* spp.) and the white pine weevil (*Pissodes strobi*).

Several species of bark beetles, *Scolytidae*, feed and breed in galleries between the bark and wood. The spruce beetle (*Dendroctonus rufipennis*) may attack trees of normal vigour, particularly those which are large-diameter and slow-growing, and has killed large areas (Ostaff and Newell 1981; Hard *et al.* 1983; Werner and Holsten 1984). Generally, though, bark beetles are considered secondary pests, attacking trees weakened by other means such as budworm epidemics, and may be thought of as beneficial in that their feeding hastens the return of wood to the humus (Rose *et al.* 1994).

Warren's collar weevil (*Hylobius warreni*) causes significant damage in scattered areas, girdling smaller trees and making larger trees susceptible to root rots such as *Inonotus tomentosus* (Merler and van der Kamp 1984; Rose *et al.* 1994). The strawberry root weevil (*Otiorhynchus ovatus*) can cause injury to young seedlings, and the root-collar weevil *Hylobius congener* can cause significant

mortality to White Spruce seedlings planted on recently cut softwood sites (Pendrel 1990; Eidt and Weaver 1993).

Many insect species inhabit or feed on spruce cones and seed, as part of their life-cycle. Significant losses in natural stands and seed orchards are caused in particular by the White Spruce cone maggot (*Strobilomyia neanthracina*) and the spruce seed moth (*Cydia strobilella*), and to a lesser extent by the spruce budworm and spruce coneworm (*Dioryctria reniculelloides*), among others (Hedlin *et al.* 1980; Turgeon 1994). Only a few pathogens cause problems with cone and seed production. The spruce cone rust (*Chrysomyxa pirolata*) can cause abnormal development of the cones, reduced seed production, and decreased viability of seeds (Sutherland *et al.* 1987; Myren *et al.* 1994).

Emerging seedlings, particularly in bareroot nurseries, are commonly affected by damping-off fungi, primarily *Fusarium* but also *Pythium*, *Rhizoctonia*, *Phytophthora* and *Cylindrocladium* (Filer and Peterson 1975). Young seedlings may also suffer from Sirococcus blight (*Sirococcus strobilinus*) and infestation by the nematode *Xiphinema bakeri* (Sutherland and Van Eerden 1980).

Spruce needle rust (*Chrysomyxa ledi* and *C. ledicola*) is common wherever the alternate host, Labrador-tea, is found, but extensive damage from the fungus is rare. Spruce broom rust is common, causing abnormal proliferation of shoots to form "witches'-broom", but rarely causes death. Witches'-broom on White Spruce is sometimes caused by eastern dwarf mistletoe (*Arceuthobium pusillum*), although black spruce is more susceptible to this parasite. Scleroderris canker (*Gremmeniella abietina*) and cytospora canker (*Leucostoma kunzei*) both affect White Spruce, but cause little damage (Myren *et al.* 1994). Massive tumour-like growths are commonly observed on stems and branches in some White Spruce populations, particularly near coastal areas, but their etiology is not known (De Torok and White 1960).

Many rot fungi produce stem, butt and root rot in White Spruce, including red ring rot (*Phellinus pini*), red belt fungus (*Fomitopsis pinicola*) and Armillaria root rot (*Armillaria mellea* complex). Tomentosus root rot (*Inonotus tomentosus*) and brown cubical rot (*Phaeolus schweinitzii*) infect root systems and can reduce quality and growth, even if direct mortality is often light (Myren *et al.* 1994).

White Spruce forest stands commonly provide cover for many species of animals. Some, like moose, deer, black bear and many other fur-bearers, seek shelter in forest habitats but rarely feed on White Spruce. Porcupines (*Erethizon dorsatum*) also seek shelter in White Spruce forests and may kill small numbers of trees by feeding on the bark (Rose *et al.* 1994). Snowshoe hares (*Lepus americanus*), which commonly feed on foliage of young trees, tend to favour many other conifer species over White Spruce (Bergeron and Tardif 1988; Rangen *et al.* 1994), although planted White Spruce seedlings are preferred over natural regeneration (Sampson and Wurtz 1994). Many small mammals such as squirrels, mice, voles, chipmunks and shrews are heavy consumers of White Spruce seed and can have a major impact on regeneration, while the impact of seed-eating birds, including chickadees, grossbeaks, crossbills, juncos and sparrows, is relatively small (Radvanyi 1974). Many more bird species feed on the many species of insects that inhabit or feed on White Spruce trees and associated species.

The hybrid between white and Sitka spruce, *Picea* × *lutzi*, also occurs naturally where these species are sympatric. The hybrid has frequently been made artificially with parents from outside the sympatric area (Fowler 1987a), often in the hope of imparting the resistance of White Spruce to the white pine weevil (*Pissodes strobi* Peck). The degree of cold hardiness of the hybrid is related to the proportion of White Spruce germplasm (Ying and Morgenstern 1982), and growth performance of the hybrid depends greatly on the origin of the parents (Sheppard and Cannell 1985).

Many other artificial hybrids have been made successfully (*e.g.* Wright 1955; Jeffers 1971; Bongarten and Hanover 1982). The hybrid with Himalayan spruce (*P. smithiana*) was inferior to native White Spruce when field tested in New Brunswick, but superior in Wisconsin (Nienstaedt and Fowler 1982). Generally speaking, few of these hybrids have shown promise and none has achieved commercial importance (Nienstaedt and Zasada 1990).

8. Summary

White Spruce is an enormously important tree species in North America. It occupies a dominant role in several forest types that span the breadth of the continent, from the northern tree limit south to the Lake States and New England. The species has been successful as both a pioneering and climax type, and is genetically broadly adapted and highly variable. It is an outcrossing, wind-pollinated species that can transfer genes rapidly, and yet it tolerates higher levels of inbreeding when found in small populations.

The ecology of White Spruce is extremely diverse, given its tremendous geographic distribution and its genetic plasticity. The typical White Spruce ecosystem has a diverse mixture of associated tree species, vascular flora, bryophytes, insects, fungi, birds and animals. Only a very small number of these associated species pose a major threat by competition or direct damage, and White Spruce is well-adapted to this complex coexistence.

White Spruce is well-suited to artificial regeneration. It is the most commonly planted forest species throughout its natural range. Tree breeding programmes have a long history, and improved material from seed orchards now constitutes a significant portion of deployed reforestation material in some areas. While White Spruce reforestation is currently based on seed propagation, vegetative propagation techniques for cuttings and regeneration of somatic embryos are well-advanced, making it a logical target for implementation of transgenic biotechnologies and the use of cloning in both breeding and deployment strategies.

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Part 4

CONSENSUS DOCUMENTS ON MICRO-ORGANISMS

SECTION 1
BACULOVIRUSES

I. General Considerations

1. Subject of Document: Species Included and Taxonomic Considerations

A. Taxonomic considerations

Baculoviuses are a family of arthropod-specific, rod-shaped (baculum = rod), enveloped viruses with a circular double-stranded DNA genome. Until recently, the family Baculoviridae was divided into two subfamilies, the Eubaculovirinae and the Nudibaculovirinae (Francki *et al*., 1991). Based on the type of virion occlusion (see below) the Eubaculovirinae comprised the genera (i) nuclear polyhedrosis virus (NPV) and (ii) granulovirus (GV). The subfamily Nudibaculovirinae contained the only genus non-occluded baculovirus (NOB) which differed from the Eubaculovirinae in the lack of occlusion body formation and virion morphology (for review see Burand, 1991).

Recently, the International Committee on Taxonomy of Viruses (ICTV) revised the classification of baculoviruses (Murphy *et al*., 1995). The family Baculoviridae is now divided into two genera (i) Nucleopolyhedrovirus (formerly nuclear polyhedrosis virus) and (ii) Granulovirus (formerly granulosis virus). The NOB including the *Oryctes rhinoceros* virus (OrV) and the *Heliothis (=Helicoverpa) zea* virus 1 (HzV-1) has been removed from this family and are not assigned to any virus family.

Some properties used for the taxonomy and classification criteria for baculoviruses are summarised as follows (Murphy *et al*., 1995) (Fig. 1 in A 2.1).

Baculoviruses exclusively have been isolated from arthropods, primarily from 4 insect orders as Lepidoptera, Hymenoptera, Diptera and Coleoptera (Martignoni and Iwai, 1986b; Adams and Bonami, 1991).

During the replicative cycle of baculovirus, two virion phenotypes are produced. One virion phenotype, called occlusion derived virus (ODV), is embeded into a crystalline protein matrix, the occlusion body. Occlusion bodies are polyhedral and contain numerous virions (genus *Nucleopolyhedrovirus*) or ovicylindrical and contain only one (rarely two) virions (genus *Granulovirus*). The ODVs of granuloviruses contain only one nucleocapsid within the viral envelope, whereas NPV ODVs can harbour a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) per virion. A second virus phenotype, called budded virus (BV), is generated during early stages of infection. BV consist of single nucleocapsids which bud through the plasma membrane of infected cells into the extracellular fluid. Their membrane envelopes are loose-fitting and contain peplomers of a viral encoded glycoprotein (Fig. 1 in A 2.1).

The rod-shaped nucleocapsids are 30-55 nm in diameter and 250-300 nm in length and contain a single supercoiled, closed circular doublestranded DNA of 90-160 kb.

B. Species included

Among the 633 potential baculovirus species compiled by the ICTV, 15 NPV were categorised as assigned species whereas 483 NPV are tentative species. The GV contains 5 assigned and 131 tentative species (Table 4.1). In general, the name of a given baculovirus consists of two parts, the name of the host insect where the baculovirus was isolated from and the type of occlusion body formed, *e.g.* the multiple nucleocapsid nucleopolyhedrovirus of the alfalfa looper *Autographa califonica* is termed *Autographa califonica* MNPV or AcMNPV.

Table 4.1 List of assigned baculovirus species

Family: Baculoviridae	
1. Genus Nucleopolyhedroviruses	***NPV***
Autographa californica MNPV (type species)	AcMNPV
Anticarsia gemmatalis MNPV	AgMNPV
Bombyx mori NPV	BmNPV
Choristoneura fumiferana MNPV	CfMNPV
Galleria mellonella MNPV	GmMNPV
Helicoverpa zea SNPV	HzSNPV
Lymantria dispar MNPV	LdMNPV
Mamestra brassicae MNPV	MbMNPV
Orgyia pseudotsugata MNPV	OpMNPV
Orgyia pseudotsugata SNPV	OpSNPV
Rachiplusia ou MNPV	RoMNPV
Spodoptera exigua MNPV	SeMNPV
Spodoptera frugiperda MNPV	SfMNPV
Trichoplusia ni MNPV	TnMNPV
Trichoplusia ni SNPV	TnSNPV
2. Genus Granulovirus	***GV***
Plodia interpunctella GV (type species)	PiGV
Artogeia rapae GV	ArGV
Cydia pomonella GV	CpGV
Pieris brassicae GV	PbGV
Trichoplusia ni GV	TnGV

Source : Murphy *et al.*, 1995

The subject of this document includes the nucleopolyhedroviruses and granuloviruses with emphasis on those that have been used for insect control. Investigations on potential improvements of application strategies and biological properties predominantly concentrate on species/strains that are infective for lepidopteran hosts.

2. Characteristics of the Organism Which Permit Identification, and the Methods Used to Identify the Organism

Baculoviruses form a distinct and well characterised group of arthropod-specific viruses which can be distinguished from other viruses by a number of unique properties described in the following.

A. Morphological and physicochemical characteristics

The most prominent characteristic of baculoviruses is the formation of occlusion bodies (OB). The OB are formed in the nuclei of infected cells and can be easily detected by light microscopy (phase-contrast or dark-field) as highly refractile particles.

Nucleopolyhedroviruses form polyhedra-like occlusion bodies of 0.15 to 15µm in size and contain many enveloped virions. The major component of the occlusion body is a single, viral encoded protein of Mr 25-33 x 10^3, called polyhedrin (Hooft van Iddekinge *et al*., 1983). Polyhedral occlusion bodies normally band at 54-56% sucrose on 40-65% w/w sucrose gradients at 100,000 g. The buoyant density of ODVs in CsCl is 1.18-1.25 g/cm^3, that of BV in sucrose is 1.17-1.18 g/ cm^3.

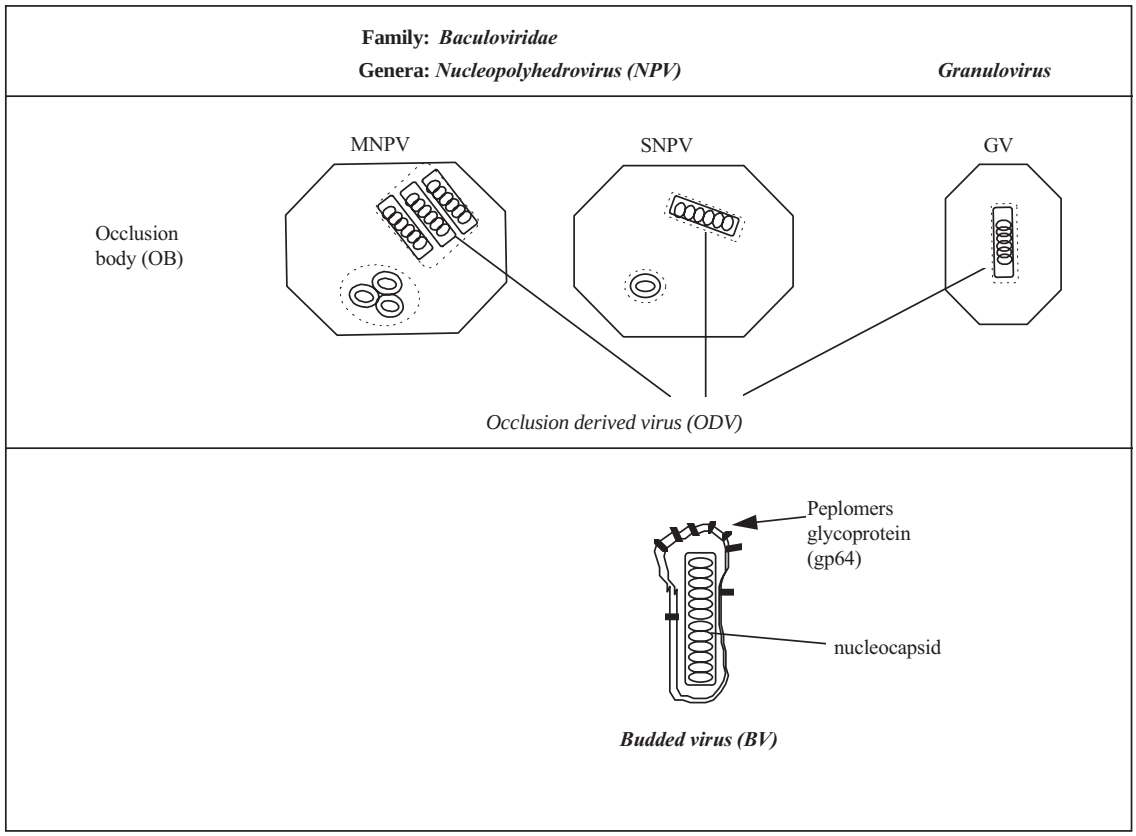
Electron microscopic observation of polyhedral inclusion bodies reveal two morphotypes: (i) single nucleocapsid nucleopolyhedroviruses (SNPV) contain only a single nucleocapsid within a virion, whereas the virions of (ii) multiple nucleocapsid nucleopolyhedroviruses (MNPV) harbour few to many nucleocapsids. Factors determining and regulating the formation of SNPV or MNPV have not been elucidated.

Granuloviruses generally form ovicylindrical (granule-like) occlusion bodies of 120-300 nm in width and 300-500 nm in length (Crook, 1991). The matrix protein, called granulin, is genetically and serologically closely related to the NPV polyhedrin.

SDS-polyacrylamide gel electrophoresis and serological techniques such as immunodiffusion, immunoelectrophoresis, radioimmunassay (RIA), enzyme-linked immunosorbent assay (ELISA) and Western-blotting have been used to identify particular NPV and GV and to study their relationship (Summers and Smith, 1975; Reinganum, 1984). ELISA was demonstrated to be a rapid, specific and sensitive method for detecting and quantifying baculoviruses (reviewed by Harrap and Payne, 1979). Polyclonal and monoclonal antisera specific against occlusion body and capsid proteins revealed a high degree of cross-reactivity among NPV and GV (Smith and Summers, 1981). These traits allowed the identification of a single baculovirus species and were used for the first phylogenetic studies of different baculoviruses.

By using monoclonal antibodies in ELISA, it was possible to detect virus antigens in NPV-infected *Helicoverpa armigera* and *Choristoneura fumiferana* larvae at about 6-9 hours after virus exposure, whereas disease symptoms of the larvae could only be observed after 5-6 days (Zhang and Kaupp, 1988; Lu *et al*., 1995). With an antiserum against the polyhedrin component of the NPV of *Mamestra brassicae,* it was possible to detect polyhedra at a concentration of 3.13 x 10^4 polyhedra/ml by means of immunoelectrophoresis, and as low as 2.44 x 10^2 polyhedra/ml by means of ELISA. Even though this antiserum was specific to MbMNPV, it also cross-reacted with the polyhedrin of *Agrotis segetum* NPV, *Lymantria monocha* NPV and *Neodiprion sertifer* NPV (Riechenbacher and Schliephake, 1988). Similarly, polyhedrin specific antisera were developed for detection of LdMNPV and *Borrelina bombycis* NPV by ELISA in infected host larvae or cultured insect cells (Ma *et al*., 1984; Shamim *et al*., 1994). Alternatively, a monoclonal antibody against the 42K protein of AcMNPV was used for virus detection in dead larvae and for safety investigations (Naser and Miltenburger, 1983).

Figure 4.1 Morphological characteristics of nucleopolyhedroviruses and granuloviruses



B. Biological characteristics

Host range

Host range and cross infectivity of many baculoviruses have been reviewed by Gröner (1986). The infectivity of NPV and GV to alternate hosts was typically evaluated on basis of virus infection and mortality of test larvae after oral virus application. However, these examinations are biased toward Lepidopteran species and economically important insects. So far, a standardisation of bioassays to determine host range and specificity is lacking (Cory *et al*., 1997).

Nucleopolyhedroviruses (NPV)

NPV are widely distributed among more than 400 arthropod species belonging to seven insect orders, which are Lepidoptera, Hymenoptera, Diptera, Coleoptera, Thysanura, and Trichoptera, as well as from Decapoda (class Crustacea) (Murphy *et al*., 1995). In general, the host range of most NPV is restricted to one or a few species of the genus or family of the host where they were originally isolated. Some of the few exceptions having a broader host range are (i) AcMNPV infecting more than 30 species from about 10 insect families, all within the order Lepidoptera, (ii) *Anagrapha falcifera* NPV infecting more than 31 species of Lepidoptera from 10 families and (iii) MbMNPV which was found to infect 32 out of 66 tested Lepidopteran species from 4 different families (Gröner, 1986; Doyle *et al*., 1990, Hostetter and Puttler, 1991).

Granuloviruses (GV)

GV infections have been reported for more than 100 insect species, however they appear to infect only members of the order Lepidoptera (Murphy *et al*., 1995). In contrast to NPV, the host range of GV appears to be even more narrow and mostly restricted to a single species. (see also C 23.3)

Gross pathology

Nucleopolyhedroviruses

The gross pathology post infection (p.i.) of NPV infecting Lepidopteran larvae can be summarised as follows:

- Day 1 - 3 p. i.: Infected larvae normally do not show obvious signs of disease.
- Day 4 - 6 p. i.: Diseased larvae only react slowly to tactile stimuli. The larvae start to appear swollen, glossy and moribund.
- Day 6 - 7 p. i.: Diseased larvae stop feeding and begin to die. Diseased larvae of some species, *e.g. Lymantria spec*., crawl to the top of the twigs (negative geotropism) on which they were feeding.
- Day 7-10 p. i.: Diseased larvae die and may liquefy, the cuticle ruptures and polyhedra arereleased.

Granuloviruses

In general three different types of gross pathology with GV can be distinguished (Federici, 1997).

- Many GV such as CpGV show a similar gross pathology in infected larvae as described for NPV.
- Some GV, esp. many Noctuid-infecting GV also pass the midgut epithelium but then infect only fat body tissue. Infected larvae do not stop feeding and grow even bigger than healthy larvae. Larval death occurs at 10-15 days.
- The third type of gross pathology has to date only been observed with *Harrisina brillians* GV. Infection is restricted to the midgut epithelium which causes heavy diarrhetic disorder and death within 4 to 7 days.

C. Pathological characteristics

Baculovirus pathogenesis has been most extensively studied for its type species AcMNPV, but appears to be similar in all other known baculoviruses (see below).

Most typically, virus replication of NPV occurs in the nuclei of infected host cells, whereas in GV-infected cells the nuclear membrane disrupts during the replication process and loses its integrity. Upon infection, the nuclei appear to become hypertrophic. The occlusion bodies produced by infected cells can be detected by light microscopy.

Divergent tissue tropism is observed with different viruses in their respective hosts. Most NPV specific for lepidopteran species as well as most GV establish a transient infection of the midgut epithelium and then invade other tissues such as fat body, epidermis, tracheal matrix, muscle, nerve, malphigian tubules, and reproductive and glandular tissues. In contrast, NPV specific for Hymenoptera, most Diptera, Trichoptera, Thysanura, and Crustacea as well as the *Harrisina brillians* GV were only found to infect midgut epithelium cells but not any other larval tissue (Federici, 1997).

D. Genetic characteristics

Restriction mapping

Early studies of the size and GV content of baculovirus DNA were based on a number of different methods, such as electron microscopy, melting point determination and reassociation kinetics, ultra-centrifugation in sucrose gradients and chemical analyses.

Restriction endonuclease analysis of isolated DNA has been for many years one of the most important and powerful tools for characterisation and identification of DNA viruses including baculoviruses (Rohrmann *et al*., 1978). This method allows investigators (i) to identify unequivocally the infecting agent, *e.g.* when a latent baculovirus is activated (Jurcovicova, 1979), (ii) to distinguish among different viruses or virus isolates, which infect the same host species and show similar biological properties (Vlak, 1980), and (iii) to recognise the identity of a virus infecting different host species (Miller and Dawes, 1978). Furthermore, restriction endonuclease analysis is indispensable for the construction of physical maps, which assign the position of the restriction fragments to each other. By convention, the smallest restriction fragment containing the gene encoding polyhedrin or granulin was chosen as the zero point of baculovirus physical maps (Vlak and Smith, 1982). To date restriction maps have been constructed for 18 NPV and 5 GV (Table 4.2).

Table 4.2 Restriction maps of different nucleopolyhedroviruses and granuloviruses

Virus species	References
Nucleopolyhedroviruses	
AcMNPV	Miller and Dawes, 1979; Smith and Summers, 1979; and 1980; Cochran *et al*., 1982; Brown *et al*., 1984
AgMNPV	Vlak, Johnson and Maruniak, 1989
Amsacta albistriga NPV	Anuradha *et al*., 1995
Anagrapha falcifera NPV	Chen *et al*., 1996
BmNPV	Maeda and Majima, 1990
Buzura suppressaria SNPV	Qi and Huang, 1987; Liu *et al*., 1993
CfMNPV	Arif *et al*., 1984
HzSNPV	Knell and Summers, 1984
Helicoverpa (=Heliothis) armigera NPV	Jin and Cai, 1987
LdMNPV	Smith *et al*., 1988; McClintock and Dougherty, 1988
MbMNPV	Possee and Kelly, 1988
OpMNPV	Chen *et al*., 1988
Panolis flammea NPV	Possee and Kelly, 1988; Weitzmann *et al*., 1992
SeMNPV	Wiegers and Vlak, 1984; Heldens *et al*., 1996
SfMNPV	Maruniak *et al*., 1984
Spodoptera littoralis NPV	Croizier *et al*., 1989
Spodoptera litura NPV	Meizhen and Yiquan, 1990
Granuloviruses	
ArGV	Smith and Crook, 1988
CpGV	Crook *et al*., 1985; Crook *et al*., 1997
Cryptophlebia leucotreta GV	Jehle *et al*., 1992
TnGV	Hashimoto *et al*., 1996
Xestia c-nigrum GV	Goto *et al*., 1992

DNA homology

Early homology studies of baculovirus strains have been accomplished by comparative restriction analysis and by the estimation of sequence relationships from the number of co-migrating restriction fragments in agarose gels by using the formula of Upholdt (1977). However, this method is only useful for closely related virus strains with more than 90% sequence homology. It does not take into

consideration that many genomic variations are due to small sequence insertions and deletions which result in differences of the restriction profiles even of very closely related virus strains.

DNA hybridisation techniques, such as Southern blot, dot blot and cross blot analysis, have been widely applied for identification and quantification of the intergenomic relationship of many baculoviruses. These methods permit the identification of heteroduplex formation of two different DNAs if they show more than 67% sequence identity (low stringency) or more than 85% identity (high stringency) (Howley *et al*., 1979).

Smith and Summers (1982) analysed the genomic interrelationship of 18 baculoviruses by restriction analysis, Southern hybridisation and semi-quantitative dot blot hybridisation. They found that under low stringency conditions all viral DNAs showed detectable cross hybridisation. Their results also corroborated the earlier classification of baculoviruses into three subgroups (nuclear polyhedrosis viruses, granulosis viruses and non-occluded baculoviruses, see A 1.1).

The combination of physical mapping and cross hybridisation indicated that the genomes of baculoviruses are similarly arranged. Leisy *et al*. (1984) showed that OpMNPV and AcMNPV are two distinct viruses with a colinear genomic arrangement. Similar observations were made for the genomes of CfMNPV and AcMNPV (Arif *et al*., 1984), MbMNPV and *Panolis flammea* NPV (Possee and Kelly, 1988) as well as for ArGV and CpGV (Crook *et al*., 1997) and for *Cryptophlebia leucotreta* GV and CpGV (Jehle *et al*., 1992).

3. Information on the Recipient Organism's Reproductive Cycle

A. In vivo and in vitro replication of baculovirus in permissive hosts

Initial stages of infection

The replication of AcMNPV has been most extensively studied in larvae of *Trichoplusia ni* and in cultured cells of *Spodoptera frugiperda* and serves as a model for NPV and GV replication in Lepidoptera (reviewed by Granados and Williams, 1986; Federici, 1997; Williams and Faulkner, 1997).

The natural route of infection is the peroral ingestion of viral occlusion bodies by larvae. In the alkaline environment of the midgut (pH > 9.5), the occlusion bodies dissolve rapidly and occlusion-derived virions (ODVs) are released. There is evidence that the dissolution of the occlusion body matrix might be facilitated by an insect derived alkaline protease which is associated with the occlusion body matrix. The ODVs pass through the peritrophic membrane (PM), a proteinaceous-chitinaceous layer which is secreted by the midgut cells to protect the midgut epithelium from direct contact with ingested material. After attachment to the microvilli of the midgut epithelium, the nucleocapsids enter the cell lumen either via fusion of the virion envelope with the epithelial membrane or by viropexis. The nucleocapsids are transported, most likely under involvement of the cellular microtubular structures, to the nucleus and become uncoated at the nuclear pore or within the nucleus where the viral DNA is released and DNA expression and replication is initiated.

The different temporal phases of gene expression

The following stages and secondary infections initiated by budded virions (BV) of host tissues or cultured cells are thought to be similar. In the early stages (8 hr p. i.) of infection the nucleus becomes hypertrophic and a virogenic stroma is formed where DNA replication and nucleocapsid assembly take place. Host cell protein synthesis is completely shut off by 24 hr p.i.

Viral gene transcription and expression follows a temporally co-ordinated cascade. Early and delayed early genes (α and β genes) are transcribed by a host dependent RNA polymerase II, which is sensitive to alpha-amanitin. These genes are necessary for regulation of viral gene transcription, for viral DNA replication, and late gene expression. Their promoters resemble those of host genes in having a CAGT transcription initiation site which is 25-31 bp downstream of TATA box and are recognized by nuclear extracts of uninfected cells.

Transcription and expression of late genes (or γ genes) involved in DNA replication, production of structural proteins and budded virions (BV) occurs between 8 and 24 hr p.i.

These genes as well as the very late (or δ genes) (see below) have a universal and invariant (A/G/T)TAAG transcription initiation site and are transcribed by a viral encoded RNA polymerae insensitive to alpha-amanitin. Five essential (*p143, ie-1, lef-1, lef-2*, and *lef-3*) and five stimulatory genes (*dnapol, p35, ie-2, lef7,* and *pe38*) were identified to be involved in AcMNPV DNA replication. In addition, transient expression studies using the chloramphenicol acetyl transferase (*cat*) reporter gene under the control of the late and very late promoters (*vp39, p6.9, polyhedrin, p10*) showed that eighteen AcMNPV genes (those necessary for DNA replication *and lef-4, lef-5, lef-6, lef-8, lef-9, lef-10, lef-11, p47,* and 39K) were essential for optimal expression from these promoters (Lu and Miller, 1995a). DNA replication and late viral gene transcription can be blocked by aphidicolin (Miller *et al*., 1981; Rice and Miller, 1986).

Expression of very late genes (or δ genes) begins about 18 - 24 hr p.i. and is characterised by a dramatic increase of *polh* and *p10* transcription and expression. *Polh* encodes the polyhedrin (its homologue in granuloviruses is called granulin gene), which is the major component of the occlusion body matrix. The *p10* gene product is associated with the formation of extensive fibrillar structures in the nucleus and cytoplasm of infected cells (Quant-Russell *et al*., 1987; van der Wilk, *et al*., 1987). Although the biological function of *p10* has not been convincingly elucidated so far, it was suggested that it might contribute to the disruption of the nuclear membrane and to the release of occlusion bodies from infected cells (van Oers *et al*., 1993; Williams *et al*., 1989).

Since both *polh* and *p10* are not required for DNA replication and are hyperexpressed during cell infection, their strong promoters were exploited for the development of the baculovirus expression vector systems. The coding region of these genes can be replaced by exogenous genes resulting in the production of high levels of the foreign protein (Smith *et al*., 1983; Vlak *et al*., 1990).

Production of budded viruses (BVs) and occlusion derived viruses (ODVs)

The replicative cycle of baculoviruses is biphasic and generates two distinct viral phenotypes, the budded virus (BV) and the occlusion derived virus (ODV). The two phenotypes are structurally distinct and destined for two different functions, both of which are essential for virus survival in nature. ODVs are released from the inclusion bodies and infect midgut epithelium cells, where a first round of virus replication takes place. The newly produced nucleocapsids traverse the nuclear membrane, the cytosol and bud through the basal lamina of the midgut cells into the hemolymph. These budded virions (BV) acquire a new envelope which consists of plasma membrane containing peplomers of a viral encoded glycoprotein, termed gp64 (see Fig. 1 in A 2.1). Gp64 appears to be pivotal for the interaction between the BV envelope and susceptible host cells through a possible interaction with a cell membrane receptor molecule and then a final fusion with the endosomal membrane (for review see Blissard, 1996). In cultured cells the production of BV peaks during the late phase of gene expression, between 10 - 20 hr p.i., whereas the very late occlusion phase can be observed between 16 - 72 hr p.i.

For most NPV and GV infecting lepidopteran host larvae, virus occlusion is not observed in midgut epithelial cells. These cells release BV into the hemolymph which then systemically spreads the virus infection among susceptible cells and tissues. In contrast, NPV of Hymenoptera, Diptera and Crustacea and the *H. brillians* GV infect only midgut cells where occluded viruses are produced (for review see Federici, 1997). However, there is also evidence that some nucleocapsids might traverse the midgut epithelial cells without replication and bud directly into the hemolymph (Granados and Lawler, 1981).

Engelhard *et al*. (1994) used a recombinant AcMNPV mutant expressing *lacZ* reporter gene (beta-galactosidase) to study the infection pathway in fourth instars of *Trichoplusia ni*. Based on the observation of early infection of midgut tracheoblast and the tracheal matrix, they postulated that the tracheol system might directly contribute to the systemic spread of BV. This finding, however, is not supported by others who used a similar approach (Flipsen *et al*., 1993; 1995).

Altogether, the spread of BV and systemic infection starts from the midgut and continues to hemocytes, tracheal cells, fat body, muscle and nerve cells as well as reproductive and glandular tissues. In the final step of infection, occlusion bodies are formed and the nuclei are packed with occlusion bodies which causes the cellular hypertrophy and swollen appearance of the infected larvae.

The different role of BVs and ODVs in the reproduction of baculoviruses can be summarised as follows: ODVs transmit infection from one larvae to another within an insect population, whereas the budded virus (BV) spreads the infection within susceptible larval tissues.

B. Behaviour in semi- and non-permissive insect cells

Baculovirus gene expression, replication and reproduction is only possible if one of the virion phenotypes (BV or ODV) is able to enter a permissive host cell. There are a number of viral factors and host responses which are necessary for a productive infection. In a non-compatible baculovirus-host cell interaction, the baculovirus gene expression and replication is blocked at a early stage. In order to analyse the genetic factors involved in host specificity and baculovirus-host cell interaction, a number of studies on the effect of virus on semi- and non-permissive insect cell lines were conducted. Infection studies with vertebrate and mammalian cells will be discussed in Section B.

When *T. ni* cells (TN-368) were infected with SfMNPV, typical early cytopathological effects such as nuclear hypertrophy and the formation of a virogenic stroma could be observed. However, only a few early transcripts and two proteins, an early 97 kD and a late 29 kD polypeptide, were synthesised. Virion assembly could not be detected. This result suggested that the block of infection occurred during the early phase of gene expression (Carpenter and Bilimoria, 1983; Bilimoria, 1991).

AcMNPV infection of *Choristoneura fumiferana* cell lines Cf124T and CF-203 is blocked after DNA replication and causes apoptotic cell death, respectively (Liu and Carstens, 1993; Palli *et al*., 1996). An apoptotic response to AcMNPV infection has also been observed for *Spodoptera littoralis* cell line SL2 (Chejanovsky and Gershburg, 1995).

By using recombinant AcMNPV expressing chloramphenicol acetyltransferase (*cat*) reporter gene under control of different temporally regulated promoters such as early, late and very late promoters, Morris and Miller (1992; 1993) showed that the activity of late and very late promoters is significantly reduced in semi- and nonpermissive cell lines of *Bombyx mori* (BmN-4), *Choristoneura fumiferana* (CF-1), *Lymantria dispar* (Ld652Y), *Mamestra brassicae* (MaBr-3) and *Drosophila melanogaster* (Dm) compared to permissive cell lines of *Spodoptera frugiperda* (Sf-21). Since there was some evidence for viral DNA replication but not for virus assembly, it was suggested that in these

cell lines progression of infection is blocked during DNA replication. By infecting different cell lines with AcMNPV expressing *cat* reporter gene controlled by the Rous sarcoma terminal repeat promoter and β-galactosidase controlled by the very late polyhedrin promoter, it was shown that nonpermissive *Drosophila* cells and permissive Sf-21 had a similar *cat* expression, whereas very late promoter activity was only observed with Sf-21 (Carbonell *et al*., 1985, Carbonell and Miller, 1987).

C. Genes involved in host range determination

So far, a number of baculovirus genes involved in differential host cell and host larval specificity have been identified. There are several examples where the expression or deletion of these host range determinants by recombinant NPV allowed the specific extension or restriction of host specificity. These results, mainly obtained with AcMNPV, provide evidence that a baculovirus which is infective to different host species relies on specific genes to establish infection and virus replication and that these sets of genes might differ slightly from host species to host species.

Although genetically closely related, AcMNPV and BmNPV have distinct host range specificities. Kondo and Maeda (1991) demonstrated that an AcMNPV mutant containing part of the BmNPV *p143* gene became infective to the normally refractile BmN cell line of *Bombyx mori*. This mutant, called eh2-AcMNPV, was obtained after coinfection of Sf-21 cells with AcMNPV (OT2) and BmNPV(T3), which are not infective for BmN and Sf21 cells, respectively. The progeny virus was subsequently passaged through BmN and Sf-21 cells thereby isolating eh2-AcMNPV which was found to be infective for both cell lines. In further experiments, the genome region responsible for host range extension was more precisely localised on a 572-bp fragment of BmNPV *p143*, which differed in 14 out of 109 amino acids as compared to AcMNPV (OT2) (Maeda *et al*., 1993). Similar results were obtained by Mori *et al*. (1992) who performed the co-transfection experiments with fragments of the BmNPV genome instead of BmNPV virions. This finding was corroborated by studies of Croizier *et al*. (1994) who demonstrated that the exchange of only three amino acids within *p143* was sufficient to expand the host range of AcMNPV to *B. mori* cells. It appears that the substitution of a single serine residue to an asparagine residue of AcMNPV *p143* is sufficient for this host range extension (Kamita and Maeda, 1997).

Infection of the *Lymantria dispar* cell line Ld652Y with AcMNPV is characterised by early cytopathic effects, transcription from all temporal classes of promoters and DNA replication but with a very low level of protein translation and no formation of infectious virions (McClintock *et al*., 1986; Guzo *et al*., 1991; 1992). However, when Ld625Y cells were coinfected with AcMNPV and LdMNPV, replication and production of AcMNPV was observed suggesting that LdMNPV encodes a trans-acting factor which rescues an abortive AcMNPV infection (McClintock and Dougherty, 1987). This trans-acting factor encoded by LdMNPV was finally mapped and identified by co-transfecting Ld625Y cells with AcMNPV genomic DNA and single cosmids of the LdMNPV genome. It was called *host range factor 1* (*hrf-1*) and was shown to extend the host range of recombinant AcMNPV expressing the factor to Ld652Y cells and to *L. dispar* larvae. This indicates that *hrf-1* determines the host range at the cell culture and larval levels (Thiem *et al*., 1996).

Transient expression studies using the *cat* reporter gene revealed that eighteen AcMNPV genes, so-called *lef* genes, were essential for optimal expression of late and very late genes in Sf21 cells (see also Chapter 3.1.2). For the permissive *T. ni* cell line TN-368, it was found that in addition to these 18 *lef* genes an additional gene, called *host cell-specific factor-1* (*hcf-1*) was required for efficient late gene expression. AcMNPV mutants lacking *hcf*-1 replicate normally in Sf21 cells and *S. frugiperda* larvae but are unable to productively infect TN-368 cells and *T. ni* larvae (Lu and Miller, 1995b; 1996; reviewed by Miller and Lu, 1997). This observation clearly indicated that *hcf*-1 has tissue specific as

well as species-specific effects on the replication of AcMNPV in cultured insect cells and in insect larvae.

Abortive replication in the permissive cell lines SF-21 (and SF-9) was observed with AcMNPV mutants lacking a functional *p35* gene. *p35* is an inhibitor of programmed cell death (apoptosis) of insect cells, which might be a defence reaction of insects against baculovirus infection at the organismal level (Clem *et al*., 1991; for review Friesen, 1997). It appears that *p35* also exerts host range function since this gene is essential for replication in SF-21-cells and *S. frugiperda* larvae but not in TN-368 and *T ni* larvae. AcMNPV *p35* mutants were 1000-fold less infective for *S. frugiperda* larvae than wildtype-AcMNPV when the virus is injected into the hemocoel and they were about 25-fold less infective in peroral infections (Clem and Miller, 1993; Clem *et al*., 1994).

4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

A. Formation of occlusion body

NPV and GV form polyhedral and granular occlusion bodies, respectively, into which the occlusion derived virions (ODV) are embedded. The occlusion bodies serve to protect the embedded virions against damaging environmental conditions and allow the virions to remain viable for many years. The occlusion bodies are solubilised in alkaline conditions of the midgut and thus deliver the ODV to susceptible columnar epithelial cells.

The crystalline matrix of the occlusion body mainly consists of a single protein, called polyhedrin and granulin, respectively. These proteins of about 245 amino acids (29 kDa) are hyperexpressed during the very late phase of virus infection and are not required for virus replication (for review see Rohrmann, 1992; Funk *et al*., 1997). To date, polyhedrin and granulin genes of about 35 different lepidopteran- and one hymenopteran-specific baculovirus species have been sequenced. The polyhedrin and granulin genes are highly conserved with at least 70% amino acid identity among lepidopteran NPV and about 50% amino acid sequence identity between NPV polyhedrins and GV granulins.

The importance of the occlusion body for the stability and maintenance of infectivity of baculoviruses in the environment has been clearly demonstrated by field tests using polyhedrin deficient AcMNPV mutants. In 1987, a field test was performed where insect larvae were infected with an AcMNPV polyhedrin minus mutant and then released into enclosed field plots. The non-occluded virus progeny rapidly lost its activity in the decaying larval carcass and no virus activity could be detected on cabbage leaves or in soil samples within two weeks after the release (Bishop *et al*., 1988a; 1988b). Wood and co-workers applied a slightly different approach by co-occluding polyhedrin-minus AcMNPV into the polyhedrin matrix of wild-type virus (Hamblin *et al*., 1990). In laboratory experiments it was shown that the persistence of such a co-occluded polyhedrin-minus mutant was significantly reduced at inoculum levels below a 100% dose. This observation was corroborated in a three-year field test in which AcMNPV polyhedra containing polyhedrin minus and wild-type AcMNPV in a ratio of 48:52 were applied on *T. ni* larvae, and the amount of polyhedrin minus mutants was analysed in the following years. It was found that the amount of polyhedrin minus mutant dropped below 20% of the virus progeny in the second and third year (Hamblin *et al*., 1990; Wood *et al*., 1994).

The occlusion bodies are not solely composed of polyhedrin. They are surrounded by an envelope, called a polyhedron calyx or polyhedron envelope (PE). Minion *et al*. (1979) reported that the PE of *Helicoverpa (=Heliothis) virescens* NPV was composed of hexose and pentose

carbohydrates. Whitt and Mannig (1988) showed that the PE of AcMNPV consisted of a phosphorylated protein which might be covalently linked to the carbohydrate component. The PE protein is encoded by a late and very late gene (*pp34*) which has been sequenced for several NPV (Gombart *et al*., 1989; Bjornson and Rohrmann, 1992). OpMNPV mutants lacking the PE gene produced unstable polyhedra with a rugged and pitted surface (Gross *et al*., 1994a).

Another protein which is normally associated with polyhedra in the infected cells is the *p10* protein. Evidence suggests that it is involved in the formation of PE and lysis of infected nuclei. AcMNPV mutants with inactivated *p10* genes failed to release the polyhedra from infected cells. *p10* negative OpMNPV and AcMNPV mutants also failed to form an intact polyhedron envelope generating fragile polyhedra with significantly reduced stability but with the peculiarity to form polyhedral aggregates (Williams *et al*., 1989; van Oers *et al*., 1993). Based on these observations, it was suggested that the function of *p10* and PE is twofold: to protect the polyhedra from mechanical damage by sealing their surface and to prevent their aggregation. Hence, these properties could be important to maximise the number of intact virions per occlusion body and to optimise virus dissemination (Gross *et al*., 1994a).

B. Enhancing of host susceptibility

Synergism between *Pseudaletia unipuncta* NPV and GV, which resulted in an increased susceptibility of *P. unipuncta* larvae for the NPV, has been described (Tanada, 1959). A protein component in the granule of PsunGV, termed synergistic factor, which interacts with the microvillar membrane of midgut cells and facilitates adsorption of the NPV virions was identified (Tanada *et al*., 1975; Zhu *et al*., 1989).

Similar factors have been found in TnGV, *Xestia c-nigrum* GV and *Helicoverpa (= Heliothis) armigera* GV (Derksen and Granados, 1988; Goto, 1990; Roelvink *et al*., 1995). These proteins are commonly known as enhancing factors or enhancin. TnGV enhancin was shown to increase the infectivity of AcMNPV to different noctuid larvae. The predicted amino acid sequences of TnGV enhancin and the synergistic factor of PSunGV-H are almost identical. TnGV enhancin is a protein of 104 kDa which most probably functions as a metalloprotease, since it can be reversibly inactivated by metal chelators (Lepore *et al*., 1996). TnGV enhancin is located in the occlusion body and causes specific degradation of the intestinal mucin component of the peritrophic membrane (Wang and Granados, 1997). The disruption of the peritrophic membrane of the larval midgut facilitates the virions access to the midgut columnar cells.

Although such a function was also found with a component of NPV polyhedra, no gene homologue to the TnGV enhancin could be identified in NPV (Derksen and Granados, 1988; Hashimoto *et al*., 1991). Observations of the synergistic enhancement of infectivity by the activity of components from different NPV, and the isolation of another synergistic factor from *Pseudaletia unipuncta* GV indicate that a somewhat heterogeneous set of genes and functions may contribute to this modulation of the infectious process (Arne and Nordin, 1995; Ding *et al*., 1995).

C. Inhibition of cellular apoptosis

Programmed cell death or apoptosis is a cellular pathway during which a cascade of responses is activated resulting in a well regulated cellular suicide. Apoptosis is the normal fate of many cells during development and metamorphosis, cellular turnover of renewing tissues and other dynamic cellular processes. There are also many examples of viral infections whose progression is blocked during early stages of replication by an apoptotic response of the infected host cell. It appears that apoptosis is a very powerful mechanism of many vertebrate and invertebrate cells to prevent viruses

from replicating and becoming persistent. On the other hand, viruses have evolved mechanisms that overcome or block this apoptotic response and so establish an infection (for review see Clem, 1997).

In baculoviruses two classes of proteins, the *p35* protein and the IAP (inhibitor of apoptosis) proteins, have been identified and characterised as anti-apoptotic agents. Expression of *p35* was found to be essential for AcMNPV replication in *S. frugiperda* cells (Clem *et al*., 1991). SF-21 cells infected with an AcMNPV 'annihilator' mutant, which was shown to contain a deletion in *p35* gene, underwent apoptotic cell death within 24 hours. The antiapoptotic effect of *p35* has been demonstrated not only in insect cells but also in many heterologous systems, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, mammalian and human cells (Sugimoto *et al*., 1994; Hay *et al*., 1994; Rabizadeh *et al*., 1993; Beidler *et al*., 1995). The competence of *p35* to prevent apoptosis in many different cell types pointed to the possibility that *p35* acts on a universal step in the apoptotic cascade. Xue and Horvitz (1995) and Bump *et al*. (1995) demonstrated that *p35* is able to inhibit cystein proteases, so-called ICE-like proteases, which play a key role in the highly conserved effector pathway of apoptotic response.

Compared to wild-type viruses, the infectivity of *p35* minus AcMNPV mutants to *S. frugiperda* larvae was dramatically reduced (by a factor of 1000 if injected into the hemocoel and by a factor of 25 if perorally applied). The yield of virus progeny from infected larvae was decreased 900-fold suggesting that the anti-apoptotic effect of *p35* may play an important role during *in vivo* infection of host larvae. In contrast to SF-21 cells, *p35* is not essential for AcMNPV to establish infection in TN-368 nor is there any difference between *p35* minus and wildtype AcMNPV in the infectivity for *T. ni* larvae. This suggests that *p35* is also involved in determining a baculovirus host range (Clem and Miller, 1993; Clem *et al*., 1994). A *p35* homologue has been also identified in BmNPV but is not present in OpMNPV (Maeda, 1994; Ahrens *et al*., 1997).

A second class of antiapoptotic genes of baculoviruses are *iap* genes, which were identified in CpGV and OpMNPV because of their ability to rescue replication and polyhedra formation of *p35* minus AcMNPV mutants in SF-21 cells (Crook *et al*., 1993; Birnbaum *et al*., 1994). All baculovirus *iap* proteins contain two cysteine-rich repeats known as BIR (baculovirus *iap* repeat) at their N-termini and a RING finger motif at the C-terminus. Further *iap* genes were identified upon genome sequencing in AcMNPV (*iap*1, *iap*2), BmNPV (*iap*1, *iap*2) and OpMNPV (*iap*1-*iap*4) (Ayres *et al*., 1994; Maeda, 1994; Ahrens *et al*., 1997). Cellular *iap* homologues were identified in humans and insects and might be ubiquitous in a wide variety of organisms (for review see Clem, 1997). Although the mode of action of *iap* is not well understood, there is strong evidence that both *iap* and *p35* inhibit defence mechanisms at the cellular and organismal levels, thus allowing baculoviruses to replicate and reproduce.

D. Arresting host development by ecdysteroid-UDP-glucosyltransferase

One of the most remarkable features of baculoviruses is their ability to impair larval moulting. In normal development, larval moulting is a physiological process regulated by ecdysteroid hormones. It was found that AcMNPV is able to inhibit larval moulting by expressing the enzyme ecdysteroid-UDP-glucosyltransferase (EGT). EGT inactivates insect ecdysteroid hormones by conjugation with glucose or galactose residues (O´Reilly and Miller, 1989). Whereas, the moulting process in healthy larvae is characterised by a cessation of feeding, baculovirus infected larvae do not show this behaviour. By generating an EGT minus AcMNPV mutant and comparing its pathogenicity with wild-type virus, O'Reilly and Miller (1989) found that EGT expression is responsible for the suppression of the host development during infection. Molecular studies showed that EGT is transcribed and expressed early during infection (O'Reilly and Miller, 1990). To date, homologous EGT genes have

been identified and sequenced from 12 nucleopolyhedroviruses and it seems very likely that it is a conserved ancestral gene present in all baculoviruses (Chen *et al*., 1997).

Since EGT minus AcMNPV mutants yielded about one fourth less virus progeny in late *S. frugiperda* instars than wild-type AcMNPV, it was proposed that EGT expression might have an important role in maximising the amount of virus progeny from an infected larva (O'Reilly and Miller, 1991, for review see O'Reilly, 1997). A further intriguing observation was that *egt* minus AcMNPV mutants killed host larvae faster than wild-type virus. The mean survival time (ST_{50}) of EGT minus AcMNPV-infected *S. frugiperda* and *T. ni* larvae was about 20-30% shorter than that of wild-type AcMNPV (O'Reilly and Miller, 1991; Eldridge *et al*., 1992). Although the reason for this increased virulence is not yet elucidated, there is apparently a correlation with the decreased amount of virus progeny. In the faster dying insects, the virus does not have enough time to produce the maximum amount of virus progeny.

The accelerated mortality of the EGT minus AcMNPV mutant accompanied by reduced feeding of the infected larvae led to a new concept of genetically engineered baculoviruses with improved insecticidal properties (O'Reilly and Miller, 1991). In recent years, EGT minus baculoviruses have been tested in the field and they are the most likely candidates to become the first registered genetically engineered baculovirus control agents.

E. Facilitating the release of occlusion bodies from larval cadavers

The final step of baculovirus infection is the breakdown of the larval cuticle and the release of the occlusion bodies into the environment. The cuticles of insect larvae consist mainly of chitin fibres embedded into a proteinacous matrix. Two baculovirus genes, encoding a chitinase (*chiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitinase is a chitin-degrading enzyme with endo- and exomolecular specificity, whereas cathepsin has cysteine proteinase activity (Slack *et al*., 1995; Hawtin *et al*., 1995).

The *chiA* and *cath* genes appear to be located next to each other in many baculovirus genomes and are expressed late in infection (for review see O'Reilly, 1997). The functional role of *chiA* and *cath* was elucidated by constructing AcMNPV and BmNPV mutants lacking either *chiA* or *cath* or both. Both single *chiA*- or *cath*-minus mutants were not able to cause liquefaction of infected larvae (Slack *et al*., 1995; Hawtin *et al*., 1995). Hence, the co-operative action of chitinase and cathepsin appears to exert an effect on the release of viruses from succumbed larvae and to facilitate the dissemination of occlusion bodies in the environment.

An additional role of *cath* was proposed by Lanier *et al.* (1996) who found that cathepsin is associated with BV of AcMNPV and is able to cleave actin in an *in vitro* assay. Whether cathepsin is also involved in the rearrangement of actin filaments during early stages of infection needs further investigation

F. Latent virus infections

Latency is the ability of a virus to persist in a host without causing disease symptoms, thereby providing the virus the possibility of vertical transmission from one generation to another. There are a number of early publications reporting circumstantial evidence of latent baculovirus infections. UV light and treatment with chemicals, rearing conditions, or superinfection with a different virus have been described as reasons for activating latent infections (Krieg, 1956; David and Gardiner, 1965; Longworth and Cunningham, 1968; Biever and Wilkinson, 1978; Podgwaite and Mazzone, 1986). However, most of these early studies were made when molecular tools for identification such as

hybridisation techniques or restriction endonuclease analysis were not available. Hence, it cannot be excluded that at least some of these reports were biased by cross-infections and contamination. Ponsen and de Jong (1964) and Jurcovicova (1979) reported activation of latent *Adoxophyes orana* SNPV by infecting *A. orana* larvae with *Barathra brassicae* MNPV and vice versa activation of latent *B. brassicae* MNPV by infection of *B. brassicae* larvae with *A. orana* SNPV. An increase in larval deaths from granulovirus infection correlated to stress from dehydrated diet or low temperature was observed in *Pieris rapae* (Biever and Wilkinson, 1978). Virulence testing indicated the identity of the stress induced virus with laboratory grown virus preparations. It was noted that larvae demonstrating symptoms of stress induced virus infection could recover to a healthy condition after relief of the stress by providing a fresh diet.

More recently, Hughes *et al.* (1993) provided first molecular evidence for activation of a latent baculovirus in *M. brassicae*. Activation of a MbMNPV was observed after feeding *Panolis flammea* NPV or AcMNPV to the laboratory culture of *M. brassicae* termed MbLC. Other (environmental) stress factors such as high and low temperatures, starvation or crowding were not effective in triggering apparent infections in this culture (Goulson unpublished data, Goulson and Cory, 1995a). Contamination of the virus inoculum was excluded by control infections of another *M. brassicae* culture (MbWS). Challenging this insect culture, established from a novel environmental isolate and adapted to laboratory growth conditions, did not result in multiplication of MbMNPV when infected with the same inoculum. Furthermore, MbMNPV-specific sequences were detected by PCR in each stage of insect development, *i.e.* in eggs, larvae, pupae and adults. When dissected larval tissues were analysed, latent virus sequences were only detected in the fat body of MbLC larvae. Cell lines established from fat body cells also harboured MbMNPV-specific sequences.

The occult state of the virus in the MbLC culture was further characterised by analysis of m-RNA in larvae, demonstrating the presence of polyhedrin specific m-RNA. Assays of transient reporter gene expression (CAT, chloramphenicol acetyl transferase) after transfection of primary cultures of MbLC fat body cells with constructs containing early, late and very late promoters, demonstrated the presence of expression factors for all of these, albeit at low levels. Furthermore, inoculation of MbWS larvae with MbLV fat body cells resulting in larval deaths from MbMNPV infection, strongly suggested the presence of viable virus particles in these cells (Hughes *et al*., 1997). The latent or occult status of MbMNPV in the insect can thus be described as a persistent infection, with a continuous production of virus proteins at a low level. Whether this state of the virus is controlled by host functions exclusively or to which degree viral regulatory functions are engaged in its establishment and support, remains an intriguing and challenging question.

A more detailed knowledge of the possibility and extent of virus persistence as a latent infection with vertical transmission to insect progeny and the potential to induce an infectious cycle through environmental conditions or stress factors, is crucial for an improved understanding of baculovirus ecology and population dynamics. So far, the contribution of this feature to the persistence of baculoviruses in natural populations, and its occurrence among insect and virus species and genotypes, is largely unknown. The perception of baculovirus population dynamics as, for example, represented in the modelling of natural and induced epizootics, almost exclusively considers the multiplication of viruses as a result of infections initiated by horizontal virus transmission.

G. Environmental factors influencing virus persistence

Virus persistence is an important factor affecting the potential of baculoviruses to interact with their hosts in the environment. Biotic and abiotic persistence mechanisms allow the virus to overcome situations of varying host density without running the risk of extinction (Evans, 1986). Baculoviruses may persist in the host population through vertical transmission of disease or by a latent infection (see

A 4.6) or in non-target organisms which may serve as vectors of virus dispersal (see C 26.2). This biotic persistence complements the maintenance of virus activity outside living organisms (abiotic persistence). In the open environment UV radiation of the sunlight has the most significant impact on abiotic persistence which also may be influenced by temperature, relative humidity and precipitation (for review, see Jacques, 1975). Half life time may be as short as 1.3 days for a purified preparation of the *Phthorimaea operculella* granulovirus (Kroschel *et al*., 1996) or, more typically, about 5 - 10 days for many NPV and GV (Bell and Hayes, 1994; Kolodny-Hirsch *et al*., 1993). Hence, a variety of formulations and additives have been tested and applied to improve the life time of preparations for pest control applications (Ignoffo *et al*., 1989; Ignoffo and Garcia, 1996; see also A.6.3). On the other hand, after spray applications of *Panolis flammea* NPV on pine foliage, only a slight decrease of infectivity was monitored over several months (Carruthers *et al*., 1988). This observation suggests that the application time of PaflNPV for control of the pine beauty moth, *Panolis flammea*, does not necessarily need a precise optimisation (Cory and Entwistle, 1990). Agricultural operations can affect the distribution of the baculoviruses in soil. Following *A. gemmatalis* NPV spraying on soybeans and subsequent epizootics, it is transported into the soil and decreases rapidly due to overwinter weathering, but enough remains near the soil surface at the beginning of the next growing season to initiate new epizootics. Persistence of the NPV in soil is not adversely affected by discing, cultivating, or other agricultural soil operations (Fuxa and Richter, 1996).

Furthermore, plant surfaces can have a distinct influence on viability and virulence as discussed in section C. 23.4.1. In soil, which can also be a potential reservoir of baculoviruses, the inactivation rate not only depends on soil type and pH but also on microbial activity (Thompson and Scott, 1979; Ignoffo and Garcia, 1966; Jaques and Huston, 1969; Undorf, 1991). Baculoviruses may persist in soil for long periods. Soil samples taken from a field treated with *Trichoplusia ni* NPV showed 15% of the original virus activity 6 years after application (Jacques, 1964). Similar results were obtained for other GVs and NPVs (for review see Jaques, 1975).

In summary, these observations denote that the physical environment, chemical conditions and the (micro)-biological composition of micro-habitats have a very significant impact on the persistence and activity of baculoviruses. The validity of comparisons of the persistence of different virus types depends on the degree of control of experimental variables. In particular cases the determination of the abundance of genotypes in artificial mixtures could be used as an approach. Parameters of persistence used in calculations of virus spread and modelling have either to account for the detailed structure of the respective environment or must include wide margins for the variation of median values. Biotic and abiotic persistence are prerequisites for mechanisms and routes of dispersal (summarised in section C.26).

5. Behaviour in Simulated Natural Environment Such as Microcosms, Growth Rooms, Greenhouses, Insectaries, etc

A. Monitoring of baculoviruses in closed environments and ecological modelling

Experiments such as bioassays (C.23.1) test well defined characteristics of baculovirus phenotypes in the laboratory. The use of microcosms or similar facilities may supplement these investigations by monitoring virus interactions in more complex environments. Microcosms may represent the variables of natural settings to a widely divergent degree, depending on their particular objective. Fraser and Keddy (1997) describe the “manipulation of an individual environmental axis” to explore its function in structuring the community as a common factor of microcosm research in ecology. Research of this kind may contribute to an improved understanding of baculovirus ecology by investigating mechanisms of fate and interactions in the environment in some detail (Cory *et al*., 1997; Cory and Hails, 1997). Increased knowledge of ecological behaviour will also generate a better

predictability and may improve safety assessments. Perspectives of safety may also trigger microcosm experimentation because some degree of confinement from the open environment is provided.

Traditionally, such testing was not usually performed with natural strains during the development of baculovirus insecticides. Baculovirus behaviour (*e.g.* pathogenicity, virulence, host range, and toxicity for particular non-target organism) is inferred from laboratory testing on one side. On the other, the world-wide application of baculoviruses for pest control together with scientific investigations on baculovirus population dynamics in the open environment contribute to the present understanding of baculovirus ecology. This background of knowledge about the mechanisms driving the dynamics of effects on insect species and about the ability to manipulate it, mostly is considered to be adequate for judgements about applications and safety in the environment. To some extent this view is reflected in the criteria of registration procedures (*e.g.* Andersen *et al*., 1989).

Conventional testing procedures using soil columns as a section of the natural environment were used to test the leaching behaviour of baculovirus preparations as a component of environmental fate in order to predict potential exposition rates of the groundwater. Rates were tested under conditions similar to pesticide registration procedures (C.26).

The perspective of using genetically modified baculoviruses seems to have changed the perception with respect to testing in "closed" environments to some degree. The first "deliberate release" of a genetically engineered baculovirus can be viewed as an experiment performed in a confined "simulated natural environment" (Bishop *et al*., 1988). Modified versions of microcosms introduced for environmental transport and fate studies in chemical risk assessment were used in this context to monitor essentially similar parameters of baculovirus fate. Modelsystems of this kind also provided some simulation of meteorological conditions (sunlight, rainfall, and air movement). Investigations included the comparison of modelsystem and field data, and of the persistence and spread of natural and genetically modified virus types. The potential of monitoring individual genotypes was demonstrated to some extent. A reduced spread and multiplication of the modified genotype in some contrast to similar biotest data was noted (Undorf, 1991). However, systematic comparisons of biotest, modelsystem, and field data, in order to analyse their correspondence, are not well documented in the scientific literature. The potentials as well as the limitations of experimentation in an intermediate scale of complexity have not been explored in great detail. This is in contrast to its use for the evaluation and modelling of the fate and effects of chemicals in the environment. The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory *et al*., 1997). Limited relevant modelling supported by experimentation is cited there to exemplify the need to analyse the correspondence between theory, closed environment experimentation and field data for an improved understanding of baculovirus population dynamics (Begon and Bowers, 1994; Begon *et al*., 1996).

For inferences, empirical data must be interpreted by theoretical structuring of knowledge in some kind of model, representing the essential components and the relations assumed to exist between them. Explicit modelling of baculovirus ecology given a mathematical form tries to calculate the dynamics of viruses and of their arthropod hosts under a given set of parameter variations. Results of such calculations are then compared with empirical data from modelsystem experimentation, field testing, or natural epizootics, to explore their present contribution to an understanding of mechanisms and to a limited degree of predictive potential, eventually. Simulated natural environments have been employed to a limited extent for testing environmental variables, to verify the formulation of interactions, and to analyse parameter values. This type of research is described in the following to account for common experimental approaches and for the correspondence with objectives to improve (predictive) knowledge of baculovirus ecology.

The work of Anderson and May (1980, 1981), cited in reviews of baculovirus ecology with some emphasis, is the key reference for the modelling of the dynamics between pathogens and their invertebrate hosts (Evans, 1986; Cory *et al*., 1997). In the Anderson and May model, the variations of forest insect pest abundances with low densities between regular extensive population increases were triggered exclusively by the dynamic relations between an insect pest population and pathogens persisting in the environment. Their model assumptions, describing the rate of disease transmission by a mass-action law with a linear relationship between pathogen/host densities and productive infection, have been criticised for incorrectness, oversimplification and lack of correspondence to reality (Bowers *et al*., 1993; D´Amico *et al*., 1996; Berryman, 1997). Whereas the statement that "it is no longer possible to claim that mathematical models provide a basis for believing that forest insect cycles might be generated by host-pathogen interactions alone" (Bowers *et al*., 1993) may be accepted or be viewed as a matter of discussion, the basic structure of the model is further employed as a starting point for modifications and adaptations of parameters and interactions. Cory *et al*. (1997) describe in some detail the modifications of the terms of transmission, virulence, yield, and persistence that are introduced or should be incorporated to generate correspondence with actual knowledge about baculovirus biology, as summarised in different sections of this paper.

Modelsystems, not intending to simulate natural environments to any significant degree, were used by Sait *et al*. (1994) and Begon *et al*. (1996) to study fluctuations in population densities of the Indian meal moth *Plodia interpunctella* including a baculovirus pathogen and a parasite. Systems varied with respect to composition: virus-free populations, infected populations continuously exposed to a granulovirus, or three-species systems including the interaction with the ichneumonid parasitoid *Venturia canescens* were used. A cyclic dynamic of abundance in all systems was recorded, with a very significant dependence of cycle periods upon the composition of the system. The correspondence of the details of the dynamical patterns with predictions from mathematical modelling was assessed only qualitatively.

The variation of viral disease transmission was analysed using different larval instars in laboratory and field studies. Modelling was used to account for different susceptibilities, feeding habits and times of larval death after infection (Goulson *et al*., 1995). The influence of the parameter of larval population density on transmission of gypsy moth NPV was investigated by D´Amico *et al*. (1996). In order to control the "environmental axis" under investigation, meshs were used to prevent the movement of larvae from their place on red oak foliage. Different numbers of larvae were used to investigate the influence on the transmission coefficient. Its density dependent variability did not correspond to predictions of different modelling approaches, demonstrating the need to redefine this process. A correspondence with data of large scale investigations (Woods and Elkinton, 1987) suggested a common driving force for the type of variability. It was concluded that the large-scale dynamics of the virus may be determined by interactions at the small scale. Further experimentation at a large scale was considered necessary in order to test this hypothesis.

Observations of virus distribution following artificially induced epizootics failed to correspond with any of the predictions included in mathematical modelling of spatial spread (Dwyer and Elkinton, 1995). The mechanism of dispersal in addition to larval movement, including ballooning of first instars, remained a matter of speculation.

In the type of research as described above, field experimentation or observations in the open environment were sometimes used directly to compare model predictions with data. The need for particular data and the convenience of their generation were the main factors determining the level of complexity for investigations. A special role of microcosm experimentation was not appreciated explicitly. Altogether, empirical testing challenges theoretical modelling, to improve its accuracy and

correspondence to reality, in this way increasing its predictive value. In general, the correspondence with data from the open environment is considered the crucial test for the validity of models.

Whereas theoretical investigations have their own merits - in particular by shaping the reflection about essential components and relations, their value for the description of real systems is that of an untested hypothesis. This character compromises the direct applicability for microbial pest control or the prediction of the fate of non-target organisms with a low susceptibility for virus infection (Bowers and Begon, 1991; Begon and Bowers, 1994).

The interrelationship of an improved understanding of baculovirus ecology on one side and their biosafety on the other is stressed in recent publications (Cory *et al*., 1997; Cory and Hails, 1997). The importance of ecological testing and modelling is highlighted and its use for inferences on the fate of novel baculovirus genotypes (is the genotype prone to extinction in time? or will it be established as a member of natural populations?) and on the fate of hosts with varying sensitivities is briefly considered. Molecular biology techniques not only offer new insights into the mechanisms of virus host interactions, they also provide methods of its modification with some impact on safety perception. The potential of strain detection and identification also provides for novel details of monitoring in space and time suitable for direct comparisons of different genotypes, differing in subtle biological parameters.

The generation of a novel quality of (predictive) knowledge about baculovirus fate and effects seems to represent a significant scientific challenge in baculovirus research. The potentials and inherent limits of an intermediate stage of microcosm research still have to be explored in response to such a challenge. There is no doubt that strategies of baculovirus use as insecticides will profit from improvements in ecological knowledge in the long run.

B. Experimentation and uses in greenhouses

Experimentation in closed environments is not generally used to improve control over experimental parameters, to confine experimentation from the open environment, or to generate predictive knowledge about the environmental behaviour of viruses. The development of a virus application in greenhouses is cited here to illustrate the stepwise extension of experience and knowledge during the conventional steps for the commercialisation as a biocide product. In this case, greenhouses merely represent the intended localisation for the application and do not constitute "simulated environments". The design of experimentation thus is not adapted to generate novel potentials of inferences about the fate and behaviour of the virus in the open environment.

The intended use in greenhouse cultures triggered experiments studying the efficacy of a nucleopolyhedrovirus, SeMNPV, for controlling larval populations of the noctuid *Spodoptera exigua*. Application of $1x10^8$ OBs per m^2 on ornamental plants and tomatoes resulted in 95% - 100% larval mortality. Mortality of early and late instar larvae was similar, but feeding damage was more pronounced when larvae were in late instars at the time of application. This is referred to as "maturation resistance". It was concluded that the virus was an effective potential control agent for *S. exigua* in greenhouses also in comparison with a use of chemical insecticides (Smits *et al*., 1987b). Superior activity of baculoviruses against pests of ornamental plants in comparison to chemical treatments has also been reported by others (Geissler and Schiephake, 1991).

Different virus strains, known or suspected to be infective to specific target insects, have been screened with respect to their virulence (lethal dose, LD_{50}). Selection of a strain for application may then include other criteria like the knowledge about host range and the possibility of cost effective production (Smits and Vlak, 1988a). Monitoring included the observation of movement and feeding

behaviour of the target organism to adapt application techniques (Smits *et al*., 1987a). After optimisation of production conditions, the product was characterised for its biological activity and its microbiological composition and purity (Smits and Vlak, 1988b). After the accumulated information was considered to be satisfactory by all parties, registration of the product was the outcome (Smits and Vlak, 1994). Data of virus dispersal, virulence, and replication together with features of the host organism (development, susceptibility of instar stages, movement) provided input for modelling. Understanding viral population dynamics in generating predictive knowledge was considered to be useful for safety assessment in general and to contribute to the optimisation of application strategies (Moed *et al*., 1990; van der Werf *et al*. 1991).

6. History of Use (Examples of Environmental Applications of the Organisms and Information Derived from These Examples)

A. Brief historical account of baculovirus detection and application

Observations of silkworm (*Bombyx mori)* cultures with descriptions of jaundiced larvae due to paralysis, as described in ancient Chinese literature, appear to be the earliest accounts describing the effect of baculovirus infections. In western literature, this is credited to the verses of the poem "De Bombyce" by the Italian bishop Marco Vida of Cremona, 1527 (Benz, 1986). Liquefaction of diseased larvae occurs in the process of converting insect biomass into progeny virus and the older literature refers to these symptoms as "melting" or "wilting".

The breakdown of the European silk industry and virus infections threatening the emerging shrimp aquaculture industry provide two of the very few examples of negative consequences from baculovirus infectivity as perceived by mankind (Chen *et al*., 1989; Chou *et al*., 1995; Momoyama and Sano, 1996). Favourable conditions for epizootics in artificial mass cultures of arthropods contribute to this type of observation. Considering present knowledge of the widespread occurrence of baculoviruses in insects, their persistence and spread by a variety of vectors which exposes soil, crop plants and plant debris with great numbers of virus particles (Heimpel *et al*., 1973), all day life can be regarded as natural "nontarget-species testing". This occurence demonstrates to some extent the lack of infectivity and pathogenicity of baculoviruses for organisms other than arthropods, in particular vertebrates including humans.

The first attempts to use baculoviruses for biological control can be dated back to the year 1892. During massive population increases of nun moths (*Lymantria monocha*, L.), a severe pine pest in Europe, the use of the infectious agent causing the so called "Wipfelkrankheit" was intended to combat the insect pest. But the "insecticide" was prepared in a manner corresponding to its identification as a bacterium at that time. Thus, the observation of diseased larvae and a final collapse of the population short after application must be attributed to a common natural outbreak of an epizootic, not to a successful application strategy (Huber, 1986).

The history of integrating novel experimental techniques and increasing knowledge in biology to finally identify the nature and features of the viruses did not proceed in a continuous and straightforward manner (Benz, 1986). The systematic exploration of the potential of baculoviruses for the control of insect pests is described by Benz (1986) and Huber (1986). Yearian and Young (1982) and Cunningham (1982) documented the use of about 50 different baculoviruses. The successful biological control of insect pests in field crops, plantation, orchard crops, and forests was demonstrated by field experimentation and applications of widely different scales all over the world. These experimentations and applications resulted in the development of fully registered baculovirus insecticides in several countries. However, registration and commercialisation of viral pesticides did

not keep pace with the development, although, it was estimated that about 30% of the major insect pest species could potentially be controlled by baculoviruses (Falcon, 1978).

A substantial increase in the use world-wide did not fundamentally change the situation regarding the registration and commercial production of baculovirus insecticides and their economic success. Although environmental policy and public awareness demand an increasingly rigorous testing for the registration of chemicals, a trend that should generally favour biologicals, the situation regarding the registration and commercial production of baculovirus insecticides has not changed fundamentally. Baculoviruses, due to their character as biologicals, are endowed with a variety of application constraints. Among these, the narrow host range, a limited life time and the slow speed of action resulting in demands on application strategies, are important factors in their failure to effectively compete with chemicals (Huber, 1986; Bohmfalk, 1986). Two noticeable developments might, in the future, contribute to a more favourable situation for baculovirus insecticides on the market.

- The progress in the development of techniques for virus production may significantly improve their economic competitiveness. Supported by the development of cell culturing techniques and a widespread use of baculovirus systems for the expression and production of a multitude of heterologous proteins, a cost effective multiplication of viruses by insect cell cultures in fermenters has become a realistic perspective (Rhodes, 1996).

- Principles and methods of molecular biology contribute to an enhanced understanding of the interaction of baculoviruses with their hosts and offer a variety of potentials to modify this interaction with the objective to relieve some of the (biological) constraints on their convenient applicability (Bonning *et al*., 1992; Crook and Winstanley, 1995).

The first trend can clearly be seen in an increasing number of publications on technical aspects of baculovirus insecticide production. Limitations on maintaining infectivity following growth in insect cell culture are becoming better understood and overcome. The exclusive emphasis given to the registration of genetically modified baculoviruses in a recent contribution on commercialisation of baculovirus insecticides, appears to underpin the importance given to the latter trend internationally (Black *et al*., 1997).

B. Types of pest control and strategies of virus selection for application

Any intended use of baculoviruses for insect pest management includes the screening for a virus strain virulent for the particular species. Isolates from diseased insects in the application area frequently are the first choice and are used as a promising and convenient starting point. If available, such isolates are in general included in the screening, but the testing for suitable viruses is not conventionally limited to those indigenous agents (Smits and Vlak, 1988a; Shapiro and Robertson, 1991). Both, Benz and Huber (1986) give a special credit to the control of the European spruce sawfly, *Gilpinia hercyniae*, introduced in the US and Canada long before the 1930s, when it became a serious pest of spruce. Population gradations frequently collapsed after the dissemination of a NPV which was probably accidentally introduced with imported parasites. Later, in the following, planned applications successfully supported the natural epizootics and the establishment of the virus finally controlled areas of minor infestations. The CpGV-isolate which is registered and commonly used for the control of the codling moth in different European countries, was originally isolated from diseased insects found in Mexico (Tanada, 1964).

This instance can be taken as an example of two types of strategies of biological control: the **introduction and establishment of a novel agent from a different geographic region**, however not planned in this case, and **inoculative augmentation**, used to enhance a natural antagonist, in order to

achieve control of the pest species. The history of baculovirus introductions or uses does not contain any documented observations of negative or unintended consequences. This is in contrast to other introductions for biological control (Ehler, 1991) and reflects the biological properties of these viruses to some extent.

The application of baculoviruses in forests can most often be characterised as an **augmentation** of an indigenous pathogen reservoir to improve natural control over high rates of insect multiplication (radiations). This type of application represents the most impressive record of the use of baculoviruses with respect to extent and success (Huber, 1986). The control of insect pests in orchards, greenhouses and annual crops, in general, demands a third type of application, **inundative augmentation**, by inoculating these areas with an amount of viruses that productively infects the number of pest organisms that must be controlled to keep the damage below economic threshold. Such applications do not rely on virus multiplication and spread to achieve the desired effect - and thus are more comparable to the use of topically applied chemicals. However, the slope of the rate effect curve and economic reasons will not allow management of the efficacy of applications by modifying (increasing) the application dose in the same way as is sometimes the case with chemicals (Huber, 1986).

The screening for virus isolates suitable for a pest management objective is generally not adapted to the intended type of control as categorised above. Infectivity and virulence, encompassing the dose-response function of the virus interaction with the pest species (C.23) make up the most important and frequently exclusive criteria for the selection of a virus and the planning of field application. Whereas bioassays serve in the analysis of the potency of a baculovirus preparation, field testing is essential to evaluate whether its use is feasible and to investigate the effect of formulations and the application conditions of the efficacy of the insecticide. World-wide, many improvements and modifications in production, formulation and application of baculovirus insecticides have been achieved and tested in recent years.

Before the era of molecular biology, the potential to modify infectivity and persistence of baculoviruses was frequently tested by conventional means of selection, eventually including mutagenesis (*e.g.* Reichelderfer and Benton, 1973). Eventually, selection resulted in a virus mutant analogous to genetically modified strains carrying a deletion of a gene for host interaction with the same objective. The description of a mutant, selected by Wood and co-workers (1981), which demonstrated a significantly reduced mean lethal time, suggests such a coherence (with genetically modified viruses lacking the *egt*-gene, see A 4.4). From experiments intending to modify host range or to adapt baculoviruses to alternate hosts, no clear cut picture has yet emerged. Experimentation before the introduction of molecular methods suffered from the difficulty of differentiating inoculum viruses from viruses persistent in the insect culture and potentially activated during the experiment. An adaptation to novel hosts has been described in several cases and/or an enhanced virulence was observed after virus multiplication in an alternative host (Shapiro *et al*., 1982; Martignoni and Iwai, 1986a; Stairs, 1990).

The testing of virus multiplication in alternative hosts may also be triggered by the objective to improve virus production with respect to cost effectiveness. For example, salt marsh caterpillars (*Estigmene acraea*) appeared to be a suitable production host for several NPVs of forest pests (*Orygia pseudotsugata* SNPV, *Choristoneura fumiferana* MNPV) by not only improving virus yield per larvae, but also by enhancing virulence (Shapiro *et al*., 1982). From experiments analysing the virulence of viruses obtained from different instar stages of larvae, it was recommended the virulence in and yield of occlusion bodies (OB) per weight of larvae for the optimisation of production yields be observed (Shapiro *et al*., 1986). Considering the potential of virus evolution during multiplication in an unnatural environment with novel selective constraints, an observation of the biological activity of production lots obviously is advisable, especially after propagation in alternative hosts (Reiser *et al*.,

1993; Maracaja *et al*., 1994). The optimisation of production in cell culture may represent an additional challenge in this respect (Lynn *et al*., 1993; Tompkins *et al*., 1988).

Apart from the modification or selection for host range or infectivity parameters, another biological feature of baculoviruses compromising their applicability in the environment seems to be prone to direct selection: that is their sensitivity for UV-radiation, being the most significant factor for the limited life time of baculovirus insecticides. Although the mechanism of improved UV adaptation is not elucidated, some reports of successful selections were published (*e.g.* Brassel and Benz, 1979; Witt and Hink, 1979; Shapiro and Bell, 1984). However, the use of such variants in field experimentation was not reported. Selective procedures were also used to isolate variants of *Spodoptera frugiperda* MNPV with an enhanced rate of vertical transmission of baculovirus infections from adults to progeny, for example, by egg contamination (Fuxa and Richter, 1991). Such variants are likely to modify the dynamics of the spread of infections in natural populations and might improve the control of pests in particular situations.

Baculoviruses have also been used in combinations of different virus strains, in order to achieve control of different pest species (Harper, 1986; Dhandapini *et al*., 1992). The synergistic effect known from certain double infections (Arne and Nordin, 1995; Ding *et al*., 1995, see section A.4.2) has not been exploited in field experimentation.

C. Modifications of chemical composition of formulations and of application techniques

In addition to approaches intending to improve the use of baculoviruses by modifying some of their biological parameters, formulations were adapted to achieve an economically feasible control. In competition with chemical insecticides, field testing and applications world-wide are evaluated for the optimisation of pest control.

The limited lifetime of baculovirus insecticides in the environment, to a great extent caused by their sensitivity for sunlight (UV), can be increased by the addition of a variety of UV-protectants. Optical brighteners, originally only tested as radioprotectants also proved to very significantly enhance virulence of virus host interactions in the laboratory (Shapiro, 1992; Shapiro and Robertson, 1992). The median lethal dose was reduced up to 4 orders of magnitude, the median lethal time was reduced for about 50% (7 instead of 14 days), and non-susceptibility was converted to susceptibility in some cases (Shapiro and Dougherty, 1994; Zou and Young, 1996). Laboratory testing was also supplemented by field testing with qualitatively corresponding results (Webb *et al*., 1994; Zou and Young, 1996). The phenomenon obviously requires the combined action of brighteners and viruses. At the highest dosage, the application of brighteners alone does not induce larval death. In field tests the increase of mortality of *Lymantria dispar* caused by the indigenous natural virus was observed (Webb *et al*., 1994).

In order to enhance the frequency and effectiveness of exposition of insect larvae, which predominantly become infected by feeding on plant material, spraying techniques have to be adapted and optimised (Smits *et al*., 1988; Payne *et al*., 1996). The addition of detergents and stickers serves for modifications of the distribution of spray droplets, and feeding attractants (*e.g.* crude sugar) are tested in order to enhance productive encounters of larvae with this distribution. The requirement to carefully test the environmental safety of formulation additives is highlighted by a field observation that molasses added as an UV protectant proved to be phytotoxic for soybean plants (Im *et al*., 1990).

A variety of approaches describe the combined use of baculoviruses and chemical insecticides at significantly reduced application dosages (1/3 - 1/10). Apparently, the chemicals reduce the tolerance of insects for other stress factors, thereby enhancing their susceptibility for baculovirus infection.

Alternatively, an impact of the virus infection on the insects ability to degrade the chemical has also been suggested as a mechanism (Huang and Dai, 1991). A synergistic (more than additive) enhancement in effectiveness of a combined use has been described (Jacques *et al*., 1988, 1989; Salama and Moawed, 1988; Peters and Coaker, 1993). This strategy was particularly recommended to compensate unsatisfactory control due to heavy insect infestations or climatic conditions (Moscardi and Corso, 1988; Ding *et al*., 1989).

D. Field testing and commercial use of baculovirus insecticides

A detailed continent-by-continent survey on the developmental, experimental and commercial use of baculovirus insecticides was recently compiled in "Insect Viruses and Pest Management" (Hunter-Fujita *et al.,* 1998). Some examples of the most important baculovirus insecticides tested and used in the field are:

- *Adoxophyes orana* GV has been extensively field tested and finally registered in Switzerland for control of the summerfruit tortix (Andermatt, 1991). This virus is also registered in Germany.

- *Agrotis segetum* GV and NPV have been tested in many countries for control of the common cutworm (*A. segetum*) and the greasy cutworm (*A. ipsilon*). They showed superior efficacy compared to chemical insecticides. *Agrotis segetum* GV-based bioinsecticides were registered in Denmark and the former Soviet Union (Huber, 1998; Lipa, 1998).

- *Anticarsia gemmatalis* MNPV has been used for control of the velvetbean caterpillar in soybean on a large scale in Brazil (Moscardi, 1990; Da Silva, 1992). The application of AgMNPV has increased from 2000 ha treated in 1982/ 1983 to 1 million ha in 1989/90. At a dose of 50 LE/ha (LE=Larval equivalents, the amount of virus prepared from one larvae) one application of the baculovirus was as efficient as chemical control (frequently requiring several applications) to maintain population densities of *Anticarsia gemmatalis* below economic threshold. The combination of the virus preparation with low dosages of chemical insecticides has also been tested and proved to enhance effectivity. It was recommended to be used in situations of heavy infestations when control cannot be achieved with the virus preparation alone (Moscardi and Corso, 1988; Da Silva, 1995). The widespread use of the viral insecticide was the reason to initiate the special monitoring of Brazilian *Anticarsia* populations for the selection of phenotypes with enhanced resistance to the virus (see C.24).

- *Cydia pomonella* GV has been extensively tested for control of the codling moth in apples, pears and walnut in the last 25 years. Meanwhile CpGV based products are registered in many European countries, *e.g.* Austria, France, Germany, Spain, Switzerland and the Netherlands and have been registered in the US since 1995. Field testing has also been conducted in Australia, Canada, Chile, the USA and other countries. Best results were obtained in cooler climates where the codling moth produces only one annual generation (Audemard *et al*., 1992, Huber, 1998).

- *Autographa californica* MNPV has a broad host range and has been tested for control of different pest insects, esp. of *H. virescens* and *T. ni.* AcMNPV has been registered in the USA since 1994. Experimental and commercial use of AcMNPV is reported from Central America, where it has been applied on several thousand hectares of cabbage and broccoli (Hunter-Fujita *et al.,* 1998). In recent years, field tests have been performed using genetically engineered AcMNPV recombinants with improved speed of kill. These recombinants, which

express insecticidal toxins and/or lack the *egt* gene, are the prototypes for other genetically engineered baculovirus insecticides (Cory *et al.,* 1994, Black, 1997).

- *Heliothis (Helicoverpa) sp*. NPV. The cotton bollworm (*Helicoverpa zea*) and the tobacco budworm (*Heliothis virescens*) are major pests of cotton in southern/south-eastern regions of the US and serious pests of many food, fibre, and forage crops world-wide. *Heliothis* SNPV based insecticides were developed during the late 1960s - 70s, registered in 1975, and were a commercial success until the early 1980s when synthetic pyrethroids were introduced. The total area in the USA sprayed with this virus was estimated to be more than 1 million hectares (C. M. Ignoffo cited in Cunningham, 1998). A novel strategy for control of *Helicoverpa ssp*. using *Heliothis* SNPV was investigated when it was found that introduced and native early season host plants (in particular the wild geranium, *Geranium dissectum)*, occupying only 5% of the Mississippi rural area, support the first generation of bollworm/budworm populations which subsequently invade the cotton fields. A control of the first and possibly second generation by applying HzSNPV to alternate hosts had been tested in small field tests as an effective potential management strategy. Large area testing followed these first experiments (Bell and Hardee, 1994; Bell and Hayes, 1994; Hayes and Bell, 1994).

- *Helicoverpa armigera* NPV has a great potential for the control of African cotton bollworm, *H. armigera*, which is one of the most deleterious insect pest in warmer climates of the Old World. Extensive studies including combinations with another biological, *Bacillus thuringiensis* (Bt), have been performed in China, India and African countries (Zhang *et al*., 1996a; 1996b, *Kunjeku et al., 1998*). A significantly reduced mean lethal time and a high efficacy (97 % of larval deaths) were observed for the combination with Bt, which also had an effect on pupal mortality in the following year. The effectiveness was found to be at least equivalent to that of recommended chemical insecticides.

- *Lymantria dispar* NPV has been widely applied for control of gypsy moth *L. dispar* in the USA, where it is registered since 1978 and where more than 11 000 ha have been treated (for review see Lewis, 1981; Podgwaite, 1985; Cunningham, 1998). It also became the most important viral insecticide in the former Soviet Union (for review see Lipa, 1998). In order to improve the control of divergent densities of the gypsy moth, the commercial product Gypchek was modified with sunlight protectants, feeding stimulants and a sticker. Two applications of 1.25×10^{12} OB/ha resulted in a reduction of egg masses by 98% and 80% in comparison with control woodlots at different sites (Podgwaite *et al*., 1992). This application dose is currently recommended for the use of LdMNPV preparations in the USA.

- *Mamestra brassicae* NPV has been tested for efficacy in a number field trials for control of the cabbage looper, *M. brassicae.* This virus has a considerably broad host range and more than 30 susceptible insect species have been identified (Doyle *et al*., 1990). Reasonable success was reported for the control of *P. xylostella, Heliothis spp, Spodoptera spp., Trichoplusia ni* and others. Commercial products were developed and registered in France and the former Soviet Union (Hunter-Fujita *et al.,* 1998).

- *Neodiprion sertifer* NPV has been successfully applied for control of the European pine sawfly, which is a serious pest on pine plantations (for review see Cunningham and Entwistle, 1981). Field work with NeseNPV has been conducted in Canada, Scandinavia, Poland, UK, the former Soviet Union and USA. Commercial products were registered in Finland (1983), UK (1985), USA (1983, but discontinued by the company in 1991) and the former Soviet Union.

- *psuedotsugata* NPV is used and has been registered since 1976 in the US and Canada for control of the Douglas fir tussock moth. It is recommended particularly for early control when population densities are relatively low because it takes five to eight weeks before the larvae stop feeding, during which time further defoliation occurs. (Defoliator Management Guidebook, Ministry of Forests, British Colombia, Canada http://www.for.gov.bc.ca/tasb/legsregs/fpc/fpcguide/guidetoc.htm).

- *Spodoptera spp. NPV.* Several specific viruses have been isolated from different *Spodoptera* species, such as SeMNPV from the beet army worm *(S. exigua*), SpexNPV from the African armyworm *(S. exempta),* SfMNPV from the fall army worm (*S. frugiperda*), SpltNPV from the Tobacco cut worm *(S. litura),* SpliNPV from the Egyptian cotton leaf worm (S. littoralis) and others. Extensive research on these viruses and field tests resulted in the development of several commercial products (Hunter-Fujita *et al.,* 1998). Field isolates of SeMNPV were found in the USA, Thailand and Spain (Caballero *et al.,* 1992). A registered product, based on the US isolate, is registered and widely used in Dutch greenhouses and in the USA, where *S. exigua* was found to be tolerant to many chemical insecticides. SfMNPV is being used in Central and South America, where *S. frugiperda* occurs as a major pest of corn and rice. It was reported that more than 20 000 ha were treated with SfMNPV in Brazil by 1992 (Oliveira *et al.,* 1998).

7. Characterisation of the Genomes (e.g. Open Reading Frames, Insertion Sequences), and Stability of These Characteristics

A. The structure of the baculovirus genome

The baculovirus genome is a double-stranded, circular DNA which varies between 90 - 160 kilo base pairs (kbp) for different members of the family Baculoviridae (Murphy *et al*., 1995). Based on the analysis of the entire genome sequence of AcMNPV, it was estimated that the genome contain between 140 - 160 genes. About half of these genes have been transcriptionally and functionally characterised (for review see Kool and Vlak, 1993, Possee and Rohrmann, 1997). The immense variation of genome size among different baculovirus species suggests that some baculovirus genomes contain significantly less genes than others. Apart of these interspecific differences of genome size, an intraspecific variation caused by natural variation (Chapter 7.2) and host transposon insertion (Chapter 7.3) can be observed. The ability of the baculovirus nucleocapsid to expand and to harbour additional genetic information was exploited and led to the development of the baculovirus expression vector system.

To date, the genomes of three baculoviruses isolated from noctuid hosts have been completely sequenced. These are AcMNPV (with a genome of 133,894 bp), BmNPV (128,413 bp) and OpMNPV (131,990 bp) (Ayres *et al*., 1994, Maeda, 1994; Ahrens *et al*., 1997). Other complete baculovirus genome sequences can be expected in the future. Partial sequence information of about 35 other baculovirus genomes is available on the GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

In general, baculovirus genomes contain non-overlapping open reading frames (ORFs) with short intergenic regions. However, a few ORFs which partially overlapped adjacent ORFs were found. For example, in the genome of several NPV and that of *Cryptophlebia leucotreta* GV, the 5' end of the adjacent, bidirectionally orientated ORFs encoding *p38* and *lef*-5 overlap to a considerable extent (Ayres *et al*., 1994, Jehle and Backhaus, 1994). Gene splicing has only been observed with the early activator *ie-0* of AcMNPV. Its coding region consists of a small exon and the ORF of *ie-1*, which are spliced together and expressed early in infection (Kovacs *et al*., 1991).

Interspersed homologous regions, known as hr regions, have been identified in many baculovirus genomes. The hr regions in the AcMNPV genome consist of multiple copies of 28-30-bp palindromic sequences flanked on either side by direct repeats of 20 bp (Cochran and Faulkner, 1983; Ayres *et al*., 1994). These regions function as cis-acting enhancing factors for the transcription of early genes by RNA polymerase II (Theilmann and Stewart, 1992, for review see Friesen 1997) and as origins of DNA replication in transient DNA replication assays (Pearson *et al*., 1992; for review see Kool *et al*., 1995).

B. Natural variability of baculovirus genomes

In the 1970s, progress of the analysis of baculovirus genomes was accelerated: (i) the development of *in vitro* and *in vivo* cloning procedures, which allowed researchers to isolate and to propagate single virus genotypes, and (ii) the introduction of restriction endonuclease analysis, which allowed isolated virus DNA to be cleaved into fragments of specific length.

The occurrence of submolar bands in the restriction enzyme profiles of isolated DNA of baculovirus field isolates provided first evidence of substantial genetic variation within the natural baculovirus population. When single genotypes were isolated using plaque purification assays, these differences were commonly mapped to point mutations, acquisition or deletion of restriction sites or to insertions or deletions of hundreds or thousands basepairs (Lee and Miller, 1978; Crook *et al*., 1985; Smith and Crook, 1993).

C. Transposon insertion into baculovirus genomes

Horizontal transfer of insect host transposable elements (TE) into baculovirus genomes is an occasionally observed event that contributes to genetic heterogeneity. Most TE insertion of AcMNPV or GmMNPV were associated with a specific alteration of plaque morphology of infected insect cells and characterised by a significantly reduced number of polyhedra per infected cell (Fraser and Hink, 1982; Fraser, 1986). These so-called “few polyhedra” or FP mutants were generated during serial passage and harboured transposon insertion within the 25K gene commonly known as FP locus (map unit 36.0 - 37.0). The role of the 25K gene has not yet been completely elucidated but it might be involved in the temporal regulation of BV and ODV production (Jarvis *et al*., 1992). AcMNPV with a mutated 25K gene was frequently impaired in virion occlusion and intranuclear nucleocapsid envelopment but released up to five-times more BV from infected SF-9 cells (Harrison and Summers, 1995). Disruption of the 25K gene apparently favours the production of budded viruses (BV). Since BV are about three orders of magnitude more infectious to cultured cell lines than ODVs, it is likely that transposon carrying FP-mutants have a strong selection advantage over wild-type viruses during the specific conditions of serial passaging in cultured cells.

A number of FP mutants of AcMNPV and GmMNPV harbouring transposons which belong to the class of (DNA)-transposons have been described (Table 4.3). These transposons originate from *T. ni* or *S. frugiperda* host cells. Although their sizes vary from 0.3 - 2.5 kbp, they share common structural characteristics as short terminal inverted repeats of 13-15 bp and a duplication of a TTAA target sequence (for review see Fraser, 1986; Friesen, 1993; Jehle, 1996). Most of these transposons lack open reading frames to encode a transposase suggesting that they are defective copies that cannot transpose autonomously. Only transposon piggyBac (formerly termed IFP2) was shown to contain an ORF which supports insertion and excision of this element (Fraser *et al*., 1995; Elick *et al*., 1996).

The conformation that transposon insertion into baculovirus genomes is not restricted to the specific conditions of cell culture but also occurs during normal infection of host larvae was provided

by Jehle *et al*. (1995) who demonstrated the insertion of two different Tc1/mariner-like transposons into CpGV genome after infection of *C. pomonella* and *C. leucotreta* larvae.

Host transposon insertion into baculovirus genomes exerts drastic effects on the genomic integrity of the virus and has possibly far reaching consequences for baculovirus evolution. The observed effects are (i) acquisition of new functional genes and/or regulatory sequences, (ii) disruption of viral genes rendering them non functional, and (iii) deletion of extended portions of the genome by self-recombination or excision (Carstens, 1987). In most cases, transposon insertion can result in reduced fitness and competitiveness of the altered virus. However, the isolation of the FP-mutants and the CpGV mutants containing a transposon have demonstrated that these viruses are viable and that transposon insertion significantly increases genetic heterogeneity.

Table 4.3 Insect host transposons found in baculovirus

Transposon	Host Virus	Insect Origin	Reference
TED (retrotransposon)	AcMNPV	*T. ni*	Miller and Miller, 1982; Friesen and Nissen, 1990
M5	AcMNPV	*S. frugiperda*	Carstens, 1987
IFP2 (piggyBac)	GmMNPV	*T. ni*	Cary *et al*., 1989
TFP3 (tagalong)	AcMNPV	*T. ni*	Wang *et al*., 1989
TFP3 (tagalong)	GmMNPV	*T. ni*	Wang *et al*., 1989
IFP2.2	AcMNPV	*S. frugiperda*	Beames and Summers, 1990
IFP1.6	AcMNPV	*S. frugiperda*	Beames and Summers, 1990
E	AcMNPV	*S. frugiperda*	Schetter *et al*., 1990
hitchhiker	AcMNPV	*T. ni*	Bauser *et al*., 1996
TCl4.7	CpGV	*C. leucotreta*	Jehle *et al*., 1995
TCp.3.2	CpGV	*C. pomonella*	Jehle, 1996

8. Genetic Transfer Capability

A. Recombination

Recombination is a decisive mechanism which is responsible for intergenomic exchange of information in all organisms, including baculoviruses. There are two different classes of recombination, a homologous and a non-homologous or illegitimate recombination. Homologous recombination means the genetic exchange of allelic sequences between two different genomes, whereas non-homologous recombination comprises all events of non-allelic sequences exchange.

Homologous recombination

With baculoviruses, extensive homologous recombination has been observed between the closely related AcMNPV, GmMNPV, RoMNPV and TnMNPV (Miller *et al*., 1980; Smith and Summers,

1980; Croizier and Quiot, 1981). After coinfection of TN-368 cells of *T. ni* with AcMNPV and RoMNPV seven out of 100 isolated plaques where identified as recombinants, each with at least one crossing over event (Summers *et al*., 1980). Intraspecific recombination was also observed when SF-9 cells were co-infected with different pairs of AgMNPV genotypes (Croizier and Ribeiro, 1992). These results indicated that the frequency of recombination among highly homologous baculoviruses is considerable.

When Croizier *et al*. (1988) co-transfected larvae of *Galleria mellonella* with isolated DNA of RoMNPV and restriction fragments of AcMNPV, a very high number of RoMNPV recombinants but no parental RoMNPV genotypes were recovered. Since replication of AcMNPV in *G. mellonella* is much more effective than that of RoMNPV, it was suggested that the RoMNPV might have acquired an unknown replication advantage by recombination with AcMNPV DNA fragments.

A high recombination rate was also found when *T. ni* larvae were co-infected with wild-type AcMNPV and a polyhedrin-negative, lacZ-expressing AcMNPV mutant. A recombination frequency of at least 6.6% was estimated by plaque assays of hemolymph of co-infected larvae and scoring for recombinant plaques (Merrywheather *et al*., 1994).

Further evidence for extensive homologous recombination among different variants of the same virus species is based on the isolation and restriction mapping of wild-type virus mixtures. By the method of *in vivo* cloning Smith and Crook (1988; 1993) isolated a number of different ArGV genotypes which differed in a few restriction fragments. Based on comparative physical mapping, it was suggested that the diversity of these variants was partly caused by recombination during natural coinfections. A rapid replacement of two closely related parental SeMNPV variants (Se-US and Se-SP2) by a recombinant (Se-SUR1) which differed from the parental strains by several cross over events was observed after coinfection of *S. exigua* larvae. This result suggested selection advantage of the new recombinant over the parental strains, though significant differences in the biological activities (LD_{50} and LT_{50}) were not observed (Munoz *et al*., 1997).

These findings underscore the fact that homologous recombination among virus variants is a normal process of genetic exchange which occurrs at a considerable frequency during *in vitro* and *in vivo* replication. The process of homologous recombination appears to be linked to and driven by the viral replicative machinery. Although the genetic factors involved in recombination have not been identified, it was suggested that genes involved in DNA replication and possibly hr regions may play a central role in the events (Martin and Weber, 1997).

Heterologous recombination

The probability of recombination between baculovirus sequences decreases drastically when the baculoviruses are less closely related. So far, recombination between different baculovirus species has only been observed in laboratory experiments where the generation of recombinants was biased by selection or replication advantages. For example, the identification of genes encoding host range factors or apoptosis inhibitor was achieved using this approach (see A 3.3 and A 4.3).

Heterologous recombination has been forced by co-transfection of restricted DNA or cloned DNA fragments with intact viruses or virus DNA. In an experiment addressing the possibility of interspecific recombination Roosien *et al*. (1986) co-transfected *S. frugiperda* cells with a polyhedrin negative AcMNPV mutant and a plasmid containing the polyhedrin gene of MbMNPV. AcMNPV mutants were isolated and contained the MbMNPV polyhedrin gene insertion at different, non-specific sites. However, these recombinants contained less virions and showed reduced infectivity to *S. frugiperda* cells than wild-type AcMNPV. Similar results were obtained when a polyhedrin minus

AcMNPV mutant was co-transfected with a cloned polyhedrin gene of SfMNPV (Gonzales *et al*., 1989). The recombinant isolated from this experiment expressed less than 25% of the level of wild-type AcMNPV polyhedrin gene.

In general, the competitiveness and biological fitness of heterologous recombinants is lower than that of wild-type viruses. One reason is that by non-specific recombination events, coding or regulatory sequence functions might become impaired resulting in a less viable virus.

II. Human Health Considerations

Baculoviruses are naturally occuring pathogens of arthropods. Their host range is exclusively restricted to arthropods. No member of this virus family is infective to plants or vertebrates. Baculoviruses are ubiquitously present in the environment and have been used for biological insect control for more than 100 years. Circumstantial evidence for the safety of baculoviruses emerges from the history of contact between baculoviruses and humans without any detrimental effect.

During the 1970s, the US Environmental Protection Agency established the Guidance for Safety Testing of Baculoviruses, which also became a guideline of baculovirus safety tests in many other countries (Anonymous, 1975; Summers *et al*., 1975). This guidance included *in vivo* and *in vitro* safety studies and was applied for commercial baculovirus insecticides, such as *Helicoverpa zea S*NPV (Elcar®), *Orgyia pseudotsugata* NPV (TM Biocontrol-1) and many others. *Helicoverpa zea* SNPV was the first commercial baculovirus insecticide and is one of the most extensively tested entomopathogenic viruses (Ignoffo, 1975). Safety tests of more than 51 entomopathogenic viruses including more than 30 baculoviruses resulted in a long and complete safety record (extensively reviewed by Ignoffo, 1973; Burges *et al*.1980a, 1980b; Gröner, 1986). No adverse effect on human health has been observed in any of these investigations indicating that the use of baculovirus is safe and does not cause any health hazards.

A. Safety tests of baculoviruses included

In vitro *and* in vivo *replication of baculoviruses in vertebrate and mammals*

The possibility of replication of baculoviruses in vertebrates and mammals was investigated by challenging many vertebrate and human cell lines with OB and BV of many baculoviruses. Although virus uptake of these cells was frequently reported, no evidences of virus replication or cytopathological effects were observed. The few early reports, which stated baculovirus replication in vertebrate cell lines (Himeno *et al*., 1967; McIntosh and Shamy, 1980) could never be demonstrated or confirmed in other laboratories. After oral uptake of baculoviruses by man, mice, chickens, rabbit, pigs, and other mammals, no specific antibody production, which would indicate replication of the virus used to challenge the host, was observed (reviewed by Gröner, 1986). In contrast, a specific immunological response against CpGV was observed in woodmice (*Apodemus sylvaticus*) which were trapped in an apple orchard sprayed with CpGV. It is conceivable that an antigenic challenge may have occurred via the nasal mucous membrane, virus replication or a negative effect to the animals was not observed (Bailey and Hunter Fujita, 1987).

Using a recombinant AcMNPV containing the *cat* gene under the control of the Rous sarcoma virus terminal repeat promoter and the β-galactosidase gene under the control of the very late polyhedrin promoter reporter gene, expression was analysed in different invertebrate and vertebrate cell lines (Carbonell *et al*., 1985; Carbonell and Miller, 1987). No *cat* or β-galactosidase activity was detected in transfected mouse or human carcinoma cells. On the other hand, recent reports showed that recombinant AcMNPV virus is efficiently taken up by human hepatocytes via an endosomal pathway.

Recombinant AcMNPV carrying the *Escherichia coli lacZ* reporter gene under control of the Rous sarcoma virus promoter and mammalian RNA processing signals showed considerable expression levels in the human liver cell line HepG2, but at very low levels, or not at all, in cell lines from other tissues (Hofmann *et al*., 1995; Boyce and Bucher, 1996). Based on these findings it was suggested that baculovirus might be exploited for liver-directed gene therapy. From the view of baculovirus safety this results also show that careful attention has to be paid to the promoters used to control heterologous gene expression in recombinant baculoviruses.

Acute oral and intraperitoneal toxicity of mammal

Acute toxicity of *Helicoverpa zea* NPV has been tested in many mammals, *e.g.* rat, mouse, rabbit, guinea pig and man, at doses from 6 x 10^9 to 3 x 10^{12} OB/kg, which is up to 1000 times the average field rate per acre. Similar tests were conducted with TnSNPV, SeMNPV, AcMNPV, LdMNPV, CpGV and others (Ignoffo, 1975).

Subacute dietary-administration to mammals

In order to test potential subacute toxicity or pathogenicity *Helicoverpa zea* NPV was fed or subcutaneously injected to mice (about 5 x 10^{10} OB/kg of animal), rats (4 x 10^9 to 4 x 10^{11} OB/kg), beagle dogs (7 x 10^9 OB/kg), rhesus monkeys (10^8 to 1.6 10^{10} OB/kg) and man (10^9 OB/day for 5 days). Similar tests were performed with rats for OpMNPV, LdMNPV and others (Ignoffo, 1975). Furthermore, health monitoring of workers who were involved in production of HzSNPV (for the viral pesticide) for extended time periods did not show any clinical symptomatology, nor any serological response or any indications that the virus is allergenic (Rogoff, 1975).

Eye- or skin-irritation to mammals

Eye irritation tests were negative, when 1 x 10^5 to 20 x 10^5 OB/eye were applied to rabbit eyes. Skin irritation sensitivity tests were conducted with *Helicoverpa zea* NPV in rabbits, guinea pigs and man at doses of 10^3 to 10^6 OB/mm^2 of skin. Dermal and eye applications have been also conducted with *Neodiprion sertifer* NPV, AcMNPV, SeMNPV and others without any adverse reactions (Ignoffo, 1975).

Cytogenetical implications as chromosomal aberrations or sister chromatid exchange to mammalian cell lines

In studies on the activation of endogenous C-type retrovirus by baculoviruses in three mammalian cell lines (mouse, rat, and man) no activation of C-type retrovirus could be detected (Schmidt, 1981). When cultured frog cells (ICT-2A) were challenged with TnSNPV no virus multiplication and no chromosome aberrations were observed over a 4-week period of time (McIntosh, 1975). No chromosome aberrations in Chinese hamster cells, mouse cells after oral uptake of BV or OB of AcMNPV and MbMNPV was observed (Miltenburger, 1978).

Carcinogenicity, teratogenicity, mutagenicity to mammals.

Potential carcinogenicity of *Helicoverpa* NPV were conducted in mice 10 x 10^9 to 4 x 10^{11} OB/kg) or rats (3.5 x 10^{12} OB/kg), teratogenicity tests were performed in rats at a dose of 10^9 OB/kg. No evidence of carcinogenic or teratogenic effects was found (Ignoffo, 1975).

III. Environmental and Agricultural Considerations

1. Natural Habitat and Geographic Distribution. Climatic Characteristics of Original Habitats

Baculoviruses are ubiquitous in the environment, their prevalence depending on the frequency of occurrence of their arthropod hosts that inhabit terrestrial and marine ecosystems. By 1986, about 1100 viruses known to infect insects had been described, more than 60% of them being baculoviruses (Martignoni and Iwai, 1986b). Natural epizootics and their survival under a variety of environmental / transport conditions provides for particularly high densities in diverse locations in the environment which may act as infection sources for pathogen multiplication (cabbage leafs, dust: Heimpel *et al*., 1973; Olofsson, 1988). Viruses from the same arthropod species from different geographic regions of the world may be as closely related as their hosts as demonstrated by the similarity of their restriction pattern (Vickers *et al*., 1991). However, different baculoviruses can also be isolated from the same species in one geographical region. Restriction endonuclease digest patterns can be used to analyse the genetic heterogeneity of baculovirus strains and of strains obtained from different geographical regions (Laitinen *et al*., 1996).

2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites

The ecological significance of baculoviruses is characterised by their impact on the population dynamics of their arthropod hosts. As deduced from experiences with epizootics of insects they can be regarded as a regulator of their host population density and multiplication. No report on a potential contribution of baculoviruses to the extinction of insect species is available. This feature does not seem to depend only on the usual evolutionary adaptation of the interaction of pathogens with their hosts, but might be a more fundamental property of their life style. Through international trade, and in the course of applications of baculoviruses for pest control to a considerable extent, strains of baculoviruses have been moved around the globe without negative observations on local faunas. The ecological traits of baculoviruses (in particular virulence and dispersal characteristics) in their combination are an apparent biological constraint on excessive population increases, potentially endangering the basis of their existence, and the survival of their arthropod hosts.

With the impact on population densities of their hosts, a secondary effect on predators, parasites and hyperparasites is evidently connected.

3. Pathogenicity – Host range, Infectivity, Toxigenicity, Virulence, Vectors

A. Bioassays for the testing of infectivity and virulence

Parameters of baculovirus host range, infectivity, and virulence are tested by bioassays which measure the response (*e.g.* mortality) of test species ("subjects") to different doses of defined virus preparations ("stimulus"). The *in vivo* bioassay is the only means by which the combined effect of all factors determining the potency of a baculovirus preparation can be measured. It is also used as a very sensitive method of monitoring the fate of baculoviruses in the field (see C.28). The titration of occlusion bodies in the light microscope offers a convenient method to compare different virus preparations with respect to the concentration of their (potentially) active structures. In principle, any comparisons between two virus preparations (*e.g.* a test substance with a standard preparation) requires that the dose response curve for both have the same shape, *i.e.* the bioactive component of the preparations is essentially similar. An understanding of the basic biological and statistical concepts of bioassays is important to fully realise their potential for analysing baculovirus interactions with other organisms and for improving their application (Hughes and Wood, 1986).

To obtain statistically meaningful results with the desired precision bioassays have to be carefully designed with respect to the **number of subjects** challenged. Optimised strategies depending on previous knowledge and model assumptions have been described (*e.g.* Hughes and Wood, 1986). Bioassays determining the yes or no (quantal) response of larval death through virus infection result in dose response relationships with the median effective dose ED_{50} (or EC_{50} if expressed in virus particles per ml) and the slope of the response curve as characteristic parameters. In addition to counting dead individuals, response data may also include the determination of particle titers in the cadavers. If the time course of larval death is recorded (time-mortality assays), as conveniently done in one experimental approach, monitoring results yield median lethal time LT_{50} (continuous exposure to virus) or median survival time ST_{50} (one inoculum at start of experiment), respectively.

To obtain meaningful and reproducible results other conditions of bioassays must be carefully observed. With respect to the **test organism,** uniform instar stages of larvae are used and reared under identical conditions (temperature, light regime, diet, group size). The ability to hatch insect larvae reproducibly with minor premature deaths (*e.g.* by bacterial infections) is one major problem for testing a great number of different test species, as required for host spectrum analysis.

Infection is frequently performed by applying the virus preparation to the surface of the natural or artificial diet - or by incorporating virus suspensions to the components of the artificial diet. Depending on the experimental design, the test species are kept on inoculated material for the duration of the assay - or the regime includes a transfer to a noninoculated diet, in order to better control inoculum and infection time. A large variety of experimental protocols for controlled virus inoculation have been described. They all suffer from inaccuracies, to which the variation of feeding behaviour of the insects also contributes (Hughes and Wood, 1986). Direct administering of virus suspensions by microinjection into the mouth of larvae, or the "measured drop feeding" method are ways to improve control over dose and inoculation time (Kunimi and Fuxa, 1996). The incubation of eggs with virus suspensions to infect neonate larvae seems to be a method for a well controlled infection of large numbers of insects at an identical developmental stage. Some of the latter methods are time consuming and have other limits (size of larvae for injection) which prevent their widespread use. Experimental protocols in general have to seek a sensible compromise between accuracy and practicability. Modifications of the assays have to accommodate the habits and peculiarities of the test species.

Data interpretation follows model assumptions. If the only interest is an estimate of the median effective dose and no inferences with respect to the absolute number of virus particles can be made, the probit model may be used. It assumes a log-normal distribution of individual effective doses (IED) for each host which invariably induces the response. The model refers to mechanistic interpretation of chemical interactions and assumes some co-operativity to be necessary for infection. The necessity to overcome host defenses can be considered as an interpretation of such assumptions in light of present knowledge. However, unlike chemicals, replicating entities such as bacteria and viruses can act independently to produce a response. In these cases, and if the hosts are reasonably uniform in their susceptibility to the pathogen, the dose response curve can be described and interpreted more adequately by an exponential model. The model describes infection as a stochastic process in which the lethal dose is the same for all individuals whereas the response rates reflect the chance to receive this dose. Essentially, one infectious virus entering the replicative stage is able to manifest the response according to such a model. Predictions of quantitative features of the infection process according to the model can be tested experimentally and results do not conflict with its interpretation, stressing the general applicability. (Hughes *et al*., 1984; Hughes and Wood, 1986; Ridout *et al*., 1993).

The method of "*in vivo*-cloning" takes advantage from the capacity of single viruses to initiate a productive infection. Administering virus titers for infection far below the LD_{50} results in a relatively large fraction of surviving larvae. The fraction of larvae, which died from replication of a single

infectious unit can then be calculated (Huber and Hughes, 1984). This method, eventually used in two consecutive steps to enhance probability of the selection, is conveniently used to obtain genetically homogeneous virus preparations (Smith and Crook, 1988).

B. Survey of non-target effects

The specificity of the interactions of baculoviruses with arthropod and the corresponding narrow range of species which are susceptible to productive infection by a particular virus, is the basis of their innocuousness for a large spectrum of nontarget organisms (Gröner, 1986). In the course of safety assessments toxicity and pathology studies have been performed on mammals, birds and other wildlife animals including beneficial insects such as the honeybee and silkworm. The studies with animals other than arthropods up to 1986 were extensively reviewed by Gröner (1986). In the following, the studies are only briefly summarised because they conclusively demonstrated the absence of any adverse effects.

- Toxicity studies on mammals with a variety of NPVs, using the spectrum of application routes as conventionally tested for chemical pesticides, never resulted in any indications of toxicity or pathogenicity using doses 10 to 100 times the per-acre field rate equated to a 70-kg man. Also, no indications of teratogenic or carcinogenic effects in mammals were found with challenges of NPVs (section B).

- No side effects on birds after oral application and on aquatic vertebrate and invertebrate animals could be observed. Such laboratory studies were supplemented by some extensive monitoring for pathological effects of wildlife birds and mammals after (aerial) applications of different NPVs.

Recently, toxic effects were observed with a larval test which was considered to be useful for assessing adverse effects of microbial pest control agents on nontarget bivalves because of its simplicity, precision, and sensitivity. Larvae of the coot clam *Mulinia lateralis* were challenged for 48 h during the straight hinged stage of development with the LdMNPV at a density of 10^6 OB/ml. Mortalities observed were significantly higher than those obtained with a heat killed control. Similar mortality was observed with a 10^{-4} dilution of a commercial mosquito larvicide based on *Bacillus thuringiensis* ssp. *israelensis* (Bti). No effect was observed with a molluscidal strain of *Bacillus alvei* or a broad-host-range fungal insect pathogen, *Metarhizium anisopliae*. Sodium dodecyl sulfate and a watersoluble fraction of a fuel oil were tested as a reference for comparison (Gormly *et al*., 1996).

- Consistent with their restriction of infectivity to the family or at least order of their original host, no infectivity or adverse effects on beneficial insects like pollinators (bees) have been observed. Baculovirus infection interferes with the multiplication of parasitoids within the same host. This interaction seems to be described most adequately as a competition for the same resource. No productive infection of the parasitoids is observed (see C.23/24).

- No genotoxic effect was observed by cytological studies after challenging mammals or cell cultures.

Similar studies with granuloviruses are smaller in number but gave the same results.

Small mammals or birds and also parasitoids feeding on insects infected by a baculovirus may take up and transport intact baculoviruses (*e.g.* in their digestive tract). Excretion of infective viruses may contribute to virus dispersal (C.26).

One experimental approach to use vectorised transport by honey bees as a nonintrusive means for virus dispersal has been field tested and indicates the perception of safety and non-target-innocuousness of baculoviruses. An applicator in a specifically designed substructure of a conventional beehive caused honey bees to take up (by surface contamination) and disseminate a talc formulation of HzSNPV into fields of *Trifolium incarnatum.* Increased HzSNPV induced mortality was observed in the clover fields foraged by the bees. A good persistence of baculovirus infectivity in honey was noted. An increased knowledge about the intersection of bee and target organism behaviour determining the virus transmission was considered to be essential, in order to further investigate the feasibility of the approach (Gross *et al*., 1994b).

C. Host range

The range of arthropods that can productively be infected by a given baculovirus can be regarded as its host range in a narrower sense. On one side, a limited host range is a feature of a rationally targeted biocontrol programme and an issue of biological safety. The control of defined pest species avoids side effects on the environment in general, and adverse effects on non-target insects including predatory and parasitoid species which provide additional control, in particular. On the other side, host range restrictions are also frequently regarded as an important application constraint if a complex of pest insects has to be managed. They determine limitations for the potential market volume of viral insecticides. Reports on particular host range testing and the selection strategies on virus strains for applications reflect this ambivalent view on host range restriction.

Among the approaches to relief application constraints for virus insecticides, prospects of modifying - and enhancing - host range by the use of molecular biology tools are being developed (Doyle *et al*., 1990; Thiem, 1997). The realisation of this objective will challenge the validity of host range testing procedures and the interpretation of their results even more than the use of genetically modified viruses does in general. An increasing knowledge about the molecular interactions involved in host range determination and limitations is the basis for such an approach. In comparison to the use of selective strategies on natural viruses such a basis may enable a design of modifications for more targeted and predictable effects on virus phenotype (Miller and Lu, 1997; see section A.3.3).

The genetic information of the virus genome, conserving evolutionary processes for perpetuation of its existence in time, is realised in the sequence of processes during an infectious cycle typically resulting in the release of high numbers of progeny virus from larval carcasses (see section A.3.1). Abortive or persistent infections indicate potential alternative strategies for persistence. A productive infection requires the specific interaction of host components with virus structure and virus encoded functions within a temporal and local arrangement of “key-lock fittings” in order to overcome host barriers and defenses and to exploit and transform host functions for viral replication. Overall, these specificities correlate with phylogenetic relationships, and the systematics of baculoviruses seems to represent the common evolutionary origin and concerted evolution with their arthropod hosts to some degree.

In detail however, evolution - and the variation and distribution of “keys” and “lockers” - follow different pathways in virus populations and in sexually reproducing animals. Genome modifications by mutations and genetic exchange are governed by different mechanisms and constraints. Thus, the degree of adaptation of a virus strain or “species” to a set of host organisms providing the requirements for productive interactions (specificities) presently is not easily discernible from its systematic position (which is defined by the degree of homology of (parts of) his genome to other viruses). In the future, the molecular analysis of the determinants (genes) for virus-host interactions and of their variability in natural populations may provide more power of inference with respect to the host range of a particular virus.

So far, the testing with host species *in vivo* provides the relevant empirical basis for judgements about host ranges. Methods of data generation with respect to the following cannot follow harmonised and accepted internationally standardised procedures:

- The control of the homogeneity of the virus inoculum.
- The administration of the virus and the range of concentrations to be used.
- The spectrum of insect or other arthropod species and cell types to be included.
- The selection and control of end points (*e.g.* larval death, virus multiplication and yield, transcription of genes, DNA replication).

Parameters of influence on testing results include:

- The particular genotype of test species.
- The developmental stage of larvae during infection.
- The diet for host.
- Other conditions challenging the general stress tolerance of the test species (*e.g.* temperature, moisture).

Descriptions of a host range spectrum based on empirical testing will always suffer from ambiguities and incompleteness, due to apparent limitations in test species selection and experimental parameter variations. Studies of virus entry, DNA replication, transcription of genes and assembly of virus progeny together with the observation of cytopathic effects in cell cultures do substantially contribute to an understanding of host specificities at the level of molecular interactions (Miller and Lu, 1997). They complement but do not supplement, so far, in vivo testing of host range limitations (Danyluk and Maruniak, 1987; Castro *et al.,* 1997). It may be stressed again, that an understanding of mechanisms is a prerequisite to improve inferences from genotype to host specificity phenotype.

Host range and parameters of virulence may be correlated to a different extent. An increase in virulence may appear as a host range extension in conventional testing using fixed doses of virus challenge. However, the spectrum of susceptible host species may not have changed to any extent. A maintenance of the order and quantitative relations of infectivity parameters (LD_{50}s) would indicate an unchanged host range. A precise definition of experimental methods and end points clearly is a prerequisite for a common understanding of host range and virulence parameters.

The modification of virulence/host range by a selective procedure in the laboratory, cited by Federici and Maddox (1996) to exemplify a natural ability to manipulate host range, can be used to illustrate the importance of both, virulence and host range determinations. Martignoni and Iwai (1986a) used large doses of the MNPV of *Orygia pseudotsugata* (tussock moth, family: Lymantriidae) to sequentially infect *Trichoplusia ni* (cabbage looper, family: Noctuidae) over a period of 12 generations. In the course of these experiments, representing a selective pressure which in its extent will not occur in natural settings with any realistic probability, the virus adapted to the novel host, the cabbage looper. Low mortalities and minor levels of infection in most tissues at the beginning were converted to increasing levels of tissue infection and a complete adaptation of the virus to the novel host by the seventh generation. The passaged virus strain, if not selected from a heterogeneous inoculum, apparently accumulated some (set of) unknown mutation(s) and also had increased its

virulence for its original host, the tussock moth, by a factor of ten. This increase in virulence, however, would not in itself necessarily result in the expansion of the host range, as observed.

An overview of the host ranges of baculoviruses, as determined in cross infectivity studies in the laboratory is given by Gröner (1986). Results presented in tables are cited with the reservation that most studies did not control the identity with the inoculum virus after challenging of different hosts. Thus, the activation of a latent virus infection followed by its replication or the selective propagation of a minor contaminating virus type cannot be excluded in most cases. In general, NPVs infect only members of the genus, or in some cases the family, of their original host. A tendency that multiple embedded NPVs have a somewhat broader host range than SNPVs and an even more limited host range of granuloviruses was noted. Among the MNPVs, the prototype AcMNPV is known for its relatively broad host range and infectivity for more than ten families within the order Lepidoptera.

The identification of virus progeny by molecular methods is recommended and used as a standard tool for host range determinations in other publications as well. Predominantly a restricted range of infectivity is corroborated (Barber *et al*., 1993). Comprehensive studies on a broad selection of potential host insects were induced by the necessity to generate an adequate background for respective studies with genetically modified baculoviruses (Doyle *et al*., 1990; Doyle and Hirst, 1991). These studies resulted in the finding that MbMNPV is infective for both, butterflies and moths (Doyle and Hirst, 1991). MbMNPV of the cabbage moth *Mamestra brassicae* (Lepidoptera: Noctuidae) also infected the small tortoiseshell butterfly, *Aglais urticae*. However, a very high virus inoculum (106 OBs) was necessary for virus multiplication and larval death. MbMNPV also infects *Cynthia cardui* (painted lady) and *Vanessa indica* (Indian red admiral), also of the Nymphalidae, but does not seem to infect other butterflies of the families Satyridae, Lycaenidae, or Pieridae (Doyle *et al*., 1990). The cross infection studies showed a somewhat broader host range of MbMNPV than previously demonstrated. Other baculoviruses are also infective for Nymphalid butterflies.

The (quantitative) interpretation of biotest data with respect to potential consequences from exposing natural populations, has not been investigated in any detail but could be regarded as a key area of judgements about baculovirus biosafety (Doyle *et al*., 1990; Cory *et al*., 1997). It is conceivable that the non-susceptibility at lower doses might normally prevent epizootics from being initiated from weak cross infectivities, *e.g.* after spray applications for biological control. But an improved potential of extrapolation from infectivity studies with respect to a potential of virus transmissions in heterologous populations is highly desirable, to more clearly define relevant borders of host range. Data from laboratory bioassays might be poor estimates for the prediction of nontarget impacts.

A multiple-embedded nucleopolyhedrovirus, AnfaMNPV, isolated from the celery looper, *Anagrapha falcipera* in central Missouri seems to have the broadest host range reported so far (Hostetter and Puttler, 1991). 31 susceptible species belong to ten lepidopteran families, and in addition to *Helicoverpa sp., Heliothis sp*., and *Spodoptera sp*. include important pests like pink bollworm (*Pectinophora gossypiella*), fall webworm (*Hyphantria cunea*), cutworms (Noctuidae), velvet bean caterpillar (*Anticarsia gemmatalis*), codling moth (*Cydia pomonella*), navel orangeworm (*Amyelois transitella*), and the diamondback moth (*Plutella xylostella*). As compared to AcMNPV, known for its relatively broad host range, the virus demonstrated equal virulence for species differing in susceptibility for AcMNPV by one order of magnitude. Relatively narrow criteria were used for differentiation of susceptibility (max. 250 OB/mm^2 of diet). The list of nonsusceptible species as published (containing also Coleoptera and Diptera) might thus include some species which are susceptible, if challenged at higher doses. Propagation of virulent AfMNPV in tobacco hornworm (*Manduca sexta*) as untypical host demonstrated extensive possibilities for production in a variety of hosts and systems. Virulence against a great number of cosmopolitan economically important insect

species was considered an encouraging result in this report and the virus became the first baculovirus patented by the US Government Patent and Trademark Office.

D. Variation of virulence and host range

By describing interactions between biological entities, virulence and host range are prone to modification by natural variability and by a variety of external "environmental" factors. Mutagenesis, recombination, and the (selective) propagation in different hosts have been analysed as causes of shifts in host range and virulence of baculoviruses, in the laboratory (sections A.3.3; A.6-8.). The same mechanisms are acting in natural or managed environments, however to an extent unknown in any detail and responding to different selection conditions. In the following, emphasis is given to some factors modulating insect responses to viral challenges that have not been described in other sections.

External (environmental) factors modulating insect response to baculoviruses

Chemicals

An additive or synergistic effect of chemicals on baculovirus infectivity has been exploited for control strategies, for example, by the use of combinations with insecticides or the addition of particular radiation protectants (section A.6).

Host plants

The plant material on which larvae are feeding may have a significant influence on their susceptibility for viral infections. Gypsy moths, feeding on *Quercus rubra* (red oak) or *Acer rubrum* (red maple) demonstrated a higher level of tolerance when infected with LdMNPV than larvae feeding on *Populus tremuloides* (quaking aspen) or *Pinus rigida* (pitch pine). The enhanced tolerance was correlated with an increased acidity and hydrolysable tannin content of leaf material (Keating and Yendol, 1987; Keating *et al*., 1988). Different tolerances for *Spodoptera littoralis* NPV were also noted in the cotton leaf worm on castor bean, alfalfa, mulberry, cotton and potato. LD_{50} values differed by a factor of about 3 between castor bean (the most "protective" feeding source) and potatoes, and the LT_{50} was also reduced by more than 10% on the latter plant (Santiago-Alvarez and Ortiz-Garcia, 1992).

A different approach was followed by Rabindra *et al*. (1994), who investigated the influence of plant surface environments on the virulence of *Heliothis armigera* SNPV. The larvae of the American bollworm were challenged with identical (microscopically controlled) titers of virus suspensions which had been exposed to the surfaces of 5 host plants (chickpea (*Cicer arietinum*), pigeon pea([syn. *dhal*] *Cajanus cajan*), lablab bean (*Dolichos lablab*), sunflower, and cotton). Bioassays used the leaf dip method with shoots of chickpea as identical feeding material for the test species. The cotton leaf surface was the most detrimental for virus activity resulting in an increase of more than three orders of magnitude in the LC_{50} value in comparison to lablab bean -exposed viruses (5×10^6 OB/ml and 1.4×10^3 OB/ml, respectively). Lablab bean even had some protective or stimulating effect on virulence in comparison to untreated NPV preparations ($LC_{50} = 8 \times 10^4$ OB/ml). Median lethal time measurements corresponded with these observations. A mortality time expanded by 25% after cotton leaf - exposure in comparison to lablab bean exposed virus was observed. This experimental approach was also used to determine the effect of adjuvants added to the virus preparations because the study together with others indicates the need to develop suitable formulations which could protect the virus from inactivating factors on plant surfaces.

These examples demonstrate the modulation of baculovirus virulence by the plant environment on which target insect larvae are feeding. This environment either indirectly alters insect response and tolerance, or interferes with the first steps of virus infection through variation of the milieu of the midgut lumen (*e.g.* pH), and/or directly modulates the specific activity (the probability of an OB in the light microscope to enter the replicative state) of virus preparations. An understanding of these kinds of interferences is valuable to improve formulations for insect control and to adapt methods of *in vivo* baculovirus production. It can also be used to reassess control strategies if applied to novel plant varieties (Beach and Todd, 1988). In an advanced stage of development of modelling the variability introduced by the respective plant canopy may be included. (Foster *et al*., 1992).

Population density

A variety of environmental factors such as temperature, light, nutrition, and humidity may compromise the capability of insects to resist baculovirus infections (Briese, 1986). Among these, rearing density has a distinct effect on their susceptibility to viral infection. Together with other phenotypic modifications (development time, weight at moulting, degree of melanisation), enhanced susceptibility is observed as a reaction to the stress of high population density of lepidopteran larvae (Goulson and Cory, 1995b). This was interpreted as a consequence of the adaptive response of accelerated development at the expense of larval weight, which compromised the ability to express resistance functions. Interestingly, larvae which were reared singly exhibited many of the same characteristics, including enhanced susceptibility to virus infection. The benefit of spending resources for resistance development may be low, if a low density of conspecifics reduces infection risk. This phenotypic variation is among the factors modulating the relationship between virus density and disease transmission.

Other infectious agents, parasitoids

The exposition of insects with different infectious agents or parasitoids is a different kind of stress, eventually enhancing baculovirus induced mortality (C.24). Superinfection of insects with a latent or persistent infection may result in larval death due to the propagation of the latent virus (see A.4.6).

Insect resistance and immunity

The varying degree of tolerance of insect larvae for baculovirus infections at different instar stages, usually increasing with age and stage of development, is but one observation suggesting the existence of particular mechanisms of pathogen tolerance (Mikhailov *et al*., 1992; Engelhard and Volkman, 1995). To assess the short-term and long-term effectivity of control strategies with baculovirus insecticides an understanding of the adaptive potential of insect populations to baculovirus infections is of paramount importance. In a review Briese gives an account on developmental and environmental factors affecting resistance (1986), the knowledge about defence mechanisms and the genetic factors conferring increased virus tolerance. Dominant and recessive autosomal genes or a multigene family may form the genetic basis of tolerance in insects. *E.g.* McIntosh and Ignoffo (1989) demonstrated that the resistance of *Helicoverpa subflexa* against *Helicoverpa* zea NPV (HzSNPV) appears to be controlled by a single non sex-linked gene.

Observations of markedly expressed virus resistance are restricted to laboratory observations of insect populations obtained under high artificial selective pressure of virus challenges, for example, by propagating the surviving fraction of infections with an LD_{80} in several subsequent generations. Somewhat conflicting results with respect to the success of selection strategies may either be caused by the general potentials of the insect species under investigation or by the particular genotype of the

strain as most obvious reasons (*e.g.* Kaomini and Roush, 1988). It is a general objection against the predictive value of laboratory selections, that insect cultures in the laboratory represent a minor fraction of the genetic heterogeneity of natural populations. The lack of documented cases of resistance in field populations of insects under control of a virus insecticide may reflect their less frequent and extensive use in comparison to chemicals, which have induced an exponentially increasing rate of resistance developments in insect populations.

There is some circumstantial evidence that the spectrum of tolerance and respective gene frequencies in natural populations may have been biased by natural virus epizootics or the application of virus insecticides (Fuxa *et al*., 1988). Laboratory selections in colonies of velvetbean caterpillar (*Anticarsia gemmatalis*) from the US and Brazil demonstrated the achievement of a significantly higher level (1000x) of resistance to AgMNPV after 13 - 15 generations in the Brazilian colonies than in the colony from Lousiana, the resistance of which levelled off at a ratio of 5x after 4 generations (Abot *et al*., 1995). AgMNPV, is used for biological control of the velvetbean caterpillar in soybean on a large scale in Brazil (see A.6.4).

Alleviation of the selection pressure by virus challenges seems to favour more competitive sensitive wild-type genotypes of insects, which are superior in their reproduction without the virus selection pressure (Fuxa and Richter, 1989). Such observations correspond to a more general perception that some cost in reduced fitness has to be paid for the expression of disease resistance. This hypothesis however, has not been tested rigorously in many cases (Gemmill and Read, 1998). Genes for virus resistance confer pleiotropic phenotypic effects including increased susceptibility to chemical insecticides (Fuxa and Richter, 1990). The exclusive restriction to the oral application route of the increased virus tolerance and enhanced insecticide susceptibility phenotypes indicates a correlation of modifications to components of the midgut interfering with early stages of virus infection.

Using the genetically modified virus AcMNPV-hsp70/lacZ for the monitoring of virus activity by the lac-Z reporter gene, the physiological basis of resistance of *Helicoverpa zea* against AcMNPV was analysed by Washburn *et al*. (1996). The larval cells were actually very susceptible to AcMNPV infection, but infected cells were encapsulated by hemocytes and subsequently cleared from the midgut lumen. Cellular immune responses of larvae seem to be a significant factor in preventing the spread of the infection and a determinant of the functional host range of baculoviruses. Based on further experiments including chemical and biological immune suppression, it was suggested to use modified viruses to express immunosuppressive genes in order to compensate for this defence mechanism.

4. Interactions with and Effects on Other Organisms in the Environment

Direct effects of baculoviruses on other organisms in the environment are restricted to their host range, and most have been observed only in their original host or in the target species of biocontrol applications. Baculovirus infection interferes with other pathogens or parasitoids feeding on a shared host. Observations on the synergistic or additive effect of particular crossinfections with other pathogens as well as interferences with parasitoids have been summarised by Harper (1986). Section A 4.2 gives some account to the synergistic co-operation between some baculoviruses. Some of such pathogen combinations have been used in field experiments to improve control of pest species.

The analysis of the interference with parasitoid developments is used to assess the possibility of adverse effects on populations providing additional natural control of pest species. The observed detrimental effect on parasitoid development, fitness or reproductive success as well as an enhanced sensitivity of parasitised insect larvae for baculovirus infection can most adequately be described with

the interspecific competition for a limited resource (Cossentine and Lewis, 1988; Hochberg, 1991; Al Fazairy *et al*., 1993; Nakai and Kunimi, 1997). The relative timings of baculovirus infection and parasitoid emergence determine the degree of interference to a large degree. No productive infection of parasitoids was observed but frequently a large number of viral occlusion bodies is present in their midgut lumen, indicating a potential mechanism of virus dispersal. A reduced level of a parasitoid population has been observed after aerial LdMNPV application (Webb *et al*., 1989).

5. Ability to Form Survival Structures

The structure of baculovirus occlusion bodies provides for their potential to persist in the environment in a variety of abiotic and biotic conditions (A.4.1/4.7). This feature is described as a key factor dominating the dynamics of virus transmission and a prerequisite of baculovirus epizootiology by Evans (1986). Viability may extend for more than 40 years in forest soil. No special survival structures are formed.

6. Routes of Dissemination, Physical or Biological

A. Physical dispersal

Transport and dispersal of baculoviruses by wind or water is considered to be of minor importance for the natural spread of baculovirus infections. Its contribution to mechanisms of primary dispersal in natural epizootics is not known in detail (Briese, 1986). In field experiments, testing the spatial spread of a baculovirus, ballooning of first instar larvae of the gypsy moth *Lymantria dispar* was a good predictor of viral spread in the first few weeks. The scale of spread and its lack of directionality at later times however, did not match the results of a mathematical modelling even when primary dispersal by ballooning and short distance larval dispersal with a high rate of disease transmission were included. Circumstantial evidence suggested that autonomous dispersal by wind of occlusion bodies did not significantly contribute to dispersal. Parasitoid vectoring of viruses was discussed as the potential relevant additional dispersal mechanism (whereas vectoring by other animals was not considered (Dwyer and Elkinton, 1995). Evidence for a dispersal of NPV from soil to pine foliage in dust was presented as a likely explanation for the observed distribution of diseased larval colonies near a forest road by Olofsson (1988).

Aerial dispersal is the predominant mechanism of distribution for insect control applications; techniques and timing have to consider meteorological conditions and other agricultural procedures like irrigation for optimal distribution (Young, 1990; Payne *et al*., 1996).

Reminiscent of traditional testing procedures for the prediction of exposition rates with chemicals, the leaching behaviour of baculoviruses in natural soil cores and columns filled with soil or sand has been tested to evaluate eventual rates of exposition of the groundwater table. Consistently, some experiments demonstrated the retention of baculoviruses in soil to be comparable or superior to that of other Viruses (Polio virus and bacteriophage f2 of *Escherichia coli* were used for comparison). Soil with higher levels of organic matter was less efficient than sand for the reference viruses but not for the baculoviruses (NPVs and GVs). Results were independent from the particular composition of percolating water. Lysimeter studies with baculoviruses did not result in any observation of positive samples of leachate taken at a depth of 1.5 m during 7 months of monitoring. The concentration of viruses applied in this experiment resulted in a surface exposition 3 - 4 orders of magnitude above concentrations used in agricultural applications (10^{14} particles of a Granulovirus in 20 L of tap water applied to a surface of 0.8 m^2). A good retention of baculoviruses by soils was concluded and this property was tentatively attributed to the particular protein envelope of virus particles consisting of polyhedrin and granulin, respectively (Lopez-Pila, 1988).

The behavioural shift of some insect larvae following baculovirus infection (*i.e.* the movement to higher and exposed positions on host plants: "Wipfelkrankheit") effects the physical distribution of baculovirus particles following death. It might be an evolutionary adaptation of the virus to increase horizontal transmission, *e.g.* by contaminating more foliage following rainfall (Vasconcelos *et al.*, 1996a; Goulson, 1997).

B. Vectorised dispersal by diseased larvae, predators and parasitoids

Virus infected larvae are an effective dispersal agent even before larval death and carcass lysis, which results in very high inoculum density. Viable virus particles are also dispersed in the environment through either defecation or regurgitation during late stages of infection (Vasconcelos, 1996). Cannibalism of infected larvae may be another possibility if it occurs at any relevant frequency. Transport and dispersal of virus particles also occurs by predators feeding on diseased larvae or by parasitoids ovipositioning on and developing within larvae (Briese, 1986; Boucias *et al.*, 1987; Vasconcelos *et al.*, 1996b). The viruses remain viable after passage of the alimentary canal of vertebrates and invertebrates, however to a widely divergent extent.

With respect to the speed and distance of the transfer of infectious viruses, the dispersal by birds deserves some special interest. Birds may take up infected or dead larvae directly or feed on non vertebrate or small vertebrate predators. Droppings may contain up to some 10^7 polyhedra. But the contribution of particular dispersal routes and mechanisms to virus epizootics is difficult to assess conclusively. Even a coincidence of novel areas with infected larvae and viruses in bird droppings is only circumstantial evidence of a significant contribution of avian transport to virus epizootics (Entwistle *et al.*, 1977; Buse, 1977; Cory *et al.*, 1988; Entwistle *et al.*, 1993). Novel baculovirus infections appearing in *Gilpinia hercyniae* (European spruce sawfly) in a previously unexposed geographical area at a time, before any virus could be monitored in avian droppings, suggests a different mode of dispersal (Buse, 1977). Even more recent experiments including trials with different virus applications at different locations and the identification of virus types in bird droppings by molecular restriction-hybridisation methods must close with the statement, that "the actual role of birds and many other biotic dispersal agents in the spread of baculoviruses and other micro-organisms remains to be demonstrated" (Entwistle *et al.*, 1993). Clearly, the knowledge of the intersection in space and time of insect behaviour and development and the ecological habit of particular bird species (including feeding - and defecation - behaviour and territoriality) must be included in such investigations.

Such intersections are also determinants of success for any approaches using insects as biotic dispersal agents for insect control. These experiments also can be regarded as a demonstration of a potential natural dispersal role of the engaged species (Biever *et al.*, 1982; Young and Yearian, 1990, 1992; Gross *et al.*, 1994b - see C.23.2). Any intentions to surmount the demonstrative character of these experiments in order to biologise application strategies for baculovirus insecticides would require the input of increased knowledge. It remains speculative whether such a manipulation of insect and baculovirus ecology for efficient pest control can be achieved in a practicable and effective manner.

7. Containment and Decontamination

Special containment conditions in order to protect workers and the environment from baculoviruses are not usually required. The establishment of confinement measures is determined by the need to protect insect and cell cultures and the experiment from unintended infections. In part, depending on laboratory experiences with outbreaks of virosis in insectaries, the extension of technical installations and working procedures is adapted to these protection objectives. Small particle size and

persistence of baculoviruses represent a challenge for effective measures. The performance of work with baculoviruses has to follow the principles of good microbiological practice. Local separation of cell culture and insect work is a minimum requirement. To change laboratory coats when beginning or leaving work with baculoviruses seems to be advisable.

Heating can be used for decontamination of laboratory material and media. Baculoviruses are inactivated at temperatures and times significantly below conventional microbiological sterilisation conditions (Martignoni and Iwai, 1977). Sodium hypochlorite and formaldehyde can be used for chemical decontamination. Sodium dodecyl sulfate was also tested for egg surface sterilisation to prevent ex ovarial (vertical) transmission. A lower impact on moth rearing and sufficient effectiveness of inactivation were noted (Ilsley *et al*., 1980).

No decontamination procedure seems to be recommendable for environmental contaminations, *e.g.* by spills from baculovirus insecticide containers. In fact, any procedure would compromise environmental quality to a greater extent than the baculovirus preparation. Soil decontamination by formaldehyde treatment as used after the first field trial with a genetically modified baculovirus or similar treatments will eventually be used according to the respective risk perception.

8. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity, and Reliability

A. Detection

Detection of baculoviruses in the environment is frequently performed by collecting (diseased or dead) larvae and making microscopic observations of stained baculoviruses. While this technique is adequate to monitor an infectious process in an insect population, other techniques are required to analyse the abundance and activity of infectious particles in the environment. Methods may consist of biotests by feeding target larvae with potentially exposed (plant) material. In order to quantify virus particles and to additionally determine their infectivity, virus particles are separated from the habitat matrix by washing or sonication. Virus particles collected by centrifugation are stained and counted by microscopy (Taverner and Connor, 1992; Carruthers *et al*., 1988). Subsequent biotesting is then performed by applying an aquaous suspension to the insect diet.

Biotesting may amplify rather low concentrations of viruses, and does not seem to generally be inferior in its sensitivity in comparison to molecular methods which can be employed to directly detect baculovirus sequences by probe hybridisations or by amplifying specific sequences via PCR. The potential of these methods has not been evaluated for the monitoring of field trials in any significant extent. A method to detect and identify baculoviruses by a combination of PCR (amplifying a homologous region of the polyhedrin gene) and subsequent differentiation by restriction analysis has been described for 8 baculoviruses (De Moraes and Maruniak, 1997). However, PCR does not differentiate between viable and non-viable viruses.

B. Identification

For the identification and differentiation of Baculovirus species/strains, the restriction analysis and/or probe hybridisations has become an established method (Trzebitzky *et al.*, 1988; Bensimon *et al.*, 1987; Doyle *et al.*, 1990; Barber *et al.*, 1993; Entwistle *et al.*, 1993; Hughes *et al.*, 1997). The feasibility of the approach for ecological investigations was conclusively demonstrated by Laitinen *et al.* (1996) who used heterogeneities in restriction endonuclease patterns among geographic isolates to assess the origin and spread of genotypes. The number of restriction enzymes used and the cutting frequency determine the degree of differentiation of divergent genotypes. A quantification of the

composition of heterogeneous mixtures is difficult to achieve by the analysis of banding heterogeneities and submolar bands. *In vivo* cloning of the genotypes of the mixture and quantification of their frequency would be a labour intensive solution.

In recent years, the PCR became an efficient tool with high sensitivity and specificity for the identification and diagnostics of micro-organisms. Primer specificities and reaction conditions determine the amplification of specific DNA fragments. De Moraes and Maruniak (1997) adopted this technique to amplify a highly conserved region of 575 bp within the polyhedrin gene of different NPVs with a specifically designed pair of primers. Restriction endonuclease digestion of the PCR product resulted in specific restriction patterns of the 8 analysed nucleopolyhedroviruses (*Anagrapha falcifera* NPV, AcMNPV, AgMNPV, BmNPV, and HzSNPV, OpMNPV, SfMNPV, and SeMNPV).

A similar approach was chosen to detect low levels of *Spodoptera littoralis* NPV from viral occlusion bodies and from infected host larvae and to differentiate between *S. littoralis* NPV and AcMNPV (Faktor and Raviv, 1996). These experiments demonstrate that PCR-based methods are useful for rapid identification and allow cost effective and sensitive monitoring of wild-type as well as genetically engineered baculoviruses.

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APPENDIX

Abbreviations

BV	budded virus/virion
cat	chloramphenicol acetyl transferase
EGT	ecdysterorid-UDP-gluceoployhedrovirus
ELISA	enzyme linked immunosorbent assay
FP	few polyhedra
GV	granulovirus
hr	homologus regions
IAP	inhibitor of apoptosis
ICTV	International Committee on Taxonomy of Viruses
IED	individual effective dose
LC_{50}	lethal concentration (50%)
LD_{50}	lethal dose (50%)
LT_{50}	lethal time (50%)
MNPV	multiple nucleocapsid nucleopolyhedrovirus
NPV	nucleopolyhedrovirus
NOB	non-occluded baculovirus
OB	occlusion body
ODV	occlusion derived virus
ORF	open reading frame
PCR	polymerase chain reaction
PE	polyhedron envelope
p. i.	post-infection
SNPV	single nucleocapsid nucleopolyhedrovirus
TE	transposable element

SECTION 2
PSEUDOMONAS

I. General Introduction

This document presents information that is accepted in the scientific literature concerning the known characteristics of fluorescent members of the genus *Pseudomonas* (rRNA group I pseudomonads). Regulatory officials may find this information useful in evaluating and establishing the properties of environmental applications of biotechnology which involve those micro-organisms which are the focus of this document. Consequently, a wide range of information is provided without prescribing when the information would or would not be relevant to a specific risk assessment. This document represents a "snapshot" of current information that may potentially be relevant to such assessments. However, Member countries have not yet attempted to put together an exhaustive literature review on all aspects of these organisms.

The genus *Pseudomonas* may potentially be utilised in a number of different engineering applications. These include *in situ* applications such as groundwater reinjection, air sparging, and bioventing. They also include *ex situ* applications such as landfarming, slurry phase remediation, and biopiles. Many of the potential uses under development or envisioned for the genus *Pseudomonas* involve improvement of air, soil or water quality, or cleanup of otherwise intractable environmental contaminants.

In considering information that should be presented on this taxonomic grouping, the Task Group for Environmental Applications of Modern Biotechnology discussed the list of topics developed in the "Blue Book", *Recombinant DNA Safety Considerations* (OECD, 1986), and attempted to pare down that list to eliminate duplications, as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered so they would be easier to understand and use (see Section III).

This effort at refining the exposition of safety considerations in the "Blue Book" for application to the genus *Pseudomonas* has also recognized the importance of a thorough understanding of the characteristics of the particular application for which these organisms will be used. Worker and other human exposures, and environmental exposures, will differ depending on the method of application. This knowledge is likely to affect the types of information on particular taxa that regulatory officials deem relevant in specific risk assessments. Group I pseudomonads are known to display a range of pathogenic and toxicological characteristics in regard to humans, animals and plants. However, even though some of the rRNA group I pseudomonads are known to exhibit pathogenic properties, exposures of and potential impacts on humans, animals and plants may be relatively limited in some circumstances, *e.g.* when the micro-organisms are used in bioreactors of various sorts that have suitable controls on liquid and gaseous emissions, or when other specific mitigation or containment measures are in place. The factors discussed in this document may, therefore, have varying levels of impact on individual risk assessments, depending upon how and where the particular micro-organisms are used, *i.e.* depending on the likely exposures presented by the application.

Given the breadth of information contained in this document, it is hoped that it will be useful not only to regulatory officials as a general guide and reference source, but also to industry and to scientists involved in research.

This document is a consensus document for environmental applications involving fluorescent members of the genus *Pseudomonas* (rRNA group I). Section II is an introduction to the genus *Pseudomonas* and to the species which are the subject of the document. The format of the information is described in Section III, and the information is presented in Section IV. Section V contains the References.

II. Introduction to the Genus Pseudomonas

1. Taxonomy

Pseudomonas is part of a large, heterogeneous and ubiquitous group of micro-organisms generally referred to as pseudomonads. The pseudomonads are characterised as being highly metabolically versatile, bioactive, and prolific colonisers of surfaces. Pseudomonads are gram-negative, straight or slightly curved rods with polar flagella; they are chemo-organotrophs with a respiratory, non-fermentative type of metabolism and are usually catalase and oxidase-positive. The taxonomy of the group has been clarified using 16S ribosomal RNA sequence analysis (Table 4.4).

The genus *Pseudomonas* corresponds to rRNA group I (Table 4.4). The type species for the genus is *Pseudomonas aeruginosa*. Strains are metabolically diverse, as well as having the capacity for denitrification and arginine degradation under anaerobic conditions. *P. aeruginosa* has been studied in more detail than any other pseudomonad using genetic techniques. Physical and genetic chromosome maps have been described (Romling *et al*., 1989; Ratnaningsih *et al*., 1990).

2. Applications

Pseudomonads have been identified to be of importance in bioremediation as a result of their tremendous capacity for biodegradation. They also offer considerable promise in agronomic applications, since many strains are bioactive, fast-growing, prolific colonisers of plant surfaces and are able to suppress or out-compete pathogenic and other deleterious micro-organisms.

A. Pseudomonads as candidates for bioremediation

Nutritional versatility is exhibited widely amongst the pseudomonads. Combined with the presence or acquisition of catabolic plasmids by large numbers of strains, pseudomonads have the potential to mineralise a wide range of natural organic compounds, including aromatic hydrocarbons. This versatility allows for the rapid evolution of new metabolic pathways for the degradation of synthetic compounds (xenobiotics), leading to their complete oxidation and mineralisation. The complexity of the catabolic routes indicates sophisticated systems of regulation to control the expression and achieve the co-ordination of catabolic activities. Although the degradative pathways of pseudomonads vary considerably, the metabolic routes are convergent and lead to a limited number of common intermediates such as catechols. These represent key intermediates for aromatic compound degradation.

It is also anticipated that the nutritional versatility of pseudomonads and the application of molecular genetic techniques will be harnessed in the design of catabolic pathways for environmental purposes (Ensley, 1994; Timmis, 1994). For example, a *Pseudomonas* strain was recently isolated that can utilise TNT (2,4,6-trinitrotoluene) as a sole nitrogen source, producing toluene, aminotoluene and nitrotoluenes as end products. This organism was, however, unable to utilise toluene as a carbon source for growth. By introducing the entire toluene degradation pathway carried on the TOL plasmid pWWO-Km, an organism was produced that could potentially completely mineralise TNT (Ensley, 1994). Despite some of the TNT being completely mineralised, the formation of some dead-end metabolites by reduction of the nitrotoluenes to aminotoluenes remains a problem.

B. Agronomic applications

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. Furthermore, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These traits include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to compete aggressively with other micro-organisms for niches and to exclude phytopathogens.

III. General Considerations

1. Subject of the Document: Species Included and Taxonomic Considerations

A. Species included

The subject of this document is a subset of seven species within the genus *Pseudomonas*, most of which produce fluorescent pigments. Many members of this set have been, or are likely to be, employed in various biotechnological applications in the environment. The seven species are: *P. aeruginosa*, *P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi, P. putida, P. syringae,* and *P. tolaasii.*

B. Taxonomic considerations

The genus Pseudomonas

Prior to 1973, *Pseudomonas* was seen as one large heterogenous genus with members sharing a few phenotypic features. Palleroni *et al*. (1973) concluded that five groups approximating genera, which were established on the basis of rRNA sequence homologies (Table 4.4), appeared phylogenetically distant from each other. Though these groupings were confirmed through DNA hybridisation experiments (Johnson and Palleroni, 1989), it nonetheless took a decade to transform them into discrete taxonomic units based on both phenotypic and genotypic associations. The groupings now comprise units of larger than genus rank. Species once called *Pseudomonas* are now classified as members of at least a dozen genera found within the original five homology groups (Table 4.4; Yabuuchi and De Vos, 1995a; Yabuuchi and De Vos, 1995b). The genus *Pseudomonas* is now strictly confined to members of the rRNA group I (Table 4.4).

The members of this genus still represent a somewhat heterogenous collection of bacteria, but they are far more closely allied to each other than they are to species formerly having the genus name *Pseudomonas*. The type species for the genus is *Pseudomonas aeruginosa*. Strains of *P. aeruginosa* can be isolated from many environmental substrates, and appear uniform in a number of diagnostic characters (Palleroni, 1992b). It can be argued that *P. fluorescens* is more "typical" of the genus than is *P. aeruginosa*, but due to the difficulty of establishing defining characteristics for *P. fluorescens, P. aeruginosa* remains the choice for the type species (Palleroni, 1992c).

Common characteristics of the genus *Pseudomonas*:

A. Gram-negative.
B. Rod-shaped (straight, asporogenous, 0.5-1.0 X 1.5-4.0 μm).
C. Motile due to polar flagella.
D. Oxidase-positive (except for *P. syringae*).
E. Oxidative metabolism (mostly saccharolytic, some non-saccharolytic species, no gas.
F. Formation from sugars).
G. Chemo-organotrophs.

H. Catalase-positive.
I. Growth with acetate as sole carbon source, most non-fastidious, few require growth factors.
J. NO_3 reduced to NO_2 or molecular N_2.
K. Accumulate longer-chained polyhydroxyalkanoates.
L. Produce pigments.
M. Indole-negative.

The "fluorescent" subgroup

The seven species considered in this document [*P. aeruginosa, P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi, P. putida, P. syringae,* and *P. tolaasii*] are considered to be closely related to each other except for *P. aeruginosa* and *P. syringae* (Molin and Ternström, 1986; Janse *et al*., 1992). These seven species are considered as the fluorescent subgroup of the rRNA group I, although *P. fragi* includes non-fluorescent strains.

P. fluorescens, P. putida and *P. chlororaphis* are seen as forming a complex, intertwined by a continuum of transitional strains (Molin and Ternström, 1986; Barrett *et al*., 1986). Complicating the classification scheme is the observation that both *P. fluorescens* and *P. putida* comprise several biovars, each of which may deserve species rank, but which are so interconnected that adequate methods have not been devised to clearly separate the member strains for each biovar. *P. chlororaphis*, which also encompasses the strains formerly called *P. aureofaciens* and *P. lundensis*, a recently described species, were once considered to belong to separate biovars of *P. fluorescens*.

Also closely associated with the *fluorescens-chlororaphis-putida* complex is *P. fragi*. This species has some fluorescent strains, but is primarily non-fluorescent. *P. fragi* is also a complex of different phenotypes, many of which are closely allied with some biovars of *P. fluorescens* and also could be misidentified as *P. putida* (Molin and Ternström, 1986).

P. aeruginosa, the type species and most clearly defined member of the genus, is seen as separate from the *fluorescens-chlororaphis-putida* complex.

P. syringae and *P. tolaasii* are pathogens in a group that also includes other pathogenic species (*e.g. P. cichorii* and *P. viridiflava*). However, *P. tolaasii* is an oxidase-positive mushroom pathogen related to, and potentially confused with, members of *the P. fluorescens* supercluster (Janse *et al*., 1992). *P. syringae* is an oxidase-negative plant pathogen comprising many pathovars derived from taxa that previously had species rank (Palleroni, 1984).

Fluorescence

Pigments often provide valuable diagnostic characters, since their production invariably correlates well with other group properties. Fluorescent pigments are produced abundantly in media with a low iron content; fluorescence varies from white to blue-green upon excitation with ultraviolet radiation. King's medium B is frequently used for the isolation of pseudomonads, especially by plant pathologists (King *et al*., 1954). Fluorescent species of *Pseudomonas* produce pyoverdin and/or phenazine pigments. Pyoverdin production is characteristic of most species. Palleroni (1984) indicates that *P. fluorescens* biovars II and V, along with *P. chlororaphis* and *P. putida* biovar B, have variable (11-89% positive) pyoveridin production. Although positive pyocyanin production is diagnostic for *P. aeruginosa*, the reverse is not necessarily true.

Table 4.4 Phylogeny and current classification of the pseudomonads

Proteobacteria subclass	rRNA group	Original name	Current classification	Characteristics
Gamma	I	* *P. aeruginosa*	*Pseudomonas*	type species; opportunistic pathogen
		* *P. fluorescens*		fluorescent supercluster; oxidase positive, mostly fluorescent, saprophytic or opportunistic pathogens
		* *P. chlororaphis*		
		P. lundensis	* *P. chlororaphis*	name reclassified
		* *P. putida*		
		* *P. tolaasii*		mushroom pathogen
		P. marginalis	* *P. fluorescens*	name reclassified
		P. aureofaciens	* *P. chlororaphis*	name reclassified
		* *P. fragi*		some strains non-fluorescent
		* *P. syringae*		fluorescent, plant or mushroom pathogen. *P. syringae* and *P. viridflava* are oxidase-negative. *P. syringae* comprises many pathovars
		P. viridiflava		
		P. cichorii		
		P. agarici		
		P. asplenii		
		P. flavescens		fluorescent
		P. alcaligenes		non-fluorescent
		P. citronella		
		P. mendocina		
		P. oleovorans		
		P. pseudoalcaligenes		
		P. stutzeri		
Gamma	V	*P. maltophila*	*Stenotrophomonas maltophilia*	related to *Xanthomonas*
		P. marina	*Delaya marina*	
Beta	III	*P. acidovorans*	*Comamonas*	
		P. terrigena		
		P. testosteroni		
		P. avenae	*Acidovorax*	facultatively autotrophic species (hydrogen pseudomonad)
		P. delafieldii		
		P. facials		
		P. flava	*Hydrogenophaga*	facultatively autotrophic species (hydrogen pseudomonad)
		P. palleroni		
		P. pseudoflava		
		P. taenispiralis		
		P. saccharophila	*Pseudomonas*	
		P. ruhlandii	*Alcaligenes xylosoxidans*	
Beta	II	*P. cepacia*	*Burkholderia*	heterogenous genus
		P. caryophylli		
		P. gladioli		
		P. mallei		
		P. pickettii		
		P. pseudomallei		

Proteobacteria subclass	rRNA group	Original name	Current classification	Characteristics
		P. solancearum		
Alpha	IV	*P. paucimobilis*	*Sphingomonas paucimobilis*	
		P. carboxidovorans	*Oligotropha carboxidovorans*	
		P. aminovorans	*Aminobacter aminovorans*	
		P. mesophilica	*Methylobacterium mesophilicum*	
		P. sp.	*Chelatobacter heintzii*	
		P. compransoris	*Zavarzinia compransoris*	
		P. diminuta	*Brevundimonas diminuta*	very distantly related to rRNA group I
		P. vesicularis	*Brevundimonas vesicularis*	

Source : Compiled from Palleroni, 1992b and 1992c; Molin and Ternström, 1986; Yabuuchi *et al*., 1995a and1995b, and Hildebrand *et al*., 1994. The species which are the focus of this document are indicated *with an asterisk.*

Plasmid-encoded characteristics

Plasmid-encoded characteristics such as antibiotic resistance, chemical resistance and metabolic capabilities are important components of the pseudomonad genome. Some examples, for the species under consideration, are given in Table 4.5. Many if not most of these characteristics are strain-specific and of little value in terms of taxonomy and identification. An exception to this rule, however, is phage susceptibility. Plasmid-encoded phage susceptibility can be important in differentiating *P. syringae* pathovars when combined with biochemical testing, and *P. aeruginosa* isolates have been typed to the subspecies level using phage sensitivity.

4. Characteristics of the Organism Which Permit Identification and the Methods Used to Identify the Organism

A. General considerations

P. aeruginosa is distinct and readily distinguished from other members of the genus, and the pathovars of *P. syringae* can be distinguished by determination of their host range.

Distinction of the other five species in the group *(P. fluorescens, P. chlororaphis, P. putida, P. tolaasii, P. fragi*) from each other is not straightforward, and the expression "continuum" is frequently used to describe their inter-relationship. Most authors agree that current methods are generally inadequate to ensure proper placement of new isolates within the related species *P. fluorescens*, and *P. putida* and their biovars (Palleroni, 1992b; Christensen *et al*., 1994; Barrett *et al*., 1986). Except for its pathogenicity, *P. tolaasii* is difficult to distinguish from *P. fluorescens* (Janse *et al*., 1992). *P. chlororaphis* is separable from *P. fluorescens* based on production of unique phenazine pigments (Palleroni, 1984), and has some distinctive substrate utilisation patterns (Barrett *et al*., 1986), but is otherwise well within the boundaries of the fluorescent supercluster (*e.g.* Janse *et al*., 1992). Finally, *P. fragi* shares many features with members of the fluorescent supercluster, but most strains are not fluorescent (Molin and Ternström, 1986).

B. Methods used for identification and classification

Numerical taxonomy

Numerical taxonomy has become the "traditional" method for classifying members of the genus *Pseudomonas*. This approach compares multiple features of the isolate, for which there is substantial discriminatory power, with a database of features of well-described members of the taxon. The accuracy of this type of approach will depend upon the quality and quantity of the data for strains comprising the reference database.

In order to achieve valid results, identical laboratory techniques need to be used for analysis of the isolate and the strains used to construct the reference database. The success of numerical taxonomy is also affected by the complexity of the relationships among the taxa being evaluated.

Use of a broad spectrum of substrates in numeric taxonomic evaluations has had some success for fluorescent species of *Pseudomonas* (*e.g.* Barrett *et al*., 1986). These techniques have permitted some assignment of strains to species and biovars within the fluorescent supercluster (a term applied to all of the species and biovars of *P. fluorescens, P. putida* and their allies, Table 4.4). However, Molin and Ternström (1986) and Janse *et al*. (1992) both reported many unclassifiable strains among those they subjected to classical numeric taxonomic analyses.

Commercial suppliers have devised simplified, automated versions of this technique. Examples of commercial kits available for identification of *Pseudomonas* on the basis of carbon source utilisation patterns, and physiological and morphological characters, are the API20E (API, 200 Express Street, Plainview, New York 11803, USA; BioMerieux, F-69280 Marcy-L'Etoile, France) and the BIOLOG (BIOLOG Inc., Hayward, California, USA) systems. For these kits, the database for *Pseudomonas* is based on mainly clinical, not environmental, strains. As a result, the kits may fail to identify all environmental isolates.

Use of these kits requires experience. In addition, most of them are designed to determine the membership of the isolate within a taxon and not to distinguish strains within a species. That is, the test profile in most cases is not unique to a particular strain. So, in most cases, test profiles will not be sufficient to distinguish the isolate from other strains of the same species. If such a distinction is being made, it must be based on the detection of properties unique within the taxon.

Details of the test methodologies and profiles of the species can be found in Palleroni (1981; 1984; 1992c).

Genotypic approaches

The current classification of the pseudomonads is based on rRNA homologies. The variable and conserved regions of the RNA molecule are both important for identification purposes. The conserved regions serve as targets for polymerase chain reaction (PCR) primer binding sites and universal hybridisation probes. The variable regions are the targets of the hybridisation probes and primers that are taxon-specific. Probes and PCR primers directed at diagnostic rRNA sequences have facilitated the classification of pseudomonads into the five rRNA groups (Table 4.4).

Strong selection pressure for the conservation of 16S and 23S rRNA molecular structure and sequence has meant that rRNA molecules are powerful evolutionary clocks for describing phylogenetic relationships between rRNA groups of pseudomonads. At present, however, they are unable to position individual strains into species groups. This is particularly true for the fluorescent rRNA group I pseudomonads (Christensen *et al*., 1994). Using 23S rDNA methods, Christensen *et al*. (1994) found that "the method

failed to provide a basis for distinguishing between *P. fluorescens, P. chlororaphis*, and *P. putida* Biovar B and to differentiate among the biovars of *P. fluorescens*." This study also showed that there did not seem to be a correspondence between taxonomies of this group based on 23S ribosomal sequences and from conventional numerical taxonomy. As pointed out by Janse *et al*. (1992), the large number of intermediate strains of all of these species shows "more variation than the present schemes (for classification) allow."

Schleifer *et al*. (1992) describe several probes for the rapid identification of members of the genus *Pseudomonas*. A 360 bp fragment of a 23S rRNA gene derived from *P. aeruginosa* (Festl *et al*., 1986) allowed differentiation of the eleven fluorescent and non-fluorescent group I species tested. A second probe was group-specific for *P. stutzeri, P. alcaligenes, P. pseudoalcaligenes* and *P. aeruginosa*. This probe comprises a 255 bp fragment of the 23S rDNA of *P. stutzeri* that is homologous to the bases 1366 to 1617 of *Escherichia coli* 23S rRNA. Both probes were tested by "dot-blot" hybridisation against genomic DNA bound to filters.

Table 4.5 Examples of plasmids responsible for the metabolism of organic compounds or resistance to heavy metals in fluorescent *Pseudomonas* species

Organism	Plasmid	Substrate	Reference
P. putida	CAM TOL	camphor xylene, toluene	Jacoby, 1975 White & Dunn, 1978
	SAL	salicylate	Korfhagen *et al.,* 1978
	NAH	napthalene	White & Dunn, 1978
	pRE4	isopropyl-benzene	Eaton & Timmis, 1986
	pEG	styrene	Bestetti *et al.,* 1984
	pCINNP	cinnamic acid	Andreoni & Bestetti, 1986
	pAC25	3-chloro-benzoic acid	Chatterjee & Chakrabarty, 1983
P. fluorescens	pQM1	mercury	Bale *et al.,* 1988
P. syringae pv. *tomato*	pPT23a; pPT23c	copper	Bender & Cooksey, 1986

Schleifer *et al*. (1992) used fluorescein or tetramethylrhodamine 5'end-labelled sequences as probes to identify *Pseudomonas* species. The first probe (P72; 5'-TTCAGTACAAGATACCTA) differentiated *P. aeruginosa, P. alcaligenes* and *P. pseudo-alcaligenes* from the other group I species. A second probe, Ps (5'-GAAGGCTAGGCCAGC), identified all species except *P. putida*. An oligonucleotide specific for the *P. putida* sequence, 5'-GAAGGUUAGGCCAGC, allowed differentiation of *P. putida,* and a mixed probe (*i.e.* both oligonucleotides) allowed detection of all species of *Pseudomonas.*

Polymerase chain reaction (PCR)

P. aeruginosa strains can be identified by PCR-based amplification of the 16S-23S rDNA internal transcribed spacer region with specific primers (Tyler *et al*., 1995).

Other biomarkers

Biomarkers such as fatty acid methyl esters (FAMES) have been used widely for the identification of bacterial species (Thompson *et al*., 1993b). A commercial identification system, the Microbial Identification System (MIDI, Newark, New Jersey, USA), offers an extensive database of strains, including many pseudomonad strains, to which the fatty acid profile of an unknown isolate may be compared. The libraries of strains contain well-described clinical, environmental and plant-pathogenic strains. Tahis system provides an identification at the species level and a diagnostic profile; however, it is unlikely to identify all environmental isolates, since many have not been described before. Whole cell fatty acid analysis was tested as a method to discriminate between members of the *Pseudomonas* fluorescent

supercluster (Janse *et al*., 1992). This analysis resulted in recognition of a large supercluster that included most *P. fluorescens* and related strains (*P. chlororaphis, P. putida* and *P. tolaasii*). In the supercluster there were no separate clusters discriminating biovars of *P. fluorescens*, the other related species, or strains received as *P. marginalis* (a name formerly applied to plant-pathogenic members of the supercluster). Thus, the resolution of this technique appears to have limitations.

Diagnostic profiles for micro-organisms may also be obtained using polyacrylamide electrophoresis of whole cell protein extracts, or DNA fingerprints produced via restriction endonuclease digestion of genomic DNA. Pseudomonads have genomes that are rich in GC DNA bases. Enzymes like SpeI that cut at sites with a high AT base composition will digest the DNA at only a few sites, producing large fragments which may be separated and analysed using pulse field gel electrophoresis.

The species of rRNA group I synthesize a ubiqionone with nine isoprene units (Q-9) in the side chain, whereas members of rRNA groups II, III, and V contain Q-8, and those of rRNA group IV a Q-10 (Oyaizu and Komgata, 1983).

Polyamine patterns are of similar utility: rRNA group I species have a high putrescine and spermidine content, rRNA group II and III species have 2-hydroxuputrescine and a high content of putrescine, rRNA group IV species only contain significant amounts of spermidine and sym-homospermidine, and rRNA group V species are characterised by high concentrations of cadaverine and spermidine (Busse and Auling, 1988; Auling, 1992; Yang *et al*., 1993).

In summary, the results of any of the methods of identification described above are only as good as the database of strains and isolates to which they are referenced. There are numerous techniques that, if applied at the optimum taxonomic level, may prove useful in identifying *Pseudomonas* and its species. Ribosomal RNA sequencing seems useful at the genus or higher level, and methods like fatty acid analysis can work at the strain and isolate level.

3. Information on the Reproductive Cycle (Sexual/ Asexual)

Pseudomonas species reproduce by cell growth and binary cell division.

4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

A. General considerations

Pseudomonas species are efficient saprophytic chemo-organotrophs which grow at neutral pH and at temperatures in the mesophilic range (optimal growth between 20° and 45°C). Some species will grow at 4°C (*P. fluorescens, P. putida)* but not at the elevated temperature of 41°C. Optimal temperatures are *25*-30°C for *P. fluorescens, P. putida and P. syringae*, 30°C for *P. chlororaphis,* and 37°C for *P. aeruginosa.* Most, if not all, species fail to grow under acid conditions (< pH 4.5) (Palleroni, 1984).

Pseudomonas species thrive under moist conditions in soil (particularly in association with plants), and in sewage sediments and the aquatic environments. Environmental conditions which will affect their growth include nutrient availability, moisture, temperature, predation, competition, UV irradiation, oxygen availability, salinity, and the presence of inhibitory or toxic compounds. As nutritional demands are modest, pseudomonads can survive and multiply for months in moist environments such as tap water, sink drains, etc. (Palleroni, 1981; Bergen, 1981).

Competition and competitive niche exclusion are likely to limit the growth of introduced pseudomonad inoculants. Competitors are likely to include closely related pseudomonads and other bacteria able to compete for the same ecological niches with similar nutritional requirements (Lindow, 1992).

A number of environmental conditions may affect the dissemination of *Pseudomonas* species including surface water runoff, wind currents, and rain splash. It is likely that insects and other animals may also serve as vectors for dispersal. For example, *P. aeruginosa* can inhabit part of the normal gut or skin microflora of humans and animals. Thus dissemination would be associated with these vectors.

B. *P. aeruginosa*

P. aeruginosa is capable of growing in conditions of extremely low nutrient content (Palleroni 1984). The species was found to survive and proliferate in water for up to 100 days or longer (Warburton *et al*., 1994). Conditions of high humidity and temperature (80-90% humidity, 27°C) favoured the colonisation of lettuce and bean plants (Green *et al*., 1974).

C. *P. chlororaphis*

P. aureofaciens (*P. chlororaphis*) is an important coloniser of the rhizosphere and phyllosphere of plants (Thompson *et al*., 1993a; Legard *et al*., 1994). Kluepfel *et al*. (1991b) reported the colonisation of wheat roots in a field release of a recombinant *P. aureofaciens* (*lac*ZY). This population of *P. aureofaciens* reached a maximum of $2x10^6$ cfu/g root two weeks after inoculation and declined steadily to reach a level below detection (<100 cfu/g root) by 38 weeks post-inoculation. Angle *et al*. (1995) found that inoculations of recombinant *P. aureofaciens* (*lac*ZY) survived approximately twice as long in wheat rhizosphere as in non-rhizosphere bulk soil.

England *et al*. (1993) compared the survival and respiratory activity of *P. aureofaciens* in sterile and non-sterile loam and sandy loam soil microcosms. Recovery of *P. aureofaciens* was greater in sterile than non-sterile soils. Respiratory activity was higher in sandy loam soil than in loam soil, but soil type had no effect on survival.

The growth of *P. aureofaciens* in the spermosphere of seed-inoculated sugarbeets exhibited long lag phases (8-12 h) and their populations increased mainly between 12 and 24 hours (Fukui *et al*., 1994). The doubling time during the exponential growth phases was 2-3 h (Fukui *et al*., 1994).

D. *P. fluorescens*

P. fluorescens is commonly found inhabiting plant rhizosphere or phyllosphere environments. The plant rhizosphere provides an environment in which the species may show improved survival and growth. *P. fluorescens* distributed homogeneously in soil can result in significantly higher numbers in the rhizosphere of young wheat plants than in non-rhizosphere soil (Trevors *et al*., 1990).

The survival of *P. fluorescens* is affected by a variety of abiotic and biotic factors. Rattray *et al*. (1993) found that temperature and soil bulk density had a significant effect on lux-marked *P. fluorescens* colonisation of wheat rhizospheres. The greatest rates of colonisation occurred at the highest temperature (22°C) and lower bulk density (0.82 g/cm^3), and 100-fold higher numbers were found in the ectorhizosphere than in the endorhizosphere. Van Elsas *et al*. (1991) found *P. fluorescens* cells were able to withstand low temperatures, and could survive better at 4°C than at 15 or 27°C following introduction into natural soil, possibly due to an inhibition of the activity of the indigenous microflora. Van Elsas *et al*. (1986) found that *P. fluorescens* cell numbers declined slowly in both silt loam and loamy sand, but

survival was better in the silt loam. Heijnen *et al*. (1993) found that *P. fluorescens* survived better in unplanted soils in the presence of bentonite clay. Stutz *et al*. (1989) demonstrated that survival of *P. fluorescens* in vermiculite clay was better than in montmorillonite, which was better than in illite.

Van Elsas *et al*. (1992) found *P. fluorescens* R2f survived above 10^7 cfu/g dry soil for up to 84 days in Ede loamy sand microcosms when encapsulated in alginate with skim milk and bentonite clay, while free cells declined below 10^5 cells/g dry soil after 21 days. Vandenhove *et al*. (1991) studied the survival of *P. fluorescens* inocula of different physiological stages in soil. Introduction of a late exponential phase inoculum into soil brought about a lower death rate compared to exponential or stationary phase inocula.

Handley and Webster (1993) studied the effect of relative humidity (RH at 20, 40, 60, and 80%) on airborne survival of *P. fluorescens* indoors. They found that *P. fluorescens,* suspended in distilled water, survived best at mid humidities and least at 80% relative humidity.

Boelens *et al*. (1994) and Bowers and Parke (1993) determined that motility of *P. fluorescens* did not affect its spread through soil. A non-motile mutant strain promoted plant growth and colonised roots as effectively as the motile strain. Water flow rates were more important than motility for dispersal through soil and rhizospheres. Knudsen (1989) developed a mathematical model for predicting aerial dispersal of bacteria during environmental release which predicted off-site dispersal patterns that were in qualitative agreement with results from a field release of a genetically engineered *P. fluorescens* in California.

Thompson *et al*. (1992) studied dissemination of *P. fluorescens* by placing bacterial populations on apple or pear pollen in the entrances of hives of honey bees. In a pear orchard, 72% of the flowers within 7.6 m of the hive were colonised with *P. fluorescens* eight days after the start of the study.

E. *P. fragi*

P. fragi is commonly found on refrigerated meat and dairy products (Jay, 1992). Psychrotrophs such as *P. fragi* generally have a lower metabolic rate than mesophiles (lower Q_{10} for the same substrate) and have membranes that transport solutes more efficiently (Jay, 1992). In addition, there is a correlation between the maximum growth temperature and the temperature at which respiratory enzymes are destroyed in psychrotrophs. Nashif and Nelson (1953) reported that extracellular lipase synthesis in *P. fragi* was inactivated at 30°C. The lipase of *P. fragi* is reported active at temperatures as low as -29°C (Alford and Pierce, 1961).

P. fragi has the ability to colonise stainless steel surfaces in food processing establishments to form "biofilms" (Hood and Zottola, 1995); attachment may involve a polysaccharide and protein matrix surrounding the cells (Herald and Zottola, 1989). Attachment of *P. fragi* to stainless steel surfaces occurred in 0.5 h at 25°C and in 2 h at 4°C through the development of attachment fibrils (Stone and Zottola, 1985).

F. *P. putida*

A variety of environmental factors can affect the survival of *P. putida*. For example, plant rhizospheres can provide an environment for improved survival. Gamliel and Katan (1992) studied the chemotaxis response of *P. putida* towards seed exudates and germinating tomato seeds and suggested this may contribute to its rapid establishment in plant rhizospheres. Temperature is also an important factor. Hartel *et al*. (1994) found that *P. putida* (*lac*ZY) declined from about 10^8 to 10^3 cfu/g of soil after 35 days at 35°C, while it did not survive after three days at 40°C.

Macnaughton *et al*. (1992), using pLV1013 as a marker plasmid in *P. putida* PaW8, investigated the effect of soil texture on survival and found that introduced bacteria survived better in soils with higher clay

content. Compeau *et al*. (1988) studied survival of *P. fluorescens* and *P. putida* strains in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there were limited sites for colonisation of *Pseudomonas* species in these soils.

Lynch (1990) found that *P. putida* WCS 358 survived in distilled water (without substrate input) for over a year. It was suggested that this could result from the utilisation of dead cells within the population, and from viable cells having a very low maintenance energy requirement in a state of arrested metabolism.

Madsen and Alexander (1982) found that cells of *P. putida* were not transported below 2.7 cm in moist soil in the absence of some transporting agent or in the presence of developing plant roots. Percolating water and a burrowing earthworm enhanced the vertical transport of *P. putida*.

G. *P. syringae*

Foliar plant pathogens such as *P. syringae* are generally not adapted to survival in the soil (Lindow *et al*., 1988). However, *P. syringae* has been isolated from plant debris in the soil and can overwinter in temperate climates (Hirano and Upper, 1983). *P. syringae* pv. *syringae* R32 have pili that function as adhesions anchoring the cell to the surface of plants, thereby enhancing epiphytic colonisation. Wild-type bacteria became virtually resistant to displacement by rinsing within one day after inoculation, whereas non-pilated mutants were only partially resistant within three days (Suoniemi *et al*., 1995).

P. syringae metabolises a broad range of substrates, thus demonstrating flexibility in nutrient utilisation (Hirano and Upper, 1990). Criteria for viability of cells have been modified as the result of starvation experiments with *P. syringae*; it was determined that respiration of acetate and glycerol were more accurate determinants of viability than respiration of succinate (Cabral, 1995). The use of bactericides in agriculture (streptomycin and copper) has resulted in selection for strains resistant to these compounds; the resistance is often encoded on plasmids (Cooksey, 1990).

Plant-pathogenic strains grow to larger population sizes on susceptible plant hosts than on resistant ones (Stadt and Saettler, 1981), and therefore pathovars of *P. syringae* will grow to greater numbers on their respective hosts than on non-hosts. The presence of free water may be the most important factor contributing to the increase in population of *P. syringae* pv. *syringae* to infectious levels on bean leaves (Hirano and Upper, 1983; 1990; Beattie and Lindow, 1994). Immediately after rainfall, there is an initial decrease in population as bacteria are washed off the leaf surface, followed by a rapid increase in the population within 12 to 24 hours. Ambient temperature appears to have little effect on field populations of *P. syringae* pv. *syringae* on leaves but the age of annual crops does have an effect, with many more cells found on older leaves than on younger ones (Hirano and Upper, 1990; Jacques *et al*., 1995). *P. syringae* pv. *savastanoi* causes tumors on olive and oleander by producing the plant growth regulators indoleacetic acid (IAA) and cytokinins following infection; mutants deficient in IAA production grew as well as the wild type in culture and on plants, but the wild type reached a higher population density and maintained its maximum density at least nine weeks longer than the mutant populations.

Rainfall plays an important role in redistributing *P. syringae* within the plant canopy by washing bacteria from upper leaves onto lower ones, and by allowing individual bacterial cells to move using their flagella and find protected micro-sites on the surface of the leaf (Beattie and Lindow, 1994). Rainfall efficiently removed bacteria from foliar surfaces, but most of the cells were washed onto the soil; only a small portion were washed a relatively short distance from the source (Butterworth and McCartney, 1991).

P. syringae is also dispersed on seeds (Hirano and Upper, 1983). When cells of *P. syringae* were applied as a spray to plots, an exponential decrease in numbers of cells was observed; some cells were detected 9.1 m downwind within 20 minutes of the spray application. When applied to oat plants (a non-

host), viable cells could be detected for up to 16 days and were detected on plants up to 27 m downwind. In contrast to the plants, viable cells could not be detected in the upper layers of soil after two days (Lindow *et al*., 1988).

H. *P. tolaasii*

In the production of commercial mushrooms, *P. tolaasii* probably survives between crops on structural surfaces, in debris, and on equipment. It can be moved readily from one crop to another on the hands of pickers, on materials or equipment used in harvesting, and by insects, mites, water droplets and mushroom spores.

Conditions of high relative humidity and surface wetness encourage the expression of symptoms of brown blotch, an important mushroom disease, caused by *P. tolaasii*. Dispersal of the micro-organisms occurs readily upon watering once the disease is established (Howard *et al*., 1994).

5. Behaviour in Simulated Natural Environments Such as Microcosms, Growth Rooms, Greenhouses, Insectaries, etc.

A. *P. aeruginosa*

In a study by Sturman *et al*. (1994), the growth rate of *P. aeruginosa* appeared to be important in determining interspecies competition within packed-bed bioreactors filled with diatomaceous earth pellets.

B. *P. chlororaphis*

Angle *et al*. (1995) found that an intact soil core microcosm closely simulated survival results obtained from a field release of a recombinant *P. aureofaciens* (*lac*ZY). The strain of *P. aureofaciens* survived approximately 63 days in the bulk soil microcosm and 96 days in the rhizosphere microcosm.

C. *P. fluorescens*

Binnerup *et al*. (1993) found that kanamycin-resistant cells of *P. fluorescens* DF57-3 (Tn5 modified) inoculated in soil microcosms rapidly lost their culturability, as defined by visible colony formation on Kings B agar supplemented with kanamycin. After 40 days, only 0.02 to 0.35% of the initial inoculum was culturable. It was determined that about 20% of the initial inoculum represented viable, but non-culturable cells.

Compeau *et al*. (1988) studied survival of *P. fluorescens* and *P. putida* in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there are limited sites for colonisation of *Pseudomonas* species in these soils. Similarly, Al-Achi *et al*. (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested that there was competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

D. *P. fragi*

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

E. *P. putida*

Winstanley *et al*. (1993) studied the survival of *P. putida,* with an xy/E marker insert, in soil and lake water microcosms. When released into these microcosms, populations of the marked *P. putida* had a steady decline with little or no apparent division of cells. The rate of decline of *P. putida* in soil microcosms was significantly greater at 35% than at 50% field capacity water content, indicating that water content of the soil had an effect on survival. Similarly, Iwasaki *et al*. (1993; 1994) reported that the density of *P. putida* decreased rapidly to less than 10^2 to 10^3 level within five days in lake water and soil microcosms. The survival was influenced by protozoa density, light intensity, and soil water content. The addition of *P. putida* (10^7 cfu/ml) into natural water and soil had no effect on the density of indigenous micro-organisms and enzyme activities.

Williams *et al*. (1992) studied the fate and effects of *P. putida* PPO200 genetically engineered for both nalidixic acid and kanamycin resistance in freshwater and marine microcosms. The freshwater microcosm contained fish (*Poecilia latipinna*), annelid worms (*Tubifex tubifex*), snails (*Gyraulus* sp.), freshwater mussels (*Margaritifera margaritifera*), freshwater shrimps (*Palaemonetes kadiakensis*), and plants (*Elodea canadensis*). The marine microcosm contained fish (*Cyprinodon variegatus*), sea anenomae (*Bunodosoma californica*), snails (*Turbo fluctosus*), oysters (*Crassostrea gigas*), estuarine shrimp (*Palaemonetes pugio*), shorefly larvae (*Ephydra* sp.), and plants (*Salicornia biglovii*). *P. putida* could be detected in the tissues of some non-target organisms, *i.e.* the bacterium survived. However, gross signs, survival, and the histological study of control and exposed non-target organisms indicated that there were no adverse effects.

Doyle *et al*. (1991) and Short *et al*. (1991) observed reductions in CO_2 evolution and the number of fungal propagules, as well as the enhancement of dehydrogenase activity in soil amended with 2,4-dichlorophenoxyacetate (2,4-D) and inoculated with *P. putida* PPO301(pRO103) genetically engineered to degrade 2,4-D. These unanticipated effects were not observed: (a) in uninoculated soil; (b) when the homologous, plasmidless parent *P. putida* PPO301 was inoculated; or (c) in the presence of the genetically engineered *P. putida* when 2,4-D was not added. Moreover, the effects were not predictable from the phenotype of this genetically engineered *P. putida*. While long-term, statistically significant differences were detected in some microbial populations and processes, the majority of the differences were transient.

The effects of *P. putida*, on nitrogen transformations and nitrogen-transforming microbial populations were studied in a soil perfusion system by Jones *et al*. (1991). Neither the genetically engineered strain nor its homologous plasmidless host had a significant effect on ammonification, nitrification or denitrification in the soil, or on the population dynamics of the micro-organisms responsible for these processes.

F. *P. syringae*

Wendtpotthoff *et al*. (1994) monitored the fate of a genetically engineered strain of *P. syringae* applied to the leaves of bush beans in a planted soil microcosm. *P. syringae* established on the bean leaves at between 5×10^3 and 4×10^6 cfu/gm^{-1} fresh weight. During senescence of the bean plants, the strain was no longer detectable by selective cultivation and subsequent colony hybridisation.

Significant differences within *P. syringae* strain MF714R were detected when the bacterium was cultured on agar or in broth or collected from colonised leaves and subsequently inoculated onto greenhouse-grown plants in growth chambers or in the field or onto field grown plants. Bacterial cells cultured in liquid medium survived the least well after inoculation under all conditions, whereas cells cultured on solid media exhibited the highest percent survival and desiccation tolerance in the growth chamber but survived less well in the field than did cells harvested from plants. Cells harvested from plants

and inoculated onto plants in the field usually had the highest percent survival, started to increase in numbers earlier, and reached a higher number than did cells cultured *in vitro* (Wilson and Lindow, 1993a).

Wilson and Lindow (1993b) indicated that greenhouse-grown plants support larger epiphytic populations of an inoculated strain of *P. syringae* than do field-grown plants.

G. *P. tolaasii*

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

6. History of Use (Examples of Environmental Applications of the Organism and Information Derived from These Examples)

A. General considerations

Pseudomonads have been identified to be of importance in bioremediation as a result of their metabolic versatility. This metabolic versatility, and the ability to acquire additional versatility via plasmids, provides the potential for the rapid evolution of novel metabolic ability in *Pseudomonas* species. Examples of useful, or potentially useful, environmental applications of *Pseudomonas* isolates are given in Table 4.6. Some pseudomonad species have been introduced into the environment in bioremediation studies and have provided valuable information pertaining to characteristics such as survival. For example, Thiem *et al*. (1994) injected *Pseudomonas* sp. strain B13, a chlorobenzoate degrader, into a subsurface aquifer and found they could detect the strain 14.5 months after its environmental introduction.

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. For example, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to aggressively compete with other micro-organisms for niches and exclude phytopathogens. The possibility of the environmental application of strains to minimise frost damage on crop plants has also been investigated (Lindow and Panopoulos, 1988).

B. *P. aeruginosa*

Some strains of *P. aeruginosa* have been shown to produce biosurfactants which have potential uses in bioremediation for washing hydrocarbons from soil (Van Dyke *et al*., 1993). Jain *et al*. (1992) found that biosurfactants produced by *P. aeruginosa,* when added to soil, significantly enhanced the degradation of tetradecane, hexadecane and pristane.

Degradation of pentachlorophenol by *P. aeruginosa* has been investigated in shake-cultures. The bacteria were able to completely degrade pentachlorophenol up to 800 mg/l in six days with glucose as a co-substrate (Premalatha and Rajakumar, 1994). *P. aeruginosa* has also been found to degrade styrene in a continuous reactor at a rate of 293 mg $g^{-1}h^{-1}$. This could be applied to the industrial treatment of waste gas or polluted water (El Aalam *et al*., 1993).

C. *P. chlororaphis*

A strain of *P. chlororaphis,* genetically engineered to contain the *lacZY* genes, was introduced into the environment in a field trial in the United States in 1987, and its behaviour compared to the non-engineered strain (Kluepfel *et al*., 1991a, b, c). The non-engineered strain increased in number for two weeks, then declined to at or near the detection limit by 31 weeks.

Table 4.6 Examples of fluorescent species of *Pseudomonas* reported to have been used, or to have potential use, for bioremediation

Species	Strain	Target chemical	Reference
P. aeruginosa	JB2	Halogenated benzoic acids	Hickey & Focht, 1990
	PaK1	Polyaromatic hydrocarbons	Kiyohara *et al*., 1994
P. fluorescens	PHK	Phthalate	Pujar & Ribbons, 1985
		Dimethylphenol	Busse *et al*., 1989
		Isopropylbenzene	Busse *et al*., 1989
P. putida		Methyl-benzoates	Galli *et al*., 1992
		Naphthalene sulphonic acid	Zurrer *et al*., 1987
		Dimethylphenol	Busse *et al*., 1989
	OUS82	Polyaromatic hydrocarbons	Kiyohara *et al*., 1994
	G7	Polyaromatic hydrocarbons	Kiyohara *et al*., 1994

D. *P. fluorescens*

P. fluorescens has been genetically engineered and used in a number of experimental field studies, including the environmental introduction of a Tn-5 marked strain in the Netherlands in 1986 and other studies in the United States with strains engineered by deletion of the ice gene, and by introduction of *lacZY* marker genes (Wilson and Lindow, 1993b). De Leij *et al*. (1995) found that field releases of a genetically engineered *P. fluorescens,* and the unmodified wild-type strain, resulted in significant but transient perturbations of some of the culturable components of the indigenous microbial communities that inhabited the rhizosphere and phylloplane of spring wheat, but no significant perturbations of the indigenous culturable microbial populations in non-rhizosphere soil were found. The release of both of these bacteria had no obvious effect on plant growth and plant health, and the observed microbial perturbations were considered minor.

P. fluorescens can produce large and unusual proteins that are a key component of bacterial ice nuclei (Warren, 1987). Warren (1987) and Lindow and Panopoulos (1988) reviewed the practical applications connected to ice nucleation, including snow-making and the use of ice nucleation gene-deletion strains to generate biological control agents for minimizing frost damage to plants. A naturally occurring *P. fluorescens* strain, A506, has been registered commercially for the control of frost injury of pear (Wilson and Lindow, 1993b).

P. fluorescens is also one of the more common bacterial species that has been used for the control of diseases in the phyllosphere of plants, and a naturally occurring strain of this species has been registered for the commercial control of fire blight on pear (Wilson and Lindow, 1993b). Hatai and Willoughby (1988) detected *P. fluorescens* and *Saprolegnia parasitica* in rainbow trout lesions and found that *P. fluorescens* could strongly inhibit the growth of the fungus. It was suggested that *P. fluorescens*, or an antibiotic derived from it, might be used in biological control of saprolegniasis.

Snyman *et al*. (1993) found that *P. fluorescens* genetically engineered to produce the insecticidal toxin from *Bacillus thuringiensis* was toxic to *Eldana saccharina*. An LC_{50} of 1.86 mg freeze-dried bacterial powder/ml of insect diet was calculated, and it successfully reduced sugarcane boring.

P. fluorescens has been shown to have the ability to degrade a wide variety of compounds, including: 3-chlorobenzoic acid (Fava *et al*., 1993); naphthalene, phenathrene, fluorene and fluoranthene (Weissenfels *et al*., 1990); chlorinated aliphatic hydrocarbons (Vandenbergh and Kunka, 1988); styrene (Baggi *et al*., 1983); and pure hydrocarbons and crude oil (Janiyani *et al*., 1993). *P. fluorescens* can also be used in biosensor applications. For example, the recombinant *P. fluorescens* strain HK9, which lights up in the presence of contaminants such as PAHs (due to the insertion of lux genes), allows easy detection of bioavailable fractions of pollutants in soils and sediments (King *et al*., 1990).

E. *P. fragi*

No information was found on the use of *P. fragi* in environmental applications.

F. *P. putida*

P. putida is capable of eliminating phytopathogenic micro-organisms and stimulating plant growth (Vancura, 1988; Kloepper *et al*., 1988; Freitas and Germida, 1990). *P. putida* is also capable of degrading many unusual compounds by means of enzymatic systems encoded in plasmids. Chemicals degraded include polychlorinated biphenyls (PCBs) (Boyle *et al*., 1993; Lajoie *et al*., 1994); trichloroethylene (TCE) (Fujita *et al*., 1995); acetonitrile and sodium cyanide (Babu *et al*., 1994). *P. putida* has also shown the ability to remediate non-ionic sewage (Turkovskaya *et al*., 1993), pulp mill waste (black liquor) (Jain *et al*., 1993), waste gases using a biofilter (Zilli *et al*., 1993), electroplating effluent with high concentrations of copper (Cu(II)) (Wong *et al*., 1993), and high-sulphur coal (Khalid and Aleem, 1991).

G. *P. syringae*

Lindow *et al*. (1988) monitored the fate of a strain of *P. syringae* in experimental field trials in the United States. They found an exponential decrease in numbers of viable cells deposited at increasing distances from sprayed field plots. The relative rate of survival of cells sprayed directly on plants was more than ten times higher than that of cells dispersed through the air to similar adjacent plants.

Use of *P. syringae* has been proposed to enhance snowmaking and to delay frost damage in plants (Lindow, 1983; Wilson and Lindow, 1993b). *P. syringae* has also been shown to incorporate aluminium, chromium and manganese, so the bioremediation of sites contaminated with these chemicals may be a potential use (Alaoukaty *et al*., 1992).

H. *P. tolaasii*

No information was found on the use of *P. tolaasii* in environmental applications.

7. Characterisation of the Genome (*e.g.* Presence of Large Plasmids, Insertion Sequences) and Stability of These Characteristics

Members of the genus *Pseudomonas* are known for their metabolic versatility. They are capable of degrading many recalcitrant xenobiotics due to their ability to recruit new genes and alter the expression of existing ones. An understanding of the relative chromosomal position of relevant genes, the diversity of mobile genetic elements found within this genus, and the role these mobile genetic elements play in the stability and metabolic adaptation of individual isolates, can be helpful for regulatory assessments.

The chromosomes of *P. putida* and *P. aeruginosa* have been described in detail by Holloway and Morgan (1986) and by Ratnaningsih *et al*. (1990), Romling *et al*. (1989) and Holloway *et al*. (1994). Holloway *et al*. (1990a) provide genetic maps of these two species, which are useful in locating the relative positions of important genes and provide a good summary of other chromosomal and extrachromosomal

features. The sizes of the chromosomes for *P. putida* and *P. aeruginosa* vary from approximately 4,400 to 5,400 kb, with *P. aeruginosa* strain PAO having a genome size (5,400 kb) significantly larger than the 4,700 kb *E. coli* chromosome. Analysis of the distribution of chromosomal genes in pseudomonads shows that those involved in biosynthesis are not contiguous as with the enterobacteria. The genes for catabolic functions tend to be clustered on the chromosome, but are also not contiguous. Many catabolic functions are located on plasmids (*e.g.* Table 4.5); these genes, such as TOL (toluene degradation) and NAH (napthalene degradation), tend to be contiguous. This genome configuration allows for many diverse substrains within a species, each adapted to a particular environment.

Pseudomonas species contain a large variety of plasmids, insertion sequences, and transposons. The diversity of plasmids involved in degradation of organic compounds, drug resistance, and phytopathogenicity is indicated in Tables 5.4, 5.7 and 5.10. Insertion sequences (IS elements) and transposons are mobile within the genome of gram-negative bacteria, and can act as new promoters or as terminators, causing polar mutations. If two IS elements are located near each other in the appropriate orientation, they can be transposed to a second genome as a unit along with any intervening genes.

These three classes of mobile genetic elements (plasmids, insertion sequences, and transposons) can potentially interact within the same isolate, causing shifts in the positions of key catabolic genes. An example is the NAH plasmid napthalene degradative genes, which are nested within a defective but mobilizable transposon on the plasmid (Tsuda and Iino, 1990). Such shifts can result in a variable stability for some traits. For example, *P. syringae* pv. *savasatoni* mutations causing IAA deficiency were identified to have resulted from the action of two IS elements. In another case, a 150 kb plasmid (able to integrate into the chromosome) from *P. syringae* pv *phaseolicola,* when excised from the chromosome, resulted in the formation of a series of plasmids that either contained chromosomal DNA or were deletion mutants of the plasmid. These events were associated with a common repeated sequence (RS) (Coplin, 1989). In a reverse situation, components of the TOL plasmid have been shown to integrate into both the *P. putida* and *P. aeruginosa* chromosomes (Holloway *et al*., 1990b), thereby potentially stabilizing degradative genes in the genome of the isolates.

Besides affecting the stability of certain traits, mobile genetic elements allow pseudomonads to recruit new genes from replicons such as plasmids, which can lead to new metabolic capabilities. Specific examples have been given by Chakrabarty (1995) of *P. putida*'s ability to recruit new degradative genes on a transposable element. These new genes allow the organism to degrade new chemicals without the need to evolve completely new degradative pathways. This species has been able to acquire the genes needed to degrade 3-chlorobenzoate to the intermediate protocatechuate, which then is further degraded by resident chromosomal genes. In a similar fashion, the same species has been able to degrade phenol by acquisition of two genes, *pheA* and *pheB*, whose products can convert phenol to intermediates which are metabolized by a chromosomally-encoded *ortho* pathway (Chakrabarty, 1995). A transposon-like mobile element encoding a dehalogenase function has also been recently described in *P. putida* (Thomas *et al*., 1992). In the well-characterised *P. putida* mt-2 plasmid pWW0, the TOL-degradative enzymes are encoded on a 56 kb transposon which is itself part of a 70 kb transposon (Tsuda *et al*., 1989), giving rise to a family of TOL plasmids (Assinder and Williams, 1990). In addition to acquisition of degradative genes, pseudomonads can also acquire genes whose products aid in waste degradation.

The chlorosis-inducing phytotoxin coronatine, produced by *P. syringae* pvs. *tomato* and *atropurpurea* is plasmid encoded (Coplin, 1989). Other toxins (*e.g.* phaseolotoxin, syringomycin and tabtoxin) have been shown to be chromosomally encoded. *P. syringae* pv. *savasatoni* produces abnormal growths due to an imbalance of cytokinin and auxin plant hormones. The genes for their biosynthesis are plasmid encoded in oleander, but not olive pathovars. The majority of *P. solanacearum* strains contain a large (700-1000 kb) megaplasmid that contains genes for host range and pathogenicity.

8. Genetic Transfer Capability

The ability of pseudomonads to develop new metabolic pathway capabilities is often dependent on an isolate's ability to acquire DNA from other bacteria, which is then integrated into the genome in a manner dependent on the organism's environment. The three common systems for gene transfer in bacteria, namely conjugation, transduction and transformation, have been observed among members of the genus *Pseudomonas.* All three gene transfer mechanisms have been observed under laboratory and natural conditions. Gene transfer by all three mechanisms is affected by biological factors such as the nature and host range of the mobile genetic element, its transfer frequency, the concentrations of recipient and donor organisms, and the presence of other organisms which prey on donors and recipients. Abiotic factors such as temperature, moisture, and the presence of physical substrates which allow survival and/or gene transfer also affect the transfer frequency.

Even if the DNA is transferred to a new recipient, it may not be expressed. Sayre and Miller (1990) provide a detailed summary of factors associated with transposons and plasmids, the donors and recipients, and other biotic and abiotic conditions which affect gene transfer rates.

A. Conjugation

The acquisition of genetic material via conjugative plasmids represents an important evolutionary mechanism in the production of strains resistant to antibiotics and heavy metals, and with the ability to mineralise xenobiotics in selective environments. Gene transfer events may even affect the pathology of certain phytopathogens. Changes in cultivar-specificity and a loss of ability to produce fluorescent pigments of *P. syringae* pv. *pisi* were found to result upon the acquisition of IncP1 replicons such as plasmid RP4. Curing the RP4 plasmid from the strain maintained the new phenotype (Moulton *et al.,* 1993). Walter *et al*. (1987) developed a combined mating technique to measure the conjugal transfer potential of conjugative plasmids that uses four different standard mating techniques (colony cross streak, broth mating, combined spread plate, and membrane filtration), since no one technique worked best for the tested combinations of plasmids and recipients.

Conjugation between pseudomonads has been detected in both soil and aquatic environments. The transfer of conjugative plasmids has been demonstrated to occur between pseudomonads in a number of non-rhizosphere and rhizosphere soil environments, both in microcosms and *in situ* (van Elsas *et al.,* 1988; Trevors and Berg, 1989; Lilley *et al*., 1994).

Transfer frequencies were found to be enhanced by two orders of magnitude, that is, up to 10^{-2} per recipient organism, on the rhizoplane of sugarbeet *in situ* (Lilley *et al*., 1994). Soil components (such as clay, silt, organic matter and plant roots) provide excellent surfaces for the cell-to-cell contact required for bacterial conjugation (Trevors and Berg, 1989; Stotzky *et al*., 1991). In wheat plant root (van Elsas *et al*., 1988) and sugarbeet (Lilley *et al*., 1994) conjugation studies, survival of the donor and recipient, as well as frequency of plasmid transfer, decreased with increasing distance from the plant root. Transfer frequencies are also affected by soil moisture, with frequencies for R-plasmid transfer between *E. coli* isolates shown to be optimal at 60 to 80% soil moisture holding capacity (Trevors and Starodub, 1987). Conjugal transfer of broad host range plasmids between *P. aeruginosa* donor and recipient strains in lake water has been observed to occur at a lower rate in the presence of the natural microbial community (O'Morchoe *et al*., 1988). Plasmids incapable of conjugation themselves have been shown to be mobilised from a laboratory strain of *E. coli* in a laboratory-scale wastewater treatment facility by mobilizer and recipient *E. coli* strains of both laboratory and wastewater origin (Mancini *et al*., 1987).

The TOL plasmid pWWO can be transferred to other micro-organisms, and its catabolic functions for the metabolism of alkylbenzoates are expressed in a limited number of gram-negative bacteria, including

members of the rRNA group I pseudomonads and *E. coli* (Ramos-Gonzalez *et al*., 1991). Transfer of the recombinant plasmid to *Erwinia chrysanthemi* was observed, but transconjugants failed to grow on alkylbenzoates because they lost catabolic functions. Pseudomonads belonging to rRNA groups II, III, and IV, *Acinetobacter calcoaceticus*, and *Alcaligenes* sp. could not act as recipients for TOL, either because the plasmid was not transferred or because it was not stably maintained. Under optimal laboratory conditions, the frequency of transfer of pWWO from *P. putida* as a donor to pseudomonads belonging to rRNA group I was on the order of 1 to 10^{-2} transconjugants per recipient, whereas the frequency of intergeneric transfer ranged from 10^{-3} to 10^{-7} transconjugants per recipient. Intra-species, but not inter-species transfer of TOL in soils has been reported (Ramos *et al*., 1991), but it was affected by the type of soil used, the initial inoculum size, and the presence of chemicals that could affect the survival of the donor or recipient bacteria (Ramos-Gonzalez *et al*., 1991).

The *P. putida* TOL plasmid pWWO and the wide host range RP4 plasmid are able to mediate chromosomal mobilisation in the canonical unidirectional way (*i.e.* from donor to recipient cells) and bi-directionally [*i.e.* donor to recipient to donor (retrotransfer)] (Lejeune and Mergeay, 1980; Mergeay *et al*., 1987; Top *et al*., 1992; Ramos-Gonzales *et al*., 1994). Transconjugants are recipient cells that have received DNA from donor cells, whereas retrotransconjugants are donor bacteria that have received DNA from a recipient. The TOL plasmid pWWO and the pRP4 plasmid are able to directly mobilise and retromobilise a chromosomal marker integrated into the chromosome of the other *Pseudomonas* strains, and this process probably involves a single conjugational event. The rate of retrotransfer (as well as direct transfer) of chromosomal markers is influenced by the location of the marker on the chromosome, and it ranges from 10^{-3} to less than 10^{-8} retrotransconjugants per donor (transconjugants per recipient). The mobilised DNA is incorporated into the chromosome of the retrotransconjugants (transconjugants) in a process that seems to occur through recombination of highly homologous flanking regions. No interspecific mobilisation of the chromosomal marker in matings involving *P. putida* and the closely related *P. fluorescens* was observed.

It seems clear that pseudomonads can acquire plasmids from other bacteria in the environment. This premise is supported by the array of plasmids that have been recovered from members of *Pseudomonas,* some of which are listed in Tables 5.5, 5.7 and 5.10. The boundaries to gene transfer events are illustrated by plasmid RP4, originally isolated in *P. aeruginosa,* which has been shown to be transmissible to all gram-negative bacteria tested (Riley, 1989). *E. coli* has been shown to transfer plasmid-borne genetic information to over 40 genera (Stotzky *et al*., 1991). Direct evidence of pseudomonad isolate acquisition of plasmids from other bacteria in the environment is also available: Bale *et al*. (1988) showed that an introduced *P. putida* recipient acquired mercury resistance plasmids from an intact lotic epilithic bacterial community at frequencies up to 3.75×10^{-6} per recipient.

B. Transduction/bacteriophage mediated gene transfer

Two characteristics of a bacteriophage (phage) which are important in determining its ability to broadly distribute DNA were summarised in Sayre and Miller (1990). First, the host range of most phages is restricted to one species or a small number of related taxa, although broad host range phages such as phages P1 and Mu are known. Second, phages which undergo specialised transduction are likely to transfer chromosomal genes which are in close proximity to the phage integration site, while generalised transducing phages can transfer any of the bacterial genome's sequences with approximately equal frequency.

Many different lytic and temperate phages have been identified in *Pseudomonas*, and the morphological diversity among phages is at least as great as for any other bacterial genera. Transduction by temperate phage of *P. aeruginosa* chromosomal DNA has been demonstrated in fresh water microcosms

(Morrison *et al*., 1979; Saye *et al*., 1987; 1990) and the phylloplane of bean and soy bean plants (Kidambi *et al*., 1994).

P. aeruginosa has been frequently reported as subject to lysogeny, the process by which the phage chromosome becomes integrated into the bacterial host chromosome and is stably replicated with it, as a prophage. Lysogeny may lead to increased fitness of bacterial strains in the natural environment, by increasing the size and flexibility of the gene pool available to natural populations of bacteria via horizontal gene transfer. Approximately 45% of *Pseudomonas* field isolates tested positive in colony hybridisations when probed with phage isolated from the same area (Miller *et al*., 1990a). The prophage appears to contribute a major source of phage in the natural environment. In addition to mediating the transfer of genetic material within and between species, the induction of certain prophages results in transposition and mutagenesis events within the host genome.

C. Transformation

Both chromosomal and plasmid DNA are subject to natural transformation in the environment, a natural physiological process which is different from the artificial transformation techniques used in the laboratory (Stewart, 1990). In order for transformation of a cell to result in expression of the new DNA sequence, DNA must: 1) be excreted or lost from a donor cell; 2) persist in the environment; 3) be present in sufficient concentrations for efficient transformation to occur; 4) come in contact with a recipient cell which is naturally competent to receive the donor DNA; 5) be able to evade any recipient cell defences which degrade foreign DNA; and 6) integrate into a stably-maintained replicon in the recipient. Marine and soil environments have been shown to contain biologically significant levels of dissolved DNA (Paul *et al*., 1987; Lorenz *et al*., 1988). Soil environments offer protection from nuclease digestion for chromosomal and plasmid DNA (otherwise available for transformation) when bound to clay and sediment matter (Lorenz and Wackernagel, 1991; Romanowski *et al*., 1991; Khanna and Stotzky, 1992).

Natural transformation was found for P. stutzeri, P. mendocina, P. alcaligenes and P. pseudoalcaligenes, but not for P. aeruginosa, P. putida, P. fluorescens, and P. syringae strains (Carlson *et al*., 1983).

IV. Human Health Considerations

1. Diseases caused and mechanism of pathogenicity including invasiveness and virulence

General considerations

Included amongst the rRNA group I fluorescent pseudomonads are species pathogenic to humans. Those that do cause infection are generally opportunists, promoting a variety of conditions ranging from endocarditis to dermatitis (Pollack, 1992; Artenstein and Cross, 1993; Berger *et al*. 1995; Jarvis and Skipper, 1994). Individuals most at risk from *Pseudomonas* infection are the immunocompromised, in particular individuals with AIDS, patients with cystic fibrosis, and those suffering major trauma or burns (Artenstein and Cross, 1993; Neu, 1985; Bodey *et al*., 1983; Moayyedi *et al*., 1994; Schuster and Norris, 1994).

It should be stressed that only some of the Pseudomonads have been implicated in human disease and that proponents should not base assessment criteria on *P. aeruginosa,* the most problematic member of the genus. Proponents should also be aware that not all strains of a given species have been shown to promote disease, and that assessment criteria should be based upon the strain being reported rather than the species as a whole.

P. aeruginosa

The predominant pseudomonad isolated from clinical sources is *P. aeruginosa*. Evaluation of the pathogens causing nosocomial infections in hospitals in the United States (Jarvis and Martone, 1992) indicated that *P. aeruginosa* was the fourth most common pathogen isolated (10.1%) in hospital-wide surveillance and the most common (12.4%) in intensive care units. In more recent surveys, *P. aeruginosa* was found to be the fifth most common pathogen (9%) (Emori and Gaynes, 1993) and increased to 29% in intensive care facilities [European Prevalence of Infection in Intensive Care Study (EPIC) (Spencer, 1994)]. The ability of *P. aeruginosa* to persist in a number of hospital disinfectants and pharmaceuticals (Dominik *et al*., 1995; Gilardi, 1991) and in sanitary facilities (Bobhammer *et al*., 1996; Döring *et al*., 1991; Zembrzuska-Sadkowska *et al*., 1995) probably contributes to its prevalence in the hospital environment. *P. aeruginosa* causes a wide range of syndromes, involving nearly all body systems, that vary from sub-acute to chronic (Artenstein and Cross, 1993; Pollack, 1992).

Bacteremia associated with *P. aeruginosa* is restricted mainly to immunocompromised individuals with significant underlying disease (Artenstein and Cross, 1993; Askamit, 1993; Spencer, 1994). Symptoms are indistinguishable from gram-negative sepsis caused by other bacteria (Pollack, 1993). Factors associated with bacteremia include malignancy, transplants (solid organ, bone marrow), diabetes, cirrhosis of the liver, renal failure, burns, trauma, intravenous drug abuse, corticosteroid therapy, cytotoxic chemotherapy, cardiopulmonary resuscitation, immunoglobulin deficiency, AIDS, broad spectrum antibiotics, and invasive instrumentation (IV catheters, endoscopes, mechanical ventilation, etc.) (Askamit, 1993; Artenstein and Cross, 1993; Dropulic *et al*., 1995; Mallolas *et al*., 1990; Nensey *et al*., 1990). Major entry portals for *P. aeruginosa* bacteremia appear to be following infection (or colonisation) of the respiratory tract (Gallagher and Watanakunakorn, 1990; Artenstein and Cross, 1993) and the genitourinary tract (Gallagher and Watanakunakorn, 1990; Aksamit, 1993).

P. aeruginosa septicemia is primarily a condition of debilitated, immunocompromised adults and of infants. It is usually acquired in hospital, with prior broad-spectrum antibiotic therapy as a predisposing factor (Bodey *et al*., 1983; Richet *et al*., 1989), although community-acquired infections in apparently healthy children (Ros, 1989) and adults (Ishihara *et al*., 1995) have been reported. Mortality is high in immunocompromised patients (up to 50%) (Artenstein and Cross, 1993; Bisbe *et al*., 1988), with a better prognosis in "normal healthy" individuals (Ishihara *et al*., 1995).

Endocarditis due to *P. aeruginosa* has been associated with two major predisposing factors (Artenstein and Cross, 1993): the use of prosthetic heart valves and the use of illicit parenteral drugs. In drug abusers, endocarditis usually occurs in the right side of the heart and is sub-acute, although a complication, septic pulmonary emboli involving the tricuspid valve, can occur (Pollack, 1992).

Nosocomial pneumonia is the second most common hospital-acquired infection in the United States (Aksamit, 1993; Emori and Gaynes, 1993). *P. aeruginosa* is the most prevalent etiological agent for both poly- and mono-microbial pneumonia (Emori and Gaynes, 1993). Community-acquired pneumonia has also been attributed to this organism; its occurrence is rare (Artenstein and Cross, 1993), but it is more frequently recognised in AIDS patients (Dropulic *et al*., 1995; Schuster and Norris, 1994). Colonisation of the oropharyngeal and/or the upper gastrointestinal tracts is an important precursor to nosocomial pneumonia, although colonisation does not always imply infection. However, susceptibility to pneumonia is inversely related to a patient's basic health (Aksamit, 1993; Artenstein and Cross, 1993, Dick *et al*., 1988; Dropulic *et al*., 1995). Conditions predisposing to *P. aeruginosa* pneumonia are similar to those mentioned for bacteremia.

Bacteremic pneumonia, with organisms isolated from both the lung and bloodstream, resembles bacteremia and pneumonia in clinical presentation. Prognosis is bleak with this syndrome. Mortality rates

of 80-100% are observed, compared to 27-50% for bacteremia and 30-60% for pneumonia (Aksamit, 1993).

Chronic pulmonary colonisation by *P. aeruginosa* in the lungs of patients with cystic fibrosis results in frequent acute episodes of pneumonia and chronic bronchiectasis, but rarely in bacteremic pneumonia (Aksamit, 1993). Chronic infection leads to the obstruction of the airways, respiratory distress, and eventually death (Gilligan, 1991; Romling *et al*., 1994).

Otolaryngologic infections due to *P. aeruginosa* range from superficial and self-limiting to life-threatening (Artenstein and Cross, 1993). The most serious ear infection due to this organism is malignant otitis externa, usually resulting from a failure of topical therapy, and resulting in an invasive disease-destroying tissue which may progress to osteomyelitis at the base of the skull and possible cranial nerve abnormalities (Artenstein and Cross, 1993). Other ear infections associated with *P. aeruginosa* include external otitis (swimmer's ear), otitis media, chronic suppurative otitis media, and mastoiditis (Artenstein and Cross, 1993; Legent *et al*., 1994; Kenna, 1994; Pollack, 1992).

P. aeruginosa is the leading cause of gram-negative ocular infections, presenting as keratitis or endophthalmitis (Holland *et al*., 1993; Chatterjee *et al*., 1995; Bukanov *et al*., 1994). Predisposing factors include the use of contact lenses (in particular their cleaning and storage solutions), trauma, burns, ocular irradiation, compromised host defences, and systemic infections (Holland *et al*., 1993; Pollack, 1992; Imayasu *et al*., 1994; Stapleton *et al*., 1995).

Moisture is the paramount defining factor in *P. aeruginosa* growth. Normal dry skin does not support growth, whereas moist skin enables the organism to flourish. For this reason, dermatologic infections with *P. aeruginosa* tend to be more prevalent in moist tropical and subtropical climates (Bodey *et al*., 1983) or to be associated with the use of swimming pools, hot tubs or whirlpools (Gustafson *et al*., 1983; Trueb *et al*., 1994; Vesaluoma *et al*., 1995). The use of contaminated "loofah" cosmetic sponges is another source of *P. aeruginosa* infection (Bottone and Perez, 1993; 1994; Fisher, 1994). Folliculitis, pyoderma, cellulitis and ecthyma gangrenosum are all dermatologic infections in which *P. aeruginosa* has been implicated (Pollack, 1992; Artenstein and Cross, 1993; Gustafson *et al*., 1983; Fisher, 1994; Noble. 1993).

P. aeruginosa is a frequent isolate from wounds, particularly those contaminated with soil, plant material or water. Its presence may reflect colonisation as opposed to infection, which is a consequence of its ubiquitous distribution in nature (Artenstein and Cross, 1993; Pollack, 1992). Puncture wounds, particularly those penetrating to bone, may result in osteomyelitis or osteochondritis. The former is common in intravenous drug abusers (Artenstein and Cross, 1993) and the latter in puncture wounds to the foot in children and diabetics (Lavery *et al*., 1994; Pollack, 1992; Jarvis and Skipper, 1994). The wearing of tennis shoes (sneakers) at the time of puncture injury increases the chance of *P. aeruginosa* infection (Pollack, 1992; Lavery *et al*., 1994; Fisher *et al*., 1985).

In rare cases, *P. aeruginosa* has been associated with meningitis or brain abscess (Pollack, 1992) and infection of the gastro-intestinal tract (Artestein and Cross, 1993). Both conditions are nosocomially acquired, occurring in patients suffering from malignancies, invasive procedures or neutropenia (Pollack, 1992; Artenstein and Cross, 1993).

P. fluorescens

P. fluorescens has occasionally been associated with human infection. The inability of most strains to grow at normal human body temperature (Palleroni, 1992a) restricts invasion and subsequent disease promotion. This organism has the ability to grow at 4^{o}C (Gilardi, 1991). This characteristic, along with the observation that it is isolated from the skin of a small percentage of blood donors, makes it an occasional

contaminant of whole blood and blood products (Puckett *et al*., 1992; Stenhouse and Milner, 1992). Pseudobacteremia may result from the infusion of contaminated products (Scott *et al*., 1988; Simor *et al*., 1985; Gottlieb *et al*., 1991; Foreman *et al*., 1991) or from the use of contaminated equipment (Anderson and Davey, 1994).

P. fluorescens has been occasionally isolated from patients with AIDS (Franzetti *et al*., 1992; Roilides *et al*., 1992), where it caused bacteremia and urinary tract, ocular and soft tissue infections. Chamberland *et al*. (1992), in their across-Canada survey of septicemia, found that 1.5% of isolates were *P. fluorescens*. It is apparent that *P. fluorescens* can be an opportunistic pathogen in cancer patients and in others who are severely immunocompromised, but that it is of little concern to immunocompetent individuals. *P. fluorescens* is occasionally found in sputa of patients with cystic fibrosis, although its role as a pathogenic factor has yet to be resolved.

P. fragi

P. fragi is one of the pseudomonads associated with food spoilage (Barrett *et al*., 1986; Drosinos and Board, 1995; Greer, 1989) and is commonly isolated from milk products, pork and lamb. A search of the literature dating back to 1966 failed to reveal any association between *P. fragi* and human disease.

P. putida

P. putida is a rare opportunistic pathogen in immunocompromised individuals. Like *P. fluorescens*, this organism can grow at 4°C in whole blood and blood products and is consequently an occasional source of pseudobacteremia (Pitt, 1990; Taylor *et al*., 1984; Tabor and Gerety, 1984). Septicaemia and septic arthritis due to *P. putida* in immunocompromised patients have been reported (MacFarlane *et al*., 1991; Madhavan *et al*., 1973) and bacteremia in AIDS patients can occur at low frequency (Roilides *et al*., 1992). All syndromes appear to be associated with breaching of the patient's mechanical defences, either associated with transfusion or following placement of in-dwelling catheters.

P. chlororaphis, P. syringae, P. tolaasii

A search of the literature dating back to 1966 failed to reveal any association between these species and human disease. The possibility does exist that an incomplete identification has failed to speciate these organisms, and that they are reported in the literature as *Pseudomonas* sp.

2. Communicability

P. aeruginosa, the species of most concern in the rRNA group I pseudomonads, has a ubiquitous distribution at a low frequency in nature (Romling *et al*., 1994a). Outside of the hospital environment, 20 to 30% of people harbour faecal *P. aeruginosa*. This frequency increases during hospitalisation as a result of contact with an environment in which the organism is more common. Both healthy individuals and patients with P. aeruginosa infections may serve as reservoirs for infection in hospitals.

P. aeruginosa is an important cause of nosocomial infections. It is particularly a problem in burn units, neonatal units, and wards housing leukemia and other cancer patients (Bergen, 1981). Nosocomial infections may spread by transmission 1) directly between patients; 2) via medical personnel; 3) via inanimate objects which may serve as reservoirs or vectors; and 4) from the normal flora of the patient (*i.e.* autoinfection).

Most types of hospital equipment or utensils can serve as a source of infection, including pharmaceutical products, disinfectants, water jugs, table tops, trays, urine bottles, urethral catheters, anaesthetic equipment, and respiratory apparatus. Transmission may also occur via food stuffs such as

strawberries, plums and other fruit, vegetables, frozen poultry, refrigerated eggs, lemonade, raw milk, and any equipment or utensil involved in the preparation or serving the food.

3. Infective dose

Infective dose for the fluorescent pseudomonads is not really relevant, since infection usually occurs in immunosuppressed individuals. Most patients suffering from cystic fibrosis acquire a *P. aeruginosa* infection at some stage of their lives, resulting in frequent, recurrent bouts of pneumonia. Mortality in such cases may reach 100%.

4. Host range, possibility of alteration

P. aeruginosa has a broad host range which includes humans, animals, and some plants. It converts from a non-mucoid state to a mucoid, alinate-producing variant in the lungs of CF patients. The mucoid form is almost exclusive to colonisation of this site. Upon *in vitro* propagation, the mucoid strains isolated from CF lungs may undergo a spontaneous reversion to the non-mucoid form (Maharaj *et al*., 1992).

5. Capacity for colonisation

Fluorescent pseudomonads may be found in the normal bacterial flora of the intestines, mouth or skin of humans or animals. Colonisation is harmless under normal circumstances. In immunosuppressed or immunocompromised patients the capacity for colonisation by *P. aeruginosa* is high.

6. Possibility of survival outside the human host

rRNA group I fluorescent pseudomonads do not require human or animal hosts for survival. Most are common residents of soil, rhizosphere, sediment, and aquatic habitats. These generally moist environments provide natural reservoirs for the organisms. The pseudomonads have modest nutritional demands and can survive for months in tap water, distilled water, sink drains, or any other moist environment.

7. Means of dissemination

The fluorescent pseudomonads are ubiquitous micro-organisms. Anyone (not only infected individuals), or anything, may serve as a source or vector for dissemination (refer also to 10 and 26).

8. Biological stability

In *P. aeruginosa* infections of the CF lung, a transition from a non-mucoid to a mucoid, alginate producing variant is observed, indicating the pleomorphic nature of this organism. Furthermore, the level of toxin production varies with the isolate, suggesting that expression levels of chromosomally encoded genes are subject to strain differences. Recent studies indicate that this variation is attributable to the variable position of the genes on the chromosome, due at least in part to chromosome reassortment and the movement of IS-like sequences (Vasil *et al*., 1990).

9. Antibiotic-resistance patterns

P. aeruginosa

P. aeruginosa is naturally resistant to many widely used antibiotics. Resistance in part is thought to be the result of an impermeable outer membrane and the production of extracellular polysaccharides (Quinn, 1992). The organism is usually resistant to low levels of kanamycin, penicillins (with the exception of the anti-pseudomonal penicillins: carbenicillin, ticarcillin, piperacillin), most of the first and second generation

cephalosporins, chloramphenicol, nalidixic acid, tetracyclines, erythromycin, vancomycin, sulfonamide, trimethoprim and clindamycin (Wiedemann and Atkinson, 1991). Antibiotic resistance is often due to the presence of plasmids (Table 4.7). Individual strains may be resistant to antibiotics to which the species is generally susceptible. For this reason, antibiotic resistance patterns should not be relied on for species verification, but should be assessed on a case-by-case basis.

P. fluorescens and P. putida

Antibiotic resistance patterns for *P. fluorescens* and *P. putida* are difficult to assess, since only small numbers of isolates have been tested in controlled studies. The organisms tested are susceptible to low levels of kanamycin and resistant to carbenicillin and gentimicin, two of the antibiotics still in use against *P. aeruginosa* (Pitt, 1990). Again the use of antibiotic resistance/susceptibility profiles should be regarded with caution, since variation within a species may be great.

P. chlororaphis, P. fragi, P. syringae, P. tolaasii

Antibiotic susceptibility patterns for these species were not found in the literature searched.

10. Toxigenicity

The pathogenicity of *P. aeruginosa* is accredited to the wide array of virulence-associated factors produced by some if not all strains. Pili act as adhesins to a variety of cell types and enable the organism to colonise epithelial surfaces (Prince, 1992). Once established, the bacteria secrete a number of extracellular products capable of tissue damage and facilitating dissemination of the bacteria (Plotkowski *et al*., 1994). Proteases (including elastase), exotoxin-A, exoenzyme-S, phospholipase-C, exolipase, rhamnolipid, alginate, cytotoxin, high molecular weight leukocidin and endotoxin have all been implicated in pathogenesis (Fick, 1993; Govan and Nelson, 1992; Holder, 1993; Holland *et al*., 1993; Jaeger *et al*., 1991; McCubbin and Fick, 1993; Kudoh *et al*., 1994; Lutz *et al*., 1991; Noda *et al*., 1991).

Exotoxin-A (ETA) and exoenzyme-S are ADP-ribosyltransferases which inhibit protein synthesis in the eukaryotic cell. ETA is produced during the decline of the *P. aeruginosa* growth cycle. Its synthesis is dependent on the iron concentration in the growth medium (Stephen and Pietrowski, 1986). The levels of both ETA and exoenzyme-S vary with the isolate examined.

Phospholipase-C (PLC) is another extracellular enzyme produced by *P. aeruginosa* which is toxic in micro or sub-microgram levels. PLC preferentially degrades phospholipids, which are plentiful in the eukaryote cell. In addition, one of the substrate products of lipid degradation by PLC (diacylglycerol) can have toxic effects on the host animal by inducing the production of potent substances (arachronic acid metabolites and protein kinase C). These by-products alter eukaryotic cell metabolism and incite inflammatory responses.

Elastase, one of the extracellular proteases, degrades elastin, collagen, human immunoglobulin and serum α-1-proteinase inhibitor (Iglewski *et al*., 1990), activities which help evade the immune response and sponsor tissue invasion. Alkaline protease, another of the extracellular proteases, is active on IgA, cytokines (TNF-α; IFN-γ; IL-2), lactferrin and transferrin, fibrinogen, and fibrin (Shibuya *et al*., 1991; Doring *et al*., 1988; Frick *et al*., 1985; Parmely *et al*., 1990). These enzymatic activities promote disruption of respiratory cilia and increased vascular permeability, which probably contribute to establishment in the lung and resulting pneumonia.

The toxigenic potential of other species of *Pseudomonas* is less well studied. Proteases and phospholipases have been detected in some strains of *P. fluorescens* and *P. putida,* but their significance in human infection has yet to be elucidated.

11. Allergenicity

Fluorescent pseudomonads have not been described as potent allergens. However, they do possess endotoxin (lipopolysaccharide), which may precipitate an allergic response in some individuals.

Table 4.7 Examples of plasmids encoding for drug resistances in *P. aeruginosa*

Plasmid	Resistances encoded
RP1	carbenicillin, kanamycin, neomycin, tetracycline
RP1-1	carbenicillin
R9169	carbenicillin, kanamycin, neomycin, tetracycline
R6886	carbenicillin, kanamycin, neomycin, tetracycline
RP8	carbenicillin, kanamycin, neomycin, tetracycline
R2-72	carbenicillin, streptomycin, kanamycin
R38-72	tetracycline, streptomycin
R39-72	tetracycline, streptomycin
R931	tetracycline, streptomycin
R679	streptomycin, sulphonamide
R1162	streptomycin, sulphonamide
R3108	streptomycin, sulphonamide, tetracycline
R209	streptomycin, sulphonamide, gentamicin
R130	streptomycin, sulphonamide, gentamicin
R716	streptomycin
R503	streptomycin
R5265	streptomycin, sulphonamide
R64	ampicillin, carbenicillin, sulphonamide, gentimicin, kanamycin
R40a	ampicillin, anamycin, paromycinin, sulphonamide

Source : taken from Trevors (1991)

12. Availability of appropriate prophylaxis and therapies

P. aeruginosa

Antibiotic therapy for *P. aeruginosa* depends upon the site of infection and the relative susceptibility of the particular strain to the antibiotics tested. Generally, the species is susceptible to very few antibiotics. Ceftazidime, cefsulodin, imipenem, ticarcillin-clavulanic acid, azlocillin, piperacillin, the aminoglycosides, colistin and ciprofloxacin are some of the antibiotics with a high percentage of susceptible isolates (Chamberland *et al*., 1992; Wiedemann and Atkinson, 1991; Legent *et al*., 1994). Combination therapy using two effective antibiotics may increase the clinical cure rate in some infections (Lucht *et al*., 1994), and synergistic combinations of an aminoglycoside with a β-lactam (that has activity against *Pseudomonas*) have continued to be effective (Sepkowitz *et al*., 1994). The particular antibiotic regime selected will depend, however, on the strain in question and cannot be answered in a generic manner.

P. fluorescens and *P. putida*

Ceftazidime (Jones *et al*., 1989; Watanabe *et al*., 1988), imipenem (Jones *et al*., 1989) and meropenem (Jones *et al*., 1989) have been described as active against *P. fluorescens*. Antibiotics active against *P. putida* are ceftazidime, carbapenems, aminoglycosides, tetracyclines and polymixin B (Kropec

et al., 1994; Bergen, 1981; Papapetropoulou *et al*., 1994). Any possible treatment regime should be proposed for the strain in question and not based on generic information for the species.

P. chlororaphis, P. fragi, P. syringae, P. tolaasii

No antibiotic susceptibility data for these species were found in the literature searched.

V. Environmental and Agricultural Considerations

1. Natural Habitat and Geographic Distribution. Climatic Characteristics of Original Habitats

A. General considerations

Pseudomonas species have been isolated from a wide variety of habitats including soils, fresh or sea water, clinical specimens and laboratory reagents (including distilled water), food stuffs and wastes, flowers, fruit, vegetables, and diseased and healthy plants and animals. Many species appear to have a global distribution. For example, beneficial colonizers and *Pseudomonas*-incited plant diseases (such as *P. syringae*) are worldwide in distribution and involve representatives of most major groups of common plants.

Although pseudomonads are often considered to be ubiquitous, there are also many reports of niche specialisation. For instance, the number of epiphytic bacteria (such as *P. syringae*) present on the leaves of newly emerged plants is very low, indicating that the soil does not appear to serve as an important source of inoculum (Lindow, 1992) or habitat. As well, many phytopathogenic pseudomonads can only be isolated from the diseased host. For example, *P. syringae* isolates are generally only found in association with live plants or propagative material, and in these niches they appear as virtually homogeneous populations (Schroth *et al*., 1981). At present, the distribution of these pathogens in the absence of the host is unclear.

B. *P. aeruginosa*

P. aeruginosa is widely distributed in soil (Bradbury, 1986) and water (Palleroni, 1984). It is occasionally isolated from both healthy and diseased plants (Bradbury, 1986). Experiments conducted with lettuce and bean under varying conditions of temperature and humidity indicated that *P. aeruginosa* can colonise these plants under conditions of high temperature and humidity (27ºC, 80-95% humidity) (Green *et al*., 1974). The occurrence declined in lettuce and bean when the temperature and humidity were lowered (16ºC, 55-75% humidity).

Cho *et al*. (1975) studied the occurrence of *P. aeruginosa* on the foliage and in the soil of potted ornamental plants in order to determine their importance as a disseminating agent in hospital environments. They concluded that although potted plants are potential carriers for introduction of the species to hospital environments, there is no evidence that these plants constitute a primary source of bacteria for hospital infections. Results of a study to determine the prevalence of bacteria in passerines and woodpeckers suggest that *Pseudomonas* spp., including *P. aeruginosa,* are not uncommon in the gut flora of omnivorous and granivorous birds (Brittingham *et al*., 1988).

C. *P. chlororaphis*

P. aureofaciens (*P. chlororaphis*) was one of the most commonly occurring bacteria in soil, and on roots and leaves of both sugarbeet and spring wheat, during the growing season (De Leij *et al*., 1994). *P. chlororaphis* has also been isolated from water and from dead larvae of cockchafer, a large European beetle (Palleroni, 1984).

D. *P. fluorescens*

P. fluorescens is commonly found on plant surfaces, as well as in decaying vegetation, soil and water (Bradbury, 1986). It can be isolated from soil, water, plants, animals, the hospital environment, and human clinical specimens. It is commonly associated with spoilage of foodstuffs such as fish and meat (Gilardi, 1991). The presence of *P. fluorescens* in the rhizosphere of plants has been widely reported. For example, Milus and Rothrock (1993) found *P. fluorescens* to be a very good coloniser of wheat roots, and Lambert *et al*. (1990) found *P. fluorescens* to be one of the most frequently occurring bacteria on root surfaces in young sugar beet plants in Belgium and Spain.

P. marginalis (*P. fluorescens*) is ubiquitous in soil and is often an internal resident of plant tissues (Schroth *et al*., 1992). Cuppels and Kelman (1980) detected *P. marginalis* in a Wisconsin river and lake, field soils, root zones of potato plants, washwater from a potato chip processing plant, and decaying carrot and cabbage heads. Strains were found in Wisconsin soils just after the spring thaw, and thus probably overwintered there.

E. *P. fragi*

P. fragi has been found associated with refrigerated meat and dairy products (Jay, 1992).

F. *P. putida*

P. putida is very common in soils and plant rhizospheres (Palleroni, 1984). Gilardi (1991) indicated the species can be isolated from soil, water, plants, animal sources, the hospital environment, and human clinical specimens. It can be isolated from soil and water after enrichment in mineral media with various carbon sources.

P. putida appears to have a broad global distribution. Sisinthy *et al*. (1989) isolated the species from soil samples collected in, and around, a lake in Antarctica. However, particular strains may have a more restricted distribution. Chanway and Holl (1993) studied strains obtained from spruce seedling rhizospheres at two different locations in British Columbia, Canada, and found two distinct strains based upon analysis of fatty acids. When the origin of the spruce seed was matched with that of the inoculated *P. putida* strain, a significant increase in the amount and rate of seedling emergence was detected compared to unmatched tests of seedling emergence, suggesting ecotype specificity of strains.

G. *P. syringae*

P. syringae occurs naturally among the microflora that inhabit the leaf surface of plants that are typically found in temperate and Mediterranean climates (Wilson and Lindow, 1994; Bradbury, 1986). *P. syringae* survives in association with the host plant and propagative material from the host plant. There is little evidence to suggest that these bacteria survive in soil. They may, however, survive in soil in association with residues of diseased plants, having some capacity to colonise root systems (both host and non-host plant). Stone or pome fruit pathogens, such as *P. syringae,* exist in lesions, cankers or tumours. Inoculum is therefore available for dissemination under favourable environmental conditions. Most of the *P. syringae* group appears to have the capacity to survive as epiphytes on protected parts of healthy leaves, in the buds of the host, and even on non-host plants.

H. *P. tolaasii*

P. tolaasii is a natural inhabitant of peat and lime used for casing material in the production of commercial mushrooms, and can be easily isolated from compost after pasteurisation (Howard *et al*., 1994). In the commercial production of mushrooms, high relative humidity and surface wetness encourage

the expression of symptoms of brown blotch caused by *P. tolaasii* (Howard *et al*., 1994). Symptoms of brown blotch occur more frequently on mushrooms that remain wet for a long time, and in places where they touch one another (Howard *et al*., 1994). Brown blotch, the mushroom disease caused by *P. tolaasii*, has been reported on all continents except Africa (Bradbury, 1986; Suyama and Fujii, 1993).

2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites

Pseudomonads can have a significant involvement in a variety of environmental processes, including important biogeochemical cycles. For example, certain *Pseudomonas* species have the capacity for denitrification producing dinitrogen gas from nitrate. These species include *P. aeruginosa, P. fluorescens* (biotypes I and III) and *P. chlororaphis* (Palleroni, 1984).

Toxic metabolites of hazardous wastes

The microbial degradation of a hazardous waste may result in mineralisation of the parent waste, or in partial degradation of the parent waste to products which may be toxic. Some micro-organisms may not initially produce problematic metabolites. However, loss of a lower portion of a degradative pathway due to genetic instability may result in the generation of toxic metabolites. These toxic metabolites may result in death of the cell, thus limiting the metabolite's production. The metabolite may also be released from the cell to soil or water and become rapidly inactivated or mineralised by other physical or biological processes. On the other hand, some metabolites may be released from the cell, remain stable in the environment, and have toxic effects equivalent to, or greater than, the parent hazardous waste. There is also a possibility that a micro-organism will not produce a metabolite of concern when presented with a single waste, but will produce toxic metabolites in the presence of a complex mixture of related compounds.

Many examples of hazardous waste metabolites have been detected in laboratory experiments, but no well-documented field studies on metabolite formation have been conducted. In many instances, the metabolites produced by one organism will be degraded further or mineralised by others in the immediate environment. For example, TCE epoxide and phosgene are likely degradation products from methanotrophic degradation of trichloroethylene and chloroform, respectively (Alvarez-Cohen and McCarty, 1991). Although these compounds are toxic in mammalian systems, both are also highly reactive and would likely react intracellularly and/or not persist in the environment once released from the cells. Examples of the potential for hazardous metabolites include the production and accumulation of formamide from cyanide as a result of cyanide degradation by *P. fluorescens* strain NCIMB 11764 (Kunz, *et al*., 1992). As well, Castro and Belser (1990) demonstrated that *P. putida* PpG-786 can dehalogenate 1,1,2-trichloroethane by two pathways under aerobic conditions. The dominant pathway is oxidative and leads to chloroacetic acid and glyoxylic acid. However, a competitive reductive pathway occurs simultaneously and yields vinyl chloride exclusively.

Complex mixtures can result in dead-end metabolite production, or failure to degrade one of the parent compounds, even though the individual wastes can be mineralised individually. Benzene, toluene, and *p*-xylene (BTX) are common contaminants of drinking water, and each individual BTX compound can be mineralised by naturally occurring organisms. However, a combination of the three cannot be mineralised naturally, and can result in accumulation of 3,6-dimethylcatechol from *p*-xylene and a lack of degradation of benzene (Lee *et al*., 1995).

3. Pathogenicity – Host Range, Infectivity, Toxigenicity, Virulence, Vectors

A. General considerations

The fluorescent rRNA group I pseudomonads exhibit a range of pathogenicity characteristics. Some species have not been implicated in animal or plant disease. Other species may be opportunistic pathogens for weakened individuals. The fluorescent rRNA group I pseudomonads also include plant pathogens.

B. P. aeruginosa

Pathogenicity to animals

P. aeruginosa may be found as part of the normal bacterial flora of the intestines, mouth or skin of animals (*e.g.* cattle, dogs, horses, pigs). It has a broad host range among animals, which may also extend to plants. Under normal circumstances, colonisation is harmless and infection only occurs when local or general defence mechanisms are reduced. *P. aeruginosa* is usually associated with disease in individuals with low resistance to infection.

In susceptible hosts *P. aeruginosa* may cause infection at any site, particularly wounds and the respiratory tract. It can cause endocarditis, meningitis, pneumonia, otitis, vaginitis and conjunctivitis. Host defence mechanisms against *P. aeruginosa* are very low in mink and chinchilla, in which the bacterium can spread rapidly, causing fatal disease (Bergen, 1981).

P. aeruginosa has been associated with disease in pigs, sheep and horses (Hungerford, 1990), as well as cattle (Hamdy *et al*., 1974). Sheep inoculated epicutaneously with *P. aeruginosa,* and then wetted, can rapidly develop a bacterial exudative dermatitis (Hungerford, 1990). *P. aeruginosa* has been reported as the etiological agent in outbreaks of acute infectious disease in mink (Wang, 1987) and was the suspected etiological agent in a report of fatal bronchopneumonia and dermatitis in an Atlantic bottle-nosed dolphin (Diamond and Cadwell, 1979). It has also been reported to be associated with pathogenicity in ducks (Safwat *et al*., 1986), turkeys (Hafez *et al*., 1987), Japanese ptarmigan (Sato *et al*., 1986), and pheasant chicks (Honich, 1972) and to be the causal agent of a disease in broiler fowl in several countries.

P. aeruginosa was reported as one of the causative agents of infectious stomatitis or "mouthrot" in snakes (Draper *et al*., 1981), although it has been suggested that it is an opportunistic invader rather than an exogenous pathogen in snakes (Draper *et al*., 1981; Jacobson *et al*., 1981). Frogs (*Rana pipiens*) that were intraperitoneally injected with high doses (10^4-10^6 bacteria) of *P. aeruginosa* showed significant mortality under stressful conditions (Brodkin *et al*., 1992). *P. aeruginosa* has been associated with pathogenicity in Nile fish (Youssef *et al*., 1990) and catfish (*Clarias batrachus*) (Manohar *et al*., 1976) and as the etiological agent of fin rot in *Rhamdia sapo* (Angelini and Seigneur, 1988).

P. aeruginosa has been associated with pathogenicity in the tobacco hornworm (*Manduca sexta*) (Horohov and Dunn, 1984) and seven species of Lepidoptera, including the silkworms *Pericallia ricini* and *Bombyx mori* (Som *et al*., 1980). Experimental inoculation of honeybees, by dipping in a bacterial suspension of *P. aeruginosa,* resulted in a 70% death rate within 50 hours (Papadopoulou-Karabela *et al*., 1992). Dorn (1976) reported *P. aeruginosa* to be responsible for disease outbreaks in laboratory populations of the milkweed bug *Oncopeltus fasciatus*.

The abundant extracellular products of *P. aeruginosa* are thought to contribute to its adverse effects. These products include toxin A, alkaline protease, alkaline phosphatase, lipase, phospholipases and elastase. Toxin A is toxic to animals, with a mean lethal dose in mice of about 0.2 µg when injected intraperitoneally or 0.06 µg when injected intravenously (Nicas and Iglewski, 1986). Toxin A is produced

by about 90% of clinical isolates, and a chromosomal location has been established for the structural gene (Nicas and Iglewski, 1986). Most strains produce several extracellular proteases. For mice injected intravenously, the LD_{50} of the alkaline protease and the elastase is 375 and 300 µg respectively (Nicas and Iglewski, 1986). *P. aeruginosa* proteases are reported to be toxic to insects (*Galleria mellonella)* (Lysenko, 1974). *P. aeruginosa* also produces the haemolytic extracellular product phospholipase C, which causes hepatic necrosis and pulmonary edema when injected interperitoneally, and rhamnolipid, which has an LD_{50} of 5 mg when injected interperitoneally into mice (Nicas and Iglewski, 1986).

Pathogenicity to plants

P. aeruginosa has been described as an opportunistic invader of plants (Bradbury, 1986). It has been reported to cause blight disease in bean plants (El Said *et al*., 1982), and to have caused a lethal palm blight (Bradbury, 1986). Slow soft rot has been produced in plant tissue upon inoculation with strains of *P. aeruginosa* isolated from both animals and plants, and lesions and some necrosis have been found in tobacco leaves when inoculated with the bacterium (Bradbury, 1986). In a study involving 46 strains of *P. aeruginosa* isolated from human, plant and soil sources, the ratio of pathogenic to non-pathogenic strains for vegetables was 5:1 (Lebeda *et al*., 1984).

More recently, two strains of *P. aeruginosa* (a clinical isolate and a plant isolate) have been found to elicit severe soft rot symptoms in the leaves of inoculated *Arabidopsis thaliana* plants from certain ecotypes but not others (Rahme *et al*., 1995). These authors suggested that a strain that exhibited ecotype specificity would most likely be a true plant pathogen, in contrast to a strain that has no capacity to be a plant pathogen under natural settings but infects plants as a consequence of the artificial environment of a laboratory. The same two strains of *P. aeruginosa* were found to cause significant mortality in a mouse burn model. The authors identified genes encoding three virulence factors (*toxA, plcS* and *gacA*) that were required for the full expression of pathogenicity in both plants and animals.

C. *P. chlororaphis*

Pathogenicity to animals

A strain of *P. chlororaphis* has been reported to cause disease in salmon fry (*Oncorhynchus rhodurus*) and to kill trout, carp and eel, when inoculated (Egusa, 1992). This strain was judged to be pathogenic to fish (Hatai *et al*., 1975). *P. chlororaphis* has also been reported to inhibit egg hatch of the nematode, *Criconemella xenoplax*, at a concentration of $2x10^8$ cfu/ml (Westcott and Kluepfel, 1993). Shahata *et al*. (1988) reported that *P. chlororaphis* infected chickens.

Pathogenicity to plants

P. chlororaphis has been reported as the causal agent for a disease in straw mushrooms (*Volvariella volvacea*) in Puerto Rico, characterised by basal soft rot, internal water-soaking and discoloration (Hepperly and Ramos-Davila, 1986).

D. P. fluorescens

Pathogenicity to animals

P. fluorescens can infect a wide range of animals including horses (Sarasola *et al*., 1992), chickens (Lin *et al*., 1993), marine turtles (Glazebrook and Campbell, 1990), and many fish and invertebrate species. However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

P. fluorescens is considered a secondary invader of damaged fish tissues, but may also be a primary pathogen (Roberts and Horne, 1978; Stoskopf, 1993). The species causes bacterial tail rot and can affect freshwater and saltwater fishes throughout the world (Stoskopf, 1993). *P. fluorescens* releases extracellular proteases upon invasion of the fish host (Li and Flemming, 1968), and morbidity can be quite high. Outbreaks of bacterial tail rot have been reported in goldfish, silver carp, bighead, tench, grass carp, black carp, golden shiner, rainbow trout, European eels, paradise fish, and other labyrinth fishes. Egusa (1992) reviewed *Pseudomonas* diseases in fish and indicated that, in the United States, the disease termed *Pseudomonas* septicemia, due to infection by bacteria related to *P. fluorescens* (AFS-FHS, 1975), is seen in comparatively large numbers in warm-water fish.

Adverse effects associated with *P. fluorescens* in fish species often appear to be linked to stress from transportation or cultivation of fish. For example, *P. fluorescens* has been associated with disease in the cultivation of rainbow trout, *Oncorhynchus mykiss* (Barros *et al*., 1986), Atlantic salmon, *Salmo salar* (Carson and Schmidtke, 1993), chinook salmon, *Oncorhynchus tshawytscha* (Newbound *et al*., 1993), sea bream, *Evynnis japonica* (Kusuda *et al*., 1974), bighead carp, *Aristichthys nobilis*, and silver carp, *Hypophthalmichthys molitrix* (Petrinec *et al*., 1985), catfish and carp (Gatti and Nigelli, 1984), tench (Ahne *et al*., 1982), and tilapia species (Okaeme, 1989; Miyashita, 1984; Miyazaki *et al*., 1984).

Barker *et al*. (1991) found that exposure of high numbers of *P. fluorescens* to egg surfaces of rainbow trout (*Oncorhynchus mykiss*) during the initial stages of incubation poses a threat to egg survival. Conversely, *P. fluorescens* was not pathogenic when injected into brown trout (Smith and Davey, 1993) or silver mullet fish (*Mugil curema*) (Alvarez and Conroy, 1987).

P. fluorescens has also been implicated in pathogenicity to some invertebrates. James and Lighthart (1992) determined an LC_{50} for the 1st, 2nd, 3rd and 4th instar larvae of a coleopteran insect (*Hippodamia convergens*) (4.8×10^9, 2.8×10^{10}, 3.9×10^9, and 3.2×10^{11} CFU/ml, respectively) and concluded that *P. fluorescens* is a weak bacterial pathogen. *P. fluorescens* has also been reported to be associated with pathogenicity in the mosquitoes *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Murty *et al*., 1994) and in the field slug *Deroeras reticulatum* (Wilson *et al*., 1994). However, Genthner *et al*. (1993) studied the effects of *P. fluorescens* on eastern oysters (*Crassostrea virginica*) and found no signs of infectivity or pathogenicity.

Pathogenicity to plants

P. fluorescens is generally considered to be a saprophyte rather than a plant pathogen (Bradbury, 1986), although Ormrod and Jarvis (1994) considered it to be an opportunistic pathogen causing soft rot in plants. *P. fluorescens* biovar 2 (*P. marginalis*), however, is actively pectinolytic, causing soft rot of various plants, and is considered a plant pathogen (Tsuchiya *et al*., 1980; Hildebrand, 1989; Membre and Burlot, 1994; Brock *et al*., 1994). Bradbury (1986) recognised three pathovars in *P. marginalis* which cause soft rot in a wide range of vegetables and other plants.

A number of studies have reported adverse effects associated with *P. fluorescens* and plants (Gaudet *et al*., 1980; Anson, 1982; Hwang *et al*., 1989; Richardson, 1993; Ozaktan and Bora, 1994). Tranel *et al*. (1993) found that *P. fluorescens* strain D7 inhibited root growth of downy brome (*Bromus tectorum*) by production of a phytotoxin. Sellwood *et al*. (1981) confirmed pathogenicity experimentally for an atypical *P. fluorescens* biotype I on chicory plants and suggested that the group *P. fluorescens* does not solely comprise saprophytes. However, other studies have found no adverse effects on plants from inoculations with *P. fluorescens* (Arsenijevic, 1986; Arsenijevic and Balaz, 1986; Surico and Scala, 1992). At present, the epidemiology of pathogenic strains of *P. fluorescens* is not well understood (Hildebrand, 1994).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. fluorescens* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated that the *pel* gene encoding production of pectate lysase (an enzyme which contributes ability to cause soft rot in plants) is well conserved in fluorescent pseudomonads, and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Liao further indicated that saprophytic strains of *P. fluorescens* can be induced to become pathogenic and raised a concern about the safety of using the species as a biocontrol agent.

E. *P. fragi*

Pathogenicity to animals

No reports were found of *P. fragi* as an animal pathogen.

Pathogenicity to plants

No reports were found of *P. fragi* as an plant pathogen.

F. *P. putida*

Pathogenicity to animals

P. putida can infect a variety of animals including goats (Hungerford, 1990), koala (Ladds *et al*., 1990), turkey (Ononiwu, 1980) and fish (Kusuda and Toyoshima, 1976). However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

Kusuda and Toyoshima (1976) reported *P. putida* to be a pathogen to cultivated yellowtail fish. However, there have been no reports on the epizootiology, symptoms, or histological or pathological findings, and the disease has not been well-defined (Egusa, 1992). Austin and McIntosh (1991) considered *P. putida* to be one of a variety of gram-negative bacteria pathogens of potential concern to farmed and wild fish. *P. putida* has also been associated with pathogenicity in the snail, *Biomphalaria glabrata* (Cheng, 1986), the crayfish (Boemare and Vey, 1977), and the olive fly (Haniotakis and Avtizis, 1977).

Pathogenicity to plants

P. putida was included in the *Guide to Plant Pathogenic Bacteria* solely because its multiplication in the rhizosphere of paddy rice plants has been implicated in "suffocation disease", which arises under conditions of poor drainage (Bradbury, 1986). Studies have reported that *P. putida* is not pathogenic to mushrooms (Ozakatan and Bora, 1994) or crucifer plants (Shaw and Kado, 1988).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. putida* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated the *pel* gene encoding production of pectate lysase (an enzyme which contributes ability to cause soft rots in plants) is well conserved in fluorescent pseudomonads and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Homologous sequences were found in strains of *P. putida,* and Liao raised a concern about the safety of using *P. putida* as a biocontrol agent.

G. *P. syringae*

Pathogenicity to animals

No reports were found of *P. syringae* as an animal pathogen.

Pathogenicity to plants

P. syringae is principally an assemblage of foliar pathogens, although it occurs as both pathogenic and epiphytic (non-pathogenic) strains. The species has a broad range of potential plant hosts (Table 4.8). Pathogenic strains can exhibit both pathogenic (*i.e.* disease-causing) and epiphytic behaviours on susceptible hosts (Crosse, 1959). The initiation of infection results when a threshold level of bacteria is reached on the leaf surface; in the case of *P. syringae* pv. *syringae* this is reported to be 10^4 cfu gm^{-1} tissue (Hirano and Upper, 1983).

The association between rain and the onset of foliar blights caused by *P. syringae* is well recognised. Rain appears to stimulate the differential growth of pathogenic *P. syringae* isolates from the heterogeneous populations (pathogenic and non-pathogenic strains). Rain-triggered growth of *P. syringae* results in the establishment of large pathogenic populations required for disease development (Hirano and Upper, 1992).

There appears to be a distinctive set of symptoms associated with each causal agent. *Pseudomonas syringae* pv. *savasatoni* incites tumourous outgrowths on stems and leaves of oleander and olive under natural conditions. These symptoms have been found to be associated with the production of the auxin, indole acetic acid (IAA), in tissues infected with the bacterium. Furthermore, chlorosis, a common symptom when plants are infected by a number of pathogens belonging to the *P. syringae* group, is indicative of production of a toxin. For example, halo blight of beans caused by *P. syringae* pv. *phaselicola* is mediated by the toxin, phaseolotoxin. Other phytopathogenic pseudomonads producing toxins are illustrated in Table 4.9.

The *Dictionary of Natural Products* (Chapman and Hall, 1995) lists the following toxins produced by various strains of *P. syringae*: 1H-Indole-3-carboxaldehyde, octicidin (phytotoxin), phaseolotoxin (phytotoxin), N-Phosphosulfamylornithine (phytotoxin), syringomycin (phytotoxin), syringostatin A (phytotoxin), syringostatin B (phytotoxin), syringotoxin B (phytotoxin), tagetitoxin (phytotoxin), coronafacic acid (induces chlorosis in plants), halotoxin (phytotoxin), tabtoxin (phytotoxin). Coronatine (phytotoxin) is also produced by certain strains of *P. syringae* (Cuppels and Ainsworth, 1995). Gross (1985) determined that syringomycin production was stimulated by iron and suppressed by inorganic phosphate, that production occurred between 15 and 27°C, and that a slow growth rate of *P. syringae* favours toxin production.

Table 4.8 Range of plant species susceptible to infection with *P. syringue*

Plant (genus, species)	*P. syringae* pathovar
Acer spp.	aceris
Aesculus indica	aesculi
Antirrhinum majus	antirrhini
Apium graveolens	apii
Beta spp., *Heleanthus annuus*, *Tropaeolum majus*	aptata
Avena sativa, *Hordeum vulgare*, *Triticum aestivum*	atrofaciens
Agropyron spp., *Agrostis* spp., *Bromus* spp., *Dlymus* spp., *Festuca* spp., *Lolium* spp., *Phleum pratense*, *Stipa* spp.	atropurpurea
Corylus avellana	avellanae
Berberis spp.	bereridis
Cannabis sativa	cannabina
Ceratonia siliqua	ciccaronei
Avena spp., *Arrhenatherum elatius*, *Calamogrostis montanensis*, *Deschampsia caespitosa*, *Koeleria cristata*, *Phelum partense*, *Triticum* X *Secale*, *Trisetum spicatum*, *Zea mays*	coronafaciens
Delphinium spp.	delphinii
Dysoxylum spectabile	dysoxyli
Eriobotrya japonica	eriobotryae
Ficus palmata	fici
Coffea arabica	garcae
Glycine max	glycinea
Helianthus spp.	helianthi
Hordeum vulgare, Triticum aestivum	japonica
Citrullum lanatus, Cucumis spp., *Cucurbita* spp.	lachrymans
Zea mays, Sorghum bicolor	lapsa
Brassica spp., *Raphus sativus*	maculicola

Plant (genus, species)	*P. syringae* pathovar
Nicotiana tabacum	mellea
Morus spp.	mori
Prunus spp.	morsprunorum
Myrica rubra	myricae
Oryza sativa	oryzae
Panicum miliaceum	panici
Malus pumila, Pyrus communis	papulans
Passiflora edulis	passiflorae
Prunus persica	persicae
Phaseolus spp., *Pisum sativum, Pueraria lobata*	phaseolicola
Philadelphus coronarium	philadelphi
Photinia glabra	photiniae
Lathrus spp., *Pisum* spp., *Vicia* spp.	pisi
Allium porrum	porri
Primula spp.	primulae
Protea cynaroides	proteae
Ribes aureum	ribicola
Forsythia intermedia, Fraxinus spp., Ligustrum spp., *Nerium oleander*, *Olea* spp., *Nicotiana tabacum*	savastanoi
Sesamum indicum	sesami
Avena sativa, *Triticum* X *Secale*	striafaciens
many hosts	syringae
Glycine max, *Nicotiana tabacum*	tabaci
Ambrosia artemisiifolia, *Helianthus* spp., *Tagetes* spp.	tagetis
Camellia sinensis	theae
Capsicum anum, *Lycopersicon esculentum*	tomato
Ulmus spp.	ulmi
Viburnum spp.	viburni
Pseudostuga menziesii	

Source : adapted from Bradbury (1986) and Chanway and Holl (1992)

Table 4.9 Some toxins produced by phytopathogenic *Pseudomonas* spp.

Pseudomonad	Toxin(s)	Mechanism or site of action	Host plant(s)
P. syringae			
pv. *atropurpurea*	coronatine		Italian rye grass
pv. *coronafaciens*	tabtoxin-β-lactam	glutamine synthetase	oat
pv. *garcae*	tabtoxin-β-lactam	glutamine synthetase	coffee
pv. *glycinea*	coronatine/ polysaccharide		soybean
pv. *lachrymans*	extracellular polysaccharides		cucumber
pv. *maculicola*	coronatine		crucifers
pv. *morsprunorum*	coronatine		sour cherry
pv. *phaseolicola*	phaseolotoxin	ornithine transcarbamoylase	bean, kudzu
pv. *savasatoni*	IAA & cytokinins	plant growth regulators	olive, oleander
pv. *syringae*	syringomycins syringopeptins syringotoxins	plasma membrane	peach, maize
pv. *tabaci*	tabtoxin-β-lactam	glutamine synthase	tobacco
pv. *tagetis*	tagetitoxin	chloroplastic RNA polymerase	marigold
pv. *tomato*	coronatine		tomato
P. tolaasii	tolaasin	plasma membrane	mushroom

Source : taken from Durbin (1996)

H. *P. tolaasii*

Pathogenicity to animals

No reports were found of *P. tolaasii* as an animal pathogen.

Pathogenicity to plants

P. tolaasii causes Brown blotch (bacterial blotch), the most common bacterial disease of the commercial button mushroom, *Agaricus bisporus* (Howard *et al*., 1994). This disease can result in serious economic losses. *P. tolaasii* has also been found to cause disease in the oyster mushroom, *Pleurotus ostreatus,* and the shiitake mushroom, *Lentinus edodes* (Suyama and Fujii, 1993).

P. tolaasii produces a haemolytic lipodepsipeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agariculs bisporus* (Rainey *et al*., 1993). Tolaasin is phytotoxic when infiltrated into tobacco leaves (Rainey *et al*., 1991). Synthesis of tolaasin is chromosomally determined, and it is known that at least five genetic loci are required for tolaasin synthesis (Rainey *et al*., 1993).

4. Interactions with and Effects on Other Organisms in the Environment

A. *P. aeruginosa*

Certain *P. aeruginosa* strains are antagonistic to plant pathogens such as damping-off fungi (Bradbury, 1986; Buysens *et al*., 1994). Duffy and Defago (1995) found that clinical and plant isolates of *P. aeruginosa* suppressed root diseases of cucumber, maize and wheat caused by soilborne fungi *Gaeumannomyces graminis* var. *tritici*, *Phomopsis sclerotiodes*, *Pythium ultimum* and *Rhizoctonia solani*. A soil isolate of *P. aeruginosa* suppressed foliar disease on wheat caused by *Septoria tritici* (Flaishman *et al*., 1990).

P. aeruginosa can have a synergistic effect on the survival of salmonellae, enabling them to survive more than 140 days in double-distilled water (Warburton *et al*., 1994). It has also been suggested that *P. aeruginosa* may act synergistically with pectolytic bacteria that colonise vegetables, such as *P. marginalis* (*P. fluorescens*) and *Erwinia cartovora* (Bradbury, 1986). A protective immunity against *P. aeruginosa* infection has been reported in mice vaccinated with heat-killed *Lactobacillus casei* (Miake *et al*., 1985).

P. aeruginosa is known to produce 1-phenazinecarboxamide (the amide of 1-phenazinecarboxylic acid), which is active against some phytopathogenic fungi and *Candida albicans.* A related compound, 1-phenazinol, which is active against gram-positive bacteria and fungi, and which shows some viral activity, is also produced by *P. aeruginosa*. 1-phenazinol has an LD_{50} of 500 mg/kg in mice dosed intraperitoneally. Pyoluteorin and its 3'-nitro derivative are produced by *P. aeruginosa*. Both compounds have antibacterial, antifungal and herbicidal properties. The LD_{50} of the pyoluteorin to mice is 125 mg/kg (Chapman and Hall, 1995). The antibiotic, 2-heptyl-4-hydroxyquinoline N-oxide, is a metabolite of *P. aeruginosa* and is a potent 5'- lipoxygenase inhibitor, with an LD_{50} of 40 mg/kg in mice dosed intraperitoneally (Chapman and Hall, 1995).

B. *P. chlororaphis*

P. chlororaphis has been widely investigated for its ability to enhance plant growth through suppression of deleterious root-colonising bacteria. Compounds known as siderophores are produced by *P. chlororaphis*. These compounds chelate iron, thereby depriving certain root-colonising plant pathogens of iron necessary for their growth (Smirnov *et al*., 1991).

Many studies have indicated that *P. chlororaphis* has the ability to suppress plant disease. For example, *P. aureofaciens* has been investigated as a biocontrol agent to suppress take-all, the wheat root fungal disease. The ability of *P. aureofaciens* to inhibit *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all, was demonstrated *in vitro* and *in vivo* (Harrison *et al*., 1993). It is believed that disease suppression is largely due to the production of phenazine antibiotics (Thomashow and Pierson, 1991). Carruthers *et al*. (1995) tested the ability of *P. aureofaciens* to suppress root rot of *Asparagus officinalis* caused by *Phytophthora megasperma* var. *sojae*. *P. aureofaciens* significantly reduced the level of infection and disease severity. Other tests suggested that *P. aureofaciens* had a direct growth stimulatory effect on asparagus, independent of antibiotic production (Carruthers *et al*., 1995). Berg and Ballin (1994) found *P. chlororaphis* inhibited the growth of the phytopathogenic fungus *Verticillium dahliae.*

When Douglas fir seed was inoculated with *P. aureofaciens,* and grown in pasteurised soil, shoot biomass increased significantly when compared with non-inoculated controls (Chanway and Holl, 1992). *P. aureofaciens* has been found to inhibit mycelial growth of *Rhizoctonia solani* in dual culture between 15 and 30°C (Lee *et al*., 1990). Inoculation of rice seeds was found to control rice sheath blight in the early growth stages, and seedling blight caused by *R. solani, Fusarium moniliforme* and *Pythium ultimum* was suppressed by seed treatment and soil incorporation of *P. aureofaciens* (Lee *et al*., 1990). In another

experiment, the emergence of sweet corn seedlings from soil infested with *Pythium ultimum* was greatly enhanced by coating the seed with *P. aureofaciens* (Mathre *et al*., 1994). *P. aureofaciens* has also been evaluated for its ability to suppress *Pythium ultimum* damping off of cucumber seedlings (Sugimoto *et al*., 1990).

P. aureofaciens was antagonistic to *Clavibacter michiganensis* subsp. *sepedonicus*, the bacteria implicated in potato ring rot in greenhouse trials with potato seedlings (de la Cruz *et al*., 1992). *P. aureofaciens* significantly reduced populations of, and infection by, the ring rot bacteria (de la Cruz *et al*., 1992). Fukui *et al*. (1994) investigated the relationship between pericarp colonisation by *Pythium ultimum* in sugar beets and the growth of pseudomonads in the spermosphere. They found a positive correlation between the incidence of pericarp colonisation by *Pythium ultimum* and the length of the lag phase of the strain used to inoculate the seeds. England *et al*. (1993) investigated the nodulation of whitebean (*Phaseolus vulgaris* L.) by *Rhizobium phaseoli* in the presence of *P. aureofaciens*. No significant difference was found in the numbers of nodules produced in the presence of *P. aureofaciens* as a result of the symbiotic relationship between *Rhizobium phaseoli* and whitebean roots in vermiculite.

P. chlororaphis was observed to interfere with the growth of shiitake mushrooms in field experiments with shiitake cultivated logs (Raaska and Mattila-Sandholm, 1991). Siderophores were produced, however the addition of iron to *in vitro* cultures did not entirely neutralize the growth inhibition of mycelia by *P. chlororaphis.* It was concluded that although iron-binding plays an important role, it is not the only factor involved in the inhibition of shiitake by *P. chlororaphis* (Raaska and Mattila-Sandholm, 1991). A siderophore extracted from *P. aureofaciens* was found to inhibit uptake of ferric iron by maize and pea, and the synthesis of chlorophyll in these plants was reduced (Becker *et al*., 1985).

P. aureofaciens is reported to produce an antibiotic-like compound in iron-rich conditions that inhibits the growth of the plant fungal pathogen *Aphanomyces euteiches* (Carruthers *et al*., 1994). Mazzola *et al*. (1992) suggested that the production of phenazine antibiotics contributes to the ecological competence of *P. aureofaciens,* and that reduced survival of strains unable to produce the antibiotics is due to diminished ability to compete with the resident microflora. Thomashow *et al*. (1990) found that suppression of take-all is related directly to the presence of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of wheat. In another experiment, 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine were also found to be responsible for take-all suppression in wheat (Pierson and Thomashow, 1992). Pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl)-1H-pyrrole] is an antifungal compound produced by *P. chlororaphis*; its LD50 in mice dosed orally is 1 g/kg (Chapman and Hall, 1995). The antifungal compound, 1,3,6-trihydroxy-2,4-diacetophenone, has also been isolated from culture media (Harrison *et al*., 1993).

C. *P. fluorescens*

P. fluorescens has been recognised as beneficial to plant growth (Weller and Cook, 1986; Kloepper *et al*., 1988). It can enhance plant growth through production of siderophores, which efficiently complex environmental iron, making it unavailable to other components of the soil microflora. Increased plant yields achieved through the inoculation of plant roots have been mimicked by the application of the siderophore, pseudobactin, isolated from *P. fluorescens*. Antibiotic production by *P. fluorescens* has been recognised as an important factor in its ability to suppress phytopathogens. *P. fluorescens* has also been found to significantly promote nodulation, growth and nitrogen accumulation in faba beans (*Vicia faba*) (Omar and Abd-Alla, 1994). Heat-killed cells had no effect.

Certain strains of *P. fluorescens* can promote the formation of ice crystals in water at temperatures near 0°C (Lindow and Panopoulos, 1988; Lindow, 1992). Large populations of these ice^+ bacteria on plant surfaces can cause frost injury. Only 0.01 to 40% of the total bacteria on plant surfaces are sufficient to cause frost injury. In the absence of these bacteria, water on plants can cool to -40°C.

Smith and Davey (1993) found that *P. fluorescens* strains were able to inhibit *Aeromonas salmonicida* that was isolated from Atlantic salmon with furunculosis. Pre-smolts asymptomatically infected with *A. salmonicida* and bathed in a solution containing *P. fluorescens* strains were less likely to develop stress-induced furunculosis than non-treated fish. It was concluded that *P. fluorescens* inhibits *A. salmonicida* by competing for free iron, and that it protects against stress-induced furunculosis by inhibiting *A. salmonicida* on external locations. Kimura *et al*. (1990) found that a strain of *P. fluorescens* biovar I (46NW-04) isolated from the aquatic environment produced an antiviral substance that was effective against fish viruses.

D. *P. fragi*

Monitoring of microbial flora succession on minced lamb meat revealed that *P. fragi* was the dominant climax species (Drosinos and Board, 1995). Another study indicated that *P. fragi* dominated the flora on lamb carcasses at both 7 and 30°C (Prieto *et al*., 1992).

E. *P. putida*

P. putida is very common in soils and plant rhizospheres, where it seems to have a stimulating effect on plant growth (Palleroni, 1984). *P. putida* has been shown to suppress a variety of plant pathogens and to reduce the incidence of plant disease (Liao, 1989; Gamliel and Katan, 1993; Duijff *et al*., 1994; Freitas *et al*., 1991; Defago and Hass, 1990). This may be due in part to its inhibition of plant pathogenic micro-organisms by sequestering iron or producing metabolites with antibiotic properties. Formation of a siderophore complex by the plant may also be involved (Defago and Hass, 1990). Siderophore-mediated competition for iron was indicated as the mechanism of suppression of Fusarium wilt of carnation by *P. putida* (WCS358r) (Duijff *et al*., 1994) and suppression of phytopathogens to winter wheat (Freitas *et al*., 1991).

Al-Achi *et al*. (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

F. *P. syringae*

Some strains of *P. syringae* have the ability to cause ice nuclei to form at temperatures just below 0°C, thus inducing freezing injury to susceptible plants and allowing disease development to occur (Lindow, 1983). Nutritional starvation for nitrogen, phorphorous, sulphur or iron at 32°C, followed by a shift to 14-18°C, led to the rapid induction (from non-detectable to 100% in 2 to 3 h) of type I ice nuclei (Nemecek-Marshall *et al*., 1993).

Replacement series experiments on bean leaves between P. syringae and epiphytic P. fluorescens, Pantoea agglomerans (Erwinia herbicola), Stenotrophomonas maltophilia (Xanthomonas maltophilia) and Methylobacterium organophilum have demonstrated that the epiphytes were all capable of higher levels of coexistence with P. syringae than was observed with another P. syringae strain. The level of coexistence with the epiphytes was inversely correlated with the ecological similarity of the strains and with a differential preference for amino acids, organic acids and carbohydrates (Wilson and Lindow, 1994).

The invasion and exclusion abilities of 29 strains of *P. syringae* were studied on leaves in 107 pairwise combinations in which each strain was inoculated on day 0, and the second (challenge) was

inoculated on the same leaf on day 3 (Kinkel and Lindow, 1993). The presence of an established population often significantly reduced the growth of the second strain when quantified on day 6; successful invaders (challenge) were significantly less likely to exclude challenge populations than were non-successful invaders. Hirano and Upper (1993) determined that an introduced antibiotic-resistant strain of *P. syringae* spread but did not persist when applied to bean plants grown in the field; it was concluded that the introduced strain was less fit than the pool of indigenous species. Competition between indigenous soil bacteria and single cells of *P. syringae* pv. *syringae* engineered with bioluminescence genes from *Vibrio harveyi* can be monitored using charge-coupled enhanced microscopy (Silcock *et al*., 1992).

Defreitas *et al*. (1993) determined that *P. syringae* R25 inoculated on field peas (*Pisum sativum*) did not affect plant growth in plastic growth pouches but, in soil, did inhibit nitrogenase activity of nodules formed by indigenous rhizobia; *P. syringae* R25 inhibited the growth of field beans (*Phaseolous vulgaris*) in both plastic growth pouches and in soil. When peas were inoculated with both *P. syringae* R25 and *Rhizobium leguminosarum,* there was an increase in plant biomass in growth pouches but no effect was observed in soil; when beans were inoculated with both *P. syringae* R25 and *Rhizobium phaseoli*, there were severe deleterious effects on seedling emergence, plant biomass and nodulation in both growth pouches and soil.

Table 4.10 Phytopathogenic strains of *P. syringae* containing plasmids

Pathovar	Reference
P. syringae pv. *angulata*	Piwowarski and Shaw, 1982
P. syringae pv. *atrpurpurea*	Sato *et al*., 1983
P. syringae pv. *coronafaciens*	Piwowarski and Shaw, 1982
P. syringae pv. *glycinea*	Curiale and Mills, 1983
P. syringae pv. *lachrymans*	Coplin, 1989
P. syringae pv. *papulans*	Burr *et al.,* 1988
P. syringae pv. *phaseolicola*	Quant and Mills, 1984
P. syringae pv. *savastanoi*	Comai *et al.,* 1982
P. syringae pv. *striafaciens*	Beck-Von Bodmann and Shaw, 1987
P. syringae pv. *syringae*	Gonzales *et al.,* 1984
P. syringae pv. *tabaci*	Obukowicz and Shaw, 1983; 1985
P. syringae pv. *tomato*	Denny, 1988; Bender and Cooksey, 1986

G. *P. tolaasii*

P. tolaasii produces a haemolytic lipodepsipeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agariculs bisporus* (Rainey *et al*., 1993). Tolaasin is also active against a range of basidiomycetes and gram-positive bacteria (Rainey *et al*., 1991).

The nematode *Caenorhabditis elegans* is reported to decrease the spread of *P. tolaasii* in mushroom growth chambers (Grewal, 1991). *P. fluorescens* biovar *reactans* was frequently isolated from the gut of *C. elegans* along with mushroom sporophores. All the isolates of *P. fluorescens* biovar *reactans* isolated from nematodes were antagonists to *P. tolaasii*. It was suggested that, as *C. elegans* selects *P. fluorescens* biovar *reactans* rather than *P. tolaasii* as a food, it probably spreads the antagonist in the mushroom crop and may contribute to the control of mushroom blotch (Grewal, 1991). *P. fluorescens* has also been described by other researchers as antagonistic to *P. tolaasii* (Khanna and Olivier, 1989; Munjal *et al*., 1989; Nair and Fahy, 1972). Nair and Fahy (1972) reported *Enterobacter aerogenes* to be antagonistic to *P. tolaasii*.

Thorn and Tsuneda (1992) report that 23 species of wood-decay basidiomycetes attacked or lysed *P. tolaasii* when tested. Attack took the form of increased hyphal branching within the bacterial colonies, often preceded by directional growth toward them.

5. Ability to form Survival Structures (e.g. Spores, Sclerotia)

Pseudomonads are asporogenous, that is, they do not form spores or other survival structures. Pseudomonads are, however, pleomorphic and represent a tremendously diverse group of strains able to tolerate extreme environmental conditions, including the extremes of temperature.

Bacteria that do not form survival structures like spores and cysts are suspected to have other survival strategies. A number of researchers have reported the existence of dwarf or ultramicrobacteria in nutrient-stressed environments (Rosak and Colwell, 1987). These cells have been described from seawater (Amy and Morita, 1983) and soil (Casida, 1977). Cells are able to develop to their full size, once exposed to an abundant supply of nutrients.

6. Routes of Dissemination, Physical or Biological

A. Physical

Pseudomonads may be disseminated by air or water currents. For example, Trevors *et al*. (1990) used soil-core microcosms to study the movement of a *P. fluorescens* isolate through soil planted with wheat and unplanted. In the absence of ground water flow, limited movement was detectable along the soil column planted with wheat, while no movement was detected in the unplanted soil. In contrast, movement of the strain through the column was dependent on the flow rate of the water and the number of times the columns were flushed through. Water flow also affected the distribution of the inoculant along the wheat roots. Bacterial cell size has been related to the movement of cells through a soil column, with smaller bacterial cells (< 1.0μm) moving fastest through the column (Gannon *et al*., 1991).

Rain may also be an important source of inoculum and means of dispersal for pseudomonads. Rain splash has been attributed to move pseudomonads colonising leaf surfaces down the plant canopy and into the soil (Hirano and Upper, 1992; Butterworth and McCartney, 1992; McCartney and Butterworth, 1992). Large drops of artificial rain were more effective in dispersing bacteria than smaller drops (Butterworth and McCartney, 1992). Humidity correlated positively with the consequent survival of pseudomonads dispersed by rain splash (McCartney and Butterworth, 1992). However, dispersal is short range (one or a few metres) (Constantidou *et al.,* 1990). Pseudomonads (including *P. syringae* Ice^+ strains) have been found to leave plant surfaces in an aerosol-stable state and enter the troposphere during dry, warm weather (Lindemann *et al.,* 1982; Lindemann and Upper, 1985). They are then transported and washed downwards during rainfall (Constantidou *et al.,* 1990).

B. Biological

Pseudomonads are motile bacteria characterised by the presence of at least one flagellum. While there is no convincing evidence that the bacteria are flagellated in soil (Stotzky *et al.,* 1991), flagella appear to confer increased epiphytic fitness on *P. syringae* strains in association with moisture on leaf surfaces (Haefele and Lindow, 1987). The potential for certain fluorescent pseudomonads to colonise plant surfaces has been attributed to the presence of pili (Vesper, 1987; de Groot *et al.,* 1994), surface charge properties (James *et al.,* 1985), the production of agglutinin, a glycoprotein complex, released from root surfaces (Anderson, 1983), and the ability of certain saprophytic pseudomonads to adhere to the agglutinin of specific plant species (Glandorf *et al.,* 1993; 1994).

Earthworms moving through soil have been implicated in the dissemination of bacteria over short distances. As well, Johnson *et al*. (1993) have demonstrated the ability of honey bees to disseminate a biological control strain of *P. fluorescens* used against the fireblight pathogen, *Erwinia amylovora,* in apple and pear blossoms. Honey bees carrying approximately 10^4 to 10^6 cfu per bee effectively inoculated fruit tree blossoms with bacteria.

7. Containment and Decontamination

Containment plans have been proposed for microbial releases, although few of them have been used, and their efficacy is yet to be demonstrated. It is likely to be difficult to eliminate all the bacteria from a site of introduction. Many of the proposed chemical treatments have gross rather than localised effects; hence their application may have considerable impact on the natural flora, fauna and microflora at the site. Pseudomonads will colonise many laboratory and hospital disinfectants, and may exhibit broad spectrum resistance to a number of widely used antibiotics. Disinfectants based on quaternary ammonium compounds and chlorhexidine solutions have been found to be contaminated with pseudomonads. Disinfectant contaminants include *P. aeruginosa, P. fluorescens,* and *P. cepacia* (Bergen, 1981).

P. putida strains that degrade alkylbenzoates have been modified to carry a fusion of the P (lac) promoter to the gef gene, which encoded a killing protein (Molin *et al*., 1993; Ramos *et al*., 1994). Expression from P (lac) was controlled through a regulatory cascade, so that P (lac) was switched on or off by the absence or presence of alkylbenzoates respectively. Similar uncontained strains were also constructed and tested as a control. Contained and uncontained strains were genetically stable, and their survival and functionality in soil microcosms were as expected. Both contained and uncontained strains survived well in soils supplemented with alkylaromatics, whereas survival of the contained strain in soil microcosms without methylbenzoates was markedly reduced in contrast to the control strain, which survived in these soils in the absence of alkylbenzoates (Jensen *et al*., 1993; Ronchel *et al*., 1995).

8. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity and Reliability

A. Techniques mployed in the laboratory and/ or environment for detecting the presence of, and for monitoring, numbers of the organism

Information on detection and monitoring techniques is provided in this information element as well as in information element 2 and Table 4.11. Each of the well-described detection methods has limitations as well as advantages for enumeration and/or detection (Drahos, 1992). For example, under certain conditions an approach which provides reasonable sensitivity by culturing a micro-organism (*e.g.* the viable plate count) may give reliable data for culturable populations. Furthermore, many approaches are complementary; methods utilising nutritional, antibiotic and enzymatic markers rely on the ability of the target organism to express the marker genes during the selection or reculturing process. However, expression of these traits may not always be optimal, for instance under conditions of severe environmental stress. In these situations, a direct method of detection could be used.

Selective plating

Selective plating has been used widely in combination with selectable phenotypes based on antibiotic resistances (often spontaneous mutations) (Compeau *et al.,* 1988; Fredrickson *et al.,* 1989; Thompson *et al.,* 1990) or introduced genes such as *xylE* (Winstanley *et al.,* 1989; Morgan *et al.,* 1989) l*acZY* (Cook *et al.,* 1991; Drahos *et al.,* 1988), *lux* (Shaw and Kado, 1986) and *mer* gene (Iwasaki *et al*., 1993; 1994). A number of these genes have been used for marking and tracking pseudomonads. It is important to ensure

that the marker is not found in the indigenous microflora of the environment to which the micro-organism will be introduced.

Pseudomonads appear to be highly culturable on laboratory media and may be isolated from environmental samples using viable plating (Drahos, 1992). Generally, 1 g of environmental sample is homogenised or shaken in 9 ml of an appropriate dilutent such as ¼ strength Ringer's solution or physiological saline. The homogenate is serially diluted 1 in 10, 100 µl aliquots spread onto selective agar, and the plates incubated at 28°C. A number of selective media are available commercially, such as *Pseudomonas* selective agar (Oxoid) and *Pseudomonas* agar F (Difco). Both media have a low iron content, promoting the production of the iron-chelating, fluorescent siderophores. Selective agars are supplemented with antibiotics. A commercially prepared cocktail of cephalorodine, fucidin acid and cetrimide (Oxoid) is available which may be supplemented with ampicillin and the antifungal agent, cyclohexamide. Micro-organisms may be detected at or above a detection limit of 10^2 (*i.e.* one cell may be detected when a minimum of 100 are present per g of sample) (Trevors and van Elsas, 1989). Sensitivity may be increased by plating larger volumes or by using smaller dilutions, *i.e.* 1 in 2 instead of 1 in 10.

Most probable number

Most probable number (MPN) methods (Alexander, 1982) have been used to attain greater sensitivity. A serial dilution of the sample is made in an appropriate dilutent to an extinction point (Atlas, 1982). Three to ten replicates of each dilution are made and the pattern of positive and negative scores recorded (*i.e.* growth or no growth). Statistical tables are used to determine the MPN of micro-organisms present in the sample. MPNs like the viable plate count require growth and reproduction of the strains, and may be less accurate since an MPN is established with confidence limits (Jain *et al*., 1988).

Table 4.11 Examples of identification and detection techniques

Method	Reference	Sensitivity/reliability
DNA extraction followed by Polymerase Chain Reaction (PCR)	Stefan and Atlas, 1988	100 *P. cepacia* cells $100g^{-1}$ sediment, against a background of 10 non-target organisms
	Pillai *et al.,* 1991	1 to 10 *E.coli* (with *Tn*5 insert) colony forming unit (cfu)g^{-1} soil
	Tsai and Olson, 1992	3 cells *E.coli* g^{-1} soil; primers directed at 16S rRNA
	Tushima *et al.*, 1995	10 cells g^{-1} water
hybridisation using radio-labelled probes	Holben *et al.,* 1988; Stefan and Atlas, 1988	10^3 to 10^4 cells g^{-1} soil
	Jain *et al.,* 1988; Blackburn *et al.,* 1987	10^2 cells g^{-1} soil (similar to viable plate count)
direct microscopy using immuno-fluorescence	Schmidt, 1974; Bohool and Schmidt, 1980; Ford and Olson, 1988	10^6 to 10^7 cells g^{-1} soil
enzyme-linked immunosorbent assays (ELISA)	Morgan *et al.,* 1991; Scholter *et al.,* 1992	10^3 cells g^{-1} soil; $10\text{-}10^2$ cells g^{-1} soil
selective viable plating	Trevors and van Elsas, 1989; Iwasaki *et al*., 1993,1994	10^2 cfu g^{-1} soil; 1 cfu ml^{-1} water; 10 cfu g^{-1} soil
most probable number (MPN) viable counts	Alexander, 1982; De Leij *et al*., 1993	$< 10^2$ cfu g^{-1} soil; $<10^1$ cfu g^{-1} soil

Simple chemotaxonomical approach

A simple chemotaxonomical approach which avoids isolation and cultivation of micro-organisms has been used. For example, quinone profiles (Hiraishi *et al*., 1991) or polyamine patterns (Auling *et al*., 1991) have been used as biomarkers for a survey of pseudomonads (and acinetobacters) in activated sludge from sewage treatment facilities.

Immunological methods

SDS-PAGE coupled with immunological probes have been applied to identify fluorescent pseudomonads of environmental origin (Sorenson *et al*., 1992). Other possibilities for detecting pseudomonads in environmental samples include the application of phylogenetic probes applied in in situ hybridisations (DeLong *et al*., 1989), or strain or species-specific monoclonal antibodies labelled with fluorescent dyes (Bohlool and Schmidt, 1980; Conway de Macario *et al*., 1982). Blair and McDowell (1995) describe an ELISA method for detecting extracellular proteinase of P. fragi.

Microscopic examination and direct enumeration of micro-organisms *in situ* can also be used, although this type of approach is not sensitive. To detect one bacterium at a magnification of 1000, the cell density must be 10^6 to 10^7 per g soil. The approach does, however, provide information about the spatial distribution of a strain colonising an environmental substrate, and can be used to enumerate non-culturable micro-organisms.

Ramos-Gonzalez *et al*. (1992) produced highly specific monoclonal antibodies against surface lipopolysaccharides (LPS) of *P. putida* 2440 and developed a semi-quantitative dot blot immunoassay for bacteria in liquid media. This allowed the authors to detect, in complex samples, as few as 100 cells per spot by using peroxidase-conjugated antibody against the antibody that recognised *P. putida* 2440. An intrinsic limitation of this technique is the turbidity of the samples, which may limit maximum assay volume. This assay is also of limited use for bacteria introduced into soils or sediments because of intrinsic fluorescent backgrounds. *P. putida* 2440 (pWWO) released in lake mesocosms have been successfully tracked with monoclonal antibodies (Brettar *et al*., 1994; Ramos-Gonzalez *et al*., 1992).

Nucleic acid probes and primers

Nucleic acid probes and/or PCR primers may be used for the detection of gene sequences in the environment. A number of sequence hybridisation techniques including Southern, slot-blot, dot-blot, and colony hybridisation have been used for environmental isolates. These approaches would be particularly applicable to strains with traits that are not widely distributed throughout the environment under study, and against which specific probes and primers may be designed. The sensitivity of the hybridisation approach is variable and for the most part strain-specific. Generally, radioactively labelled probes provide for more sensitivity than non-radioactive probes. Sensitivity can be enhanced using PCR. However, the increased efficiency of the amplified signal obtained by the PCR assay is countered by the inefficient extraction of nucleic acids from environmental samples (Bramwell *et al*., 1994). For example, soils contain positively charged cations which are sandwiched between layers of clay, and which are able to bind negatively charged nucleic acids, making their retrieval difficult. Caution is required in using PCR as a method for the enumeration of bacteria, as the extreme sensitivity of this procedure renders quantification by target dilution difficult (Drahos, 1992). Thiem *et al*. (1994) and Zhou and Tiedje (1995) point out the complexity of using molecular techniques for monitoring pseudomonads used for subsurface bioremediation.

Denaturing gradient gel electrophoresis (DGGE) of DNA is a suitable method for those species which are difficult to culture on growth media. This method has been used by Muyzer *et al*. (1993). Whole DNA is isolated. Using two primers, one with a GC-rich end, a fragment of 16s rDNA is amplified by PCR. This results in a mixture of DNA fragments, equal in size but different in sequence, corresponding to the various organisms in the sample. The mixture is fractionated by DGGE, resulting in one band for each organism type. The bands are sequenced, and based on the sequences, the rRNA-group can be determined.

Polymerase chain reaction (PCR) based sequence amplification

A technique that is finding increasing application for specific identification of micro-organisms is the technique referred to as REP-PCR (based on PCR amplification between repetitive sequences commonly found in bacteria). This technique relies on development of adequate databases, but is used with increasing frequency (De Bruijn, 1992). Other approaches are to follow the expressed phenotype attributed to the introduction of a marker gene (*e.g.* bioluminescent genes) (Prosser, 1994), and to use competitive PCR based on introduction of an internal standard during the PCR amplification (Leser, 1995).

Arbitrary PCR primers

Identification can be facilitated based on the analysis of DNA produced from total DNA, using PCR and arbitrary primers (Welsh and McClelland, 1990; Williams *et al*., 1990).

Specific PCR primers

P. aeruginosa can be identified using PCR amplification of the 16S-23S rDNA internal transcribed spacer region (Tyler *et al*., 1995).

B. Specificity, sensitivity, reliability

The specificity of identification/monitoring methodologies will generally require some sort of experimental study to demonstrate that the method distinguishes the introduced inoculant from indigenous relatives. An approximate estimate of sensitivity for a number of methods is given in Table 8. However, sensitivity of detection is a function of the organism and of the habitat.

A problem with applying any method of detection is its dependence on extraction efficiencies. Problems are exemplified in soil. Traditionally, bacteria have been recovered from soils through the mechanical shaking of the soil in an appropriate dilutent. The ease of extracting cells or nucleic acids varies between soil types, with extraction efficiencies being higher in sand as opposed to clay-based soils. Strong chemical and physical interactions may occur between micro-organisms and the particulate matter of soil. These associations may be ionic, since bacteria are negatively charged and clay soil minerals contain positively charged cations. Dispersion of soil aggregates has been considered important, as entrapment of micro-organisms in soil aggregates is considered to be one of the most significant means by which micro-organisms are retained in soil (Hopkins *et al.,* 1991). Attempts to disrupt these soil-microbe associations to extract bacteria have utilised homogenisation, chemical dispersants, cation exchange resins, and differential centrifugation (Faegri *et al.,* 1977; Bakken, 1985; MacDonald, 1986; Herron and Wellington, 1990; Hopkins *et al.,* 1991).

Soil is a highly heterogeneous substrate with a non-uniform spatial distribution of bacterial colonies (Wellington *et al.,* 1990). Sampling strategies should consider the variability of the soil matrix under study; errors attributable to the difficulties of sampling heterogeneous substrates may be compensated for by taking composite samples (Atlas and Bartha, 1981).

Micro-organism themselves will also affect the efficiency of extraction of biological molecules such as DNA. For example, bacteria, even those quite closely related, vary in the conditions required for lysis. Hence methodologies aiming to extract the total DNA from soil will selectively recover DNA from isolates that lyse easily, making representative sampling of environmental substrates difficult.

Similar selective pressures apply to viable plating methodologies, since these methods favour the growth of bacteria that readily grow on agar plates under laboratory conditions. Furthermore, all media are selective to some extent, so that certain bacterial species will appear in different proportions, if at all, on different bacteriological agars. Sorheim *et al*. (1989) compared the populations recovered from soil on three different non-selective media. Bacterial populations exhibiting the same level of diversity were isolated on all media. Each of the media appeared to select for a different population of isolates, with 30% of the population appearing common to all three media. 20% of the isolates recovered from two of the media were distinct to that particular media, and 60% of isolates on the third media were unique to it.

The sensitivity of the viable plate count has been estimated to be 10^2 cfu/g soil (Trevors and van Elsas, 1989). However, this may be improved by combining methods to extract and concentrate the biomass from environmental material prior to plating. Detection limits as low as 10 streptomycete spores per 100 g sterile soil have been demonstrated (Herron and Wellington, 1990).

Pseudomonads are highly culturable on rich media. Their importance may therefore have been overestimated as a result of over-representation on isolation plates (Miller *et al.,* 1990b; Sorheim *et al.,* 1989). Nutritionally limiting isolation media and lower incubation temperatures with longer incubations may allow a greater diversity of bacterial isolates to be recovered from environmental substrates (Miller *et al.,* 1990b).

Ottawa' 92: The OECD Workshop on Methods for Monitoring Organisms in the Environment (OECD, 1994a) includes a review of the monitoring of micro-organisms (including *P. aureofaciens*) in the phyllosphere (Bailey *et al*., 1994) and a review of the different methods available. A companion document, *Compendium of Methods for Monitoring Organisms in the Environment* (OECD, 1994b), contains 39 methods for detecting or monitoring micro-organisms, including the following species of *Pseudomonas*: *P. aureofaciens*, *P. fluorescens*, *P. putida*, *P. aeruginosa* and *P. syringae.*

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SECTION 3
ACIDITHIOBACILLUS

I. General Introduction

This document presents information that is accepted in the literature about the known characteristics of bacteria in the genus *Acidithiobacillus*. Regulatory officials may find the technical information useful in evaluating properties of micro-organisms that have been derived for various environmental applications. Consequently, this document provides a wide range of information without prescribing when the information would or would not be relevant to a specific risk assessment. The document represents a snapshot of current information (end-2002) that may be potentially relevant to such assessments.

In considering information that should be presented on this taxonomic grouping, the Task Group on Micro-organisms has discussed the list of topics presented in the "Blue Book" (*i.e.* Recombinant DNA Safety Considerations (OECD, 1986)) and attempted to pare down that list to eliminate duplications as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered to be more easily understood (the Task Group met in Vienna, 15-16 June, 2000). This document is a first draft of a proposed Consensus Document for environmental applications involving organisms from the genus *Acidithiobacillus.*

II. General Considerations

1. Subject of Document: Species Included and Taxonomic Considerations

The four species of *Acidithiobacillus* covered in this document were formerly placed in the genus *Thiobacillus* Beijerinck. In recent years several members of *Thiobacillus* were transferred to other genera while the remainder became part of three newly created genera, *Acidithiobacillus*, *Halothiobacillus*, *Thermithiobacillus,* and to the revised genus *Thiobacillus sensu stricto* (Kelly and Harrison, 1989; Kelly and Wood, 2000). The reassignment to these three newly designated genera was based on physiological characteristics and 16S rRNA gene sequence comparisons (Kelly and Wood, 2000). *Acidithiobacillus* contains two species (*A. ferrooxidans* and *A. thiooxidans*) of the original genus *Thiobacillus* that have the potential to cause significant ecological damage. Two other species have been reassigned to this new genus, *A. caldus* and *A. albertensis*. All these species have been, or are likely to be, employed in various biotechnological applications in the environment.

2. Characteristics of the Organism: Identification and the Methods Used to Identify the Organism

A. Characterisation of the genus Acidithiobacillus

The genus was established by Kelly and Wood (2000), with *A. thiooxidans* (formerly *Thiobacillus thiooxidans)* as the type species. The four species included in the genus are Gram-negative, rod-shaped (0.4 x 2.0 μm), motile with one or two flagella, and possess the ability to use reduced sulphur compounds as electron donor for autotrophic growth, in common with various other unrelated "sulphur bacteria" (Kelly and Harrison, 1989; Kuenen *et al*., 1992). As with other *Thiobacillus* species now redistributed, members of this genus are distinguished morphologically from other colourless sulphur bacteria by

forming external rather than internal sulphur particles (Kuenen, 1989). They are strictly aerobic and obligately acidophilic (optimum pH < 4.0). Some species oxidise ferrous iron and hydrogen (Table 4.12) or use natural and synthetic metal sulphides to generate energy, while one species (*A. ferrooxidans*) can oxidise iron. The optimum temperature ranges from 30-35 ^{O}C for mesophilic species to 45 ^{O}C for moderately thermophilic species. All of the species contain ubiquinone Q-8, and the G+C content of the DNA is 52-64 mol %. *Thiobacillus sensu stricto* now contains only species belonging to the β-subclass of the Proteobacteria, but *Acidithiobacillus*, together with *Halothiobacillus* and *Thermithiobacillus*, have been assigned to the γ-subclass (Kelly and Wood, 2000). A full account of the genus is given in the section contributed by Kelly and Wood (2005) in the 2nd edition of Bergey's Manual of Systematic Bacteriology.

B. Differentiation of Acidithiobacillus from related taxa

Members of the genus are distinguished by their obligate acidophilic nature (pH < 4.0) and possession of ubiquinone Q-8.

C. Characters used in classification

Phenotypic characters

Many of the phenotypic characters of *Acidithiobacillus* such as the rod-like shape, motility, Gram-negative reaction and utilisation of sulphur compounds are shared in common with species formerly placed in *Thiobacillus.* These characters are useful for broad recognition but no longer for critical identification.

Mol% G+C content

The determination of the mol % G+C content of the DNA of bacterial isolates has been used for a long time to determine whether strains could be related to each other. It is to some extent, a negative test. While widely differing G+C values can suggest that two isolates are not related, matching G+C values do not guarantee that they are the same. G+C values for the four species of *Acidithiobacillus* are, however, often sufficiently far apart to serve as useful species characteristics.

Ubiquinones and cellular fatty acid analysis

Lane *et al*. (1985) determined that there was a correlation between ubiquinone type and physiological behaviour. Katayama-Fujimura *et al*. (1982) used types of ubiquinones and the DNA base composition to differentiate 11 species of the former genus *Thiobacillus*. The association between species and ubiquinone type was constant except for *T. perometabolis*, one strain having 8 and the other 10-isoprene units. Species presently assigned to *Acidithiobacillus* all possessed eight units. The strain of *A. ferrooxidans* examined by the later authors was unique in that it had ubiquinones with 9 as well as 8 isoprene units.

Nucleotide structure

5S rRNA sequences were obtained for thirteen species of the original genus *Thiobacillus* (Lane *et al*., 1985, 1992) and these sequences were shown to be distinct for each species. Similarities within the sequences also enabled the species also to be assigned to the α, β or γ groups of the four groups of Proteobacteria, the last group including species of *Acidithiobacillus.*

DNA homologies

DNA hybridisation studies on *A. ferrooxidans* and *A. thiooxidans,* together with *Thiobacillus thioparus* and five bacteria formerly placed in *Thiobacillus* (Huber and Stetter, 1989, 1990) established the value of these tests because they showed that there was usually a high degree of homology (>70%) between strains of the same species.

Table 4.12 *Acidithiobacillus* : Characters used in classification

Species	Optimum pH[a]	pH range	Optimum temperature	Temperature range	Ubiquinone	Mol % G+C	Subclass of Proteobacteria	References
Strictly chemolithotrophic and autotrophic								
Acidithiobacillus albertensis	3.5-4.0	2.0-4.5	28-30	ND	Q-8	61-62	ND	b, c
Acidithiobacillus ferrooxidans	2.0-2.5	1.3-4.5	30-35	2-37	Q-8	58-59	γ	b, d, e
Acidithiobacillus thiooxidans	2.0-3.0	0.5-5.5	28-30	10-37	Q-8	52	γ	b, f
Facultatively chemolithotrophic or mixotrophic with tetrathionate								
Acidithiobacillus caldus[1]	2-2.5	1-3.5	45	32-52	Q-8	63-64	γ	g

ND: not determined

[1]Moderately thermophilic

[a]Katayama-Fujimura *et al*. (1982); [b]Kelly and Harrison (1989)); [c]Bryant *et al*. (1983); [d]Leduc and Ferroni (1994); [e]McCready (1988); [f]Fliermans and Brock (1972); [g]Hallberg and Lindstrom (1994).

D. Comments on the species

Acidithiobacillus albertensis

Syn. *Thiobacillus albertensis* (Bryant *et al*., 1983; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished morphologically by a tuft of polar flagella and a glycocalyx extending outwards from the outer membrane of the bacterial cell envelope and which is used to attach itself to elemental sulphur (Bryant *et al*., 1983). These features together with the relatively high G+C content of the DNA, differentiate this species from the other three (Kelly and Wood, 2000). It has been tentatively assigned to *Acidithiobacillus* because a 16S rRNA sequence for the species is not yet available (Kelly and Wood, 2005).

Acidithiobacillus caldus

Syn. *Thiobacillus caldus* (Hallberg and Lindstrom, 1994; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished by extremely short rod-shaped cells, each with a single polar flagellum (Hallberg and Lindstrom, 1994), and by its moderately thermophilic nature. It cannot oxidise sulphidic ores, but it may be found associated with others involved in leaching. It is facultatively rather than obligately chemolithotrophic. A specific, fast and sensitive non-radioactive immuno-binding assay had been used for the detection and enumeration of this species (Amaro *et al*., 1994). Chemiluminescence or peroxidase-conjugated immunoglobulins are employed in a dot or slot blotting system. This method is very convenient for monitoring bioleaching micro-organisms in effluents from industrial bioleaching processes.

Acidithiobacillus ferrooxidans

Syn. *Thiobacillus ferrooxidans* (Temple and Colmer, 1951; Kelly and Harrison, 1989; Kelly and Wood, 2000). Morphologically this species appears to be distinguished by a single coiled flagellum in mature cells (Gonzalez and Cotoras, 1987). Also, this is the only species in the genus so far to be able to utilise iron as well as sulphur.

Serological and electrophoretic methods have been employed for the rapid detection of isolates of *A. ferrooxidans* and the differentiation of strains (Jerez *et al*., 1986). Different serotypes, characterised by specific lipopolysaccharide banding patterns in polyacrylamide gels, have been described (Koppe and Harms, 1994). A specific and very sensitive dot-immuno-binding assay for the detection and enumeration of *A. ferrooxidans* has been developed by Arredondo and Jerez (1989). Samples were spotted onto nitrocellulose membranes and first incubated with polyclonal antisera, derived from a rabbit inoculated with whole cells of *A. ferrooxidans*, and in ^{125}I-labeled protein A or ^{125}I-labelled goat anti-rabbit immunoglobulin G. The membranes were then dried, autoradiographed on Fuji Rx X-ray film and scanned at 550 nm. The antisera reacted with every strain of *A. ferrooxidans* tested but not with *A. thiooxidans* and *H. neapolitanus* and three species formerly placed in *Thiobacillus*.

A specific, fast and very sensitive immuno-electron microscopy method was also developed to identify *A. ferrooxidans* present with other iron oxidising bacteria in acidic mine waters (Coto *et al*., 1992). Polyclonal antisera, produced against whole cells of *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* gave highly specific reactions when cross-reacted with 23 strains of acidophilic bacteria using an immuno-fluorescence staining technique (Koppe and Harms, 1994). These methods have been criticised, however, because of the inability of the antisera to distinguish between dead and living cells (Khalid *et al*., 1993).

A systematic study of a large collection of strains ascribed to *A. ferrooxidans* revealed considerable diversity among them (Harrison, 1982). The members of seven DNA homology groups recognised by

Harrison (1982) were largely homologous with strains inside each group but to a lesser degree with strains in other groups. Although all strains grew between 25 and 30 ^{O}C, some were able to grow at 5 ^{O}C and others at 40 ^{O}C, and two genomic groups could be distinguished by different temperature optima. The strains in two other genomic groups (1 and 7) were apparently unable to use elemental sulphur, and their high mol% G+C values (53 and 65 respectively) were well outside the range normally accepted (see Table 4.12), giving rise to the suspicion that they might represent different species. Although strain m-1, comprising the seventh genomic group, was later shown to be able to oxidise elemental sulphur on prolonged incubation (Johnson, 1995b), it has been further distinguished from a more typical strain of *A. ferrooxidans* by its classification in the 5S rRNA sequence group III rather than in group II (Lane *et al*., 1985).

Acidithiobacillus thiooxidans

Syn. *Thiobacillus concretivorus* Parker *Thiobacillus thiooxidans* (Waksman and Joffe, 1922; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is motile by means of a single polar flagellum, and as with *A. caldus*, it cannot oxidise iron or pyrite, although it can grow on sulphur from pyrite in conjunction with *Leptospirillum ferrooxidans* (Kelly and Wood, 2000). In a study by Harrison (1982), four strains of *A. thiooxidans* were found to be largely homologous with a fifth strain but not with representatives of the seven homology groups of *A. ferrooxidans*. However, a sixth strain ascribed to *A. thiooxidans* showed no similarity to any of them. This indicates that there may be atypical representatives of *A. thiooxidans* as well as *A. ferrooxidans*.

3. Information on the Organisms' Reproductive Cycle (Sexual/ Asexual)

Thiobacillus sensu lato, in which *Acidithiobacillus* was included, was found to reproduce by binary cell division (Sokolova and Karavaiko, 1968). No spores were observed.

4. Biological Features and Environmental Conditions Which Affect Survival, Reproduction, Growth, Multiplication or Dissemination

A. Growth requirements

Most strains are able to produce colonies on appropriate media solidified with agar or agarose. The use of elemental sulphur is avoided because of its insolubility. Formation of hydrogen sulphide is potentially toxic in moderate concentrations, and the most widely used sulphur compound is thiosulphate (Smith and Strohl, 1991).

Some strains, especially those of *A. thiooxidans*, grow poorly on agar media, possibly due to the toxicity of agar hydrolysis products (Kelly and Harrison, 1989). The problem is generally solved by the use of a minimal concentration of agar, screening for suitable brands of purified agars, use of agarose, and, in the case of *A. thiooxidans*, a combination of low agar concentration with pH 2.2-2.5 and ferrous sulfate at only about 20 mM (Johnson, 1995a). The growth of strains of *A. ferrooxidans* on solid media is also difficult. However, the double-layered plates such as FeTSBo and described by Johnson (1995a) allow also the growth of most strains.

Species of *Acidithiobacillus*, in common with other former species of *Thiobacillus*, are able to use carbon dioxide as the sole source of carbon for synthesis of cell material (Kuenen, 1975). Ribulose diphosphate carboxylase, the enzyme responsible for the fixation of carbon dioxide, appears to be located in polyhedral inclusions in the cell.

In contrast to species of *Halothiobacillus* which have been recorded as either halotolerant or having a strict NaCl requirement (Sievert *et al*., 2000), *A. ferrooxidans* has been recorded as unable to grow at salt

concentrations above 1% (Lazaroff, 1963; McCready, 1987; Razzell and Trussell, 1963) whereas specific information for other species of *Acidithiobacillus* for their tolerance to salt is not available.

Acidithiobacillus ferrooxidans has a remarkable physiology that allows it to thrive in an inorganic mining environment. Its minimum growth requirements can be satisfied by water, air, an oxidisable iron or sulphur source and trace minerals. The trace elements required are usually present as impurities in the water or ore (Rawlings and Woods, 1995). This statement is also true for *A. thiooxidans*, however elucidation of these abilities for other members of *Acidithiobacillus* is still not available. *A. caldus* was found to be relatively insensitive to a number of xanthate and dithiocarbamate-based flotation reagents but sensitive to a number of mercapto-benzthiazole-based reagents (Okibe and Johnson, 2002).

B. Oxidation of hydrogen

In contrast to strains of *A. thiooxidans*, *H. neapolitanus*, *T. prosperus*, *Leptospirillum ferrooxidans* and four species formerly placed in *Thiobacillus*, three strains of *A. ferrooxidans* were found to be facultative hydrogen oxidisers, being able to use molecular hydrogen as a sole source of energy (Drobner *et al*., 1990). The ability to oxidise hydrogen was repressed by ferrous iron or sulphur and occurred only in the presence of oxygen.

C. Nitrogen

In addition to their ability to fix carbon dioxide all strains of *A. ferrooxidans* examined so far are also able to fix atmospheric nitrogen (*i.e.* they are diazotrophic)(Rawlings and Kusano, 1994).

D. Aerobic/Anaerobic growth

Acidithiobacillus species are strict aerobes with the exception of *A. ferrooxidans*, which is a facultative aerobe. In the absence of oxygen, *A. ferrooxidans* is able to grow on reduced inorganic sulphur compounds using ferric iron as an alternative electron acceptor (Pronk *et al*., 1992; Sugio *et al*., 1985).

E. Resistance to metals

Resistance to metal ions is a function of those thiobacilli tested to date. *Acidithiobacillus ferrooxidans* is resistant to a variety of metal ions such as chromium (Baillet *et al*., 1998), copper, zinc, nickel, thorium and uranium (Leduc *et al*., 1997; Tuovinen *et al*., 1971) and mercury (Takeuchi *et al*., 1999, 2001; Sugio *et al*., 2001). According to Iwahori *et al*. (2000), the resistance of *A. ferrooxidans* to mercury is ferrous iron dependent.

F. Role of Rusticyanin

Rusticyanin is a blue copper protein present in the periplasmic space of *A. ferrooxidans*. Consisting of a single polypeptide chain with one copper atom as a cofactor (Hazra *et al*., 1992), rusticyanin reportedly serves as the initial electron acceptor upon oxidation of ferrous iron (Hazra *et al*., 1992; Hutchins *et al*., 1986). An acid-stable cytochrome c was found to catalyse the reduction of rusticyanin (Blake *et al*., 1988).

G. Survival

No information on factors influencing survival of *Acidithiobacillus* in the natural environment appears to be available. According to Kelly and Harrison (1989), *A. ferrooxidans* survives in culture on pyrite (FeS_2) for very long periods when stored at 5-15 ^{o}C. Many strains have been successfully freeze-dried or have survived storage in liquid nitrogen or in glycerol suspension at -20 ^{o}C (Kelly and Harrison, 1989).

Hubert *et al*. (1994) have shown that survival rates of *A. ferrooxidans* decreased rapidly under laboratory conditions above and below the individual temperature ranges of psychrotrophic and mesophilic strains.

H. Adhesion

The ability to adhere to surfaces seems to be a peculiar feature of *Acidithiobacillus*. Myerson and Kline (1983) observed the physical adsorption of cells of *A. ferrooxidans* to the surface of different non-porous solid particles (glass, pyrite, sulphur). Selective adherence to iron containing minerals appears to occur naturally (Ohmura *et al*., 1993), and the ferrous ion, but not the ferric ion, inhibited such selective adhesion. A model of the biofilm structure has been proposed by Karamanev (1991). Intimate contact and adhesion are required for enzymatic attack by *A. ferrooxidans* on insoluble substrates such as sulphur, pyrite (FeS_2), and chalcopyrite ($CuFeS_2$), and this is brought about by either a proteinaceous surface appendage (Devasia *et al*., 1993) or by extra-cellular polymeric substances (specifically lipopolysaccharides) combined with iron (III) (Gehrke *et al*. 1998, 2001; Blais *et al*., 1994). *Acidithiobacillus ferrooxidans* does not randomly adsorb onto pyrite or other surfaces, but congregates selectively at sites where dislocations, grain boundaries and other non-uniformities in the crystal structure emerge to the surface (Andrews, 1988; Bagdigian and Myerson, 1986; Gehrke *et al*., 1998). It is possible that diffusion of sulphur atoms along dislocations in the substrate is an important part of the mechanism of microbial decomposition. This pattern of diffusion provides a great advantage to bacteria because sulphur oxidation has a much higher yield of free energy than iron oxidation. No corresponding advantage would be gained by adsorption onto pure pyrite sites because the diffusion through pyrite crystals is several orders of magnitude lower.

Growth of bacteria adhering to the mineral surface initiates the oxidation process in arsenopyrite bioleaching (Fernandez *et al*., 1995). Corrosion patterns appear, with the liberation of ferrous ions and formation of elemental sulphur. With the increase in number of the bacteria, the ferrous ions are oxidised to ferric ions with the ultimate production of ferric arsenate (Fernandez *et al*., 1995).

Adhesion structures, consisting of a filamentous matrix, have been observed in *A. thiooxidans* (Blais *et al*., 1994), linking the cells to the surface of sulphur particles. They were not observed however in *T. thioparus*, where the cells fixed directly onto the sulphur (Sokolova and Karavaiko, 1968). The latter process is the usual type of fixation observed in the species placed formerly in *Thiobacillus.*

Adhesion can be estimated using the technique of Dziurla *et al*. (1998), who developed an immuno-filtration assay (ELIFA) for this purpose. ELIFA is a modified ELISA using micro-titer plates with 0.2-µm pore-size filters in place at the bottom. Particles, either previously inoculated with bacteria or to be reacted with added bacteria are incubated in the wells and then successively filtered and washed by applying a vacuum to the bottom of the plate. The inoculated particles are retained by the filter. Polyclonal antiserum raised against a strain of *A. ferrooxidans* is added and the plates incubated at 35 °C for one hour. The polyclonal rabbit antibody used was shown to react with different *A. ferrooxidans* and *A. thiooxidans* but not with other bacterial genera. Following washing the bound antibody is detected with goat-anti-rabbit globulin conjugated to alkaline phosphatase and ρ-nitrophenylphosphate was used as the detection substrate.

I. pH and nutrition preference

Species of *Acidithiobacillus* are acidophilic as well as obligate or facultative chemolithotrophs according to the nutritional table presented by Kuenen *et al*. (1992). Species of *Acidithiobacillus* are abundant in acid mine drainage water where they oxidise and gain energy from the oxidation of metals such as iron. The optimum pH for *A. ferrooxidans* is between 2-3, but when the substrate is in large part pyritic, the pH can reach extremely low values, (less than 1). This is due to the availability of abundant

sulphur and the precipitation of ferric hydroxide when the solution reaches saturation (Morin, 1995). *Acidithiobacillus caldus* is the single mixotrophic species which can utilise sulphur or tetrathionate and yeast extract or glucose (Hallberg and Lindstrom, 1994).

Blais *et al*. (1993) have demonstrated that less acidophilic bacteria in sludge such as *Thermithiobacillus tepidarius, T. aquaesulis*, *T. denitrificans*, *T. thioparus* and other species formerly placed in *Thiobacillus*, may initiate the acidification to the point where the acidophilic species can take over. Acidophilic bacteria decreased the pH of a sulphur-containing synthetic salts medium to the level of 1.4-1.6 in 10 days. Evangelou (1995) mentioned pH 3.5 as the upper limit below which Fe^{2+} is oxidised by *A. ferrooxidans*. For the less acidophilic bacterial species, the limit of acidification was more variable, between pH 2.2 and 6.9. This has important implications for the removal of heavy metals from sludge (presented later in this document).

J. Temperature relations

Acidithiobacillus contains one thermophilic species, *A. caldus*, but little is known about its actions. *Acidithiobacillus ferrooxidans* has been traditionally regarded as mesophilic. Recently, however, psychrotrophic strains have been isolated with a growth range on iron of 2-37 °C (Leduc and Ferroni, 1994). Some Canadian isolates have a greater cold tolerance than most strains with a temperature optimum of only 20 °C (McCready, 1988). The occurrence of broad temperature range for psychrotrophic isolates of *A. ferrooxidans* has been reported by Leduc *et al*. (1993) and by Berthelot *et al*. (1993). Temperature ranges of 2-35 °C and 4-21 °C, respectively, were observed. However, psychrophiles have not been isolated from cold tailing effluents where they would be expected (Berthelot *et al*., 1994). The temperature used for bioleaching in most studies is 35 °C. Although *A. ferrooxidans* was reported to grow most rapidly at 30 °C, it oxidised iron faster at 35 °C (Holmes, 1988). This has important implications for industrial bioleaching since the oxidation of sulfides is exothermic, and therefore_cooling may be necessary to maintain a satisfactory industrial process (Morin, 1995). Industrial applications are further described in section 8.

K. Metabolic pathways: involvement of Acidithiobacillus in bioleaching

Bioleaching is a biochemical oxidation process, catalysed by a living organism, whereby an insoluble mineral is oxidised to a soluble form and recovered in a pure form. A large number of acidophilic bacteria capable of attacking mineral sulphides have been isolated from industrial leaching operations or from sites of natural leaching. *Acidithiobacillus ferrooxidans* and *A. thiooxidans* are prominent among the species isolated from leaching sites, although the redox potential and concentration of ferric iron will influence which species dominates (Rawlings *et al*., 1999).

Acidithiobacillus ferrooxidans

This bacterium derives its energy from oxidation-reduction reactions using insoluble sulphidic minerals as growth substrates, including pyrite (FeS_2), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S) and sphalerite (ZnS). This, coupled with its resistance to high concentrations of normally toxic metal ions in solution, accounts for the ubiquity of this organism in leaching systems (Cripps, 1980). Combinations of *A. ferrooxidans* and either *A. thiooxidans* or *Acidiphilium acidophilum* and *Leptospirillum ferrooxidans* have been associated with degradation of pyrite and chalcopyrite. Metals can be released from sulphidic ores by direct or indirect leaching or by galvanic conversion (Hutchins *et al*., 1986).

Direct leaching

This involves oxidation of the substrate by the bacterium and may require physical attachment of the bacteria to particles of the mineral sulphide (see section 4.8). The process can be described in general by a simplified reaction:

$MS + 2O_2 \Rightarrow MSO_4$ where M represents a bivalent metal [1]
(Hutchins *et al*., 1986)

Several different metal sulphides can be acted on directly by cells of *A. thiooxidans* (Leduc and Ferroni, 1994). These include sulphides of copper, nickel, lead, iron, gallium, cobalt and zinc. Examples of direct leaching include the following:

$CuS + 2O_2 \Rightarrow CuSO_4$ (Woods and Rawlings, 1989) [2]
Covellite

$2FeAsS + 7O_2 + 2H_2O \Rightarrow 2FeAsO_4 + 2H_2SO_4$ (Brierley, 1995) [3]
Arsenopyrite

Indirect leaching

This occurs through the production of an oxidative reagent or lixiviant that causes solubilisation to occur. The use of iron pyrites by *A. thiooxidans* as an energy source is a good example of how both direct and indirect leaching processes work together. The overall reaction describing pyrite oxidation is usually written as:

$4FeS_2 + 15O_2 + 2H_2O \Rightarrow 4Fe_2(SO_4)_3 + 2H_2SO_4$ (Rawlings and Woods, 1995) [4]

However, the actual pathway for the oxidation of pyrite is not a simple one-step reaction, but a series of reactions, passing through a number of intermediates. Under natural conditions, the two main oxidising agents that act on the pyrite are oxygen and the ferric ion. The reaction can be catalyzed by *A. ferrooxidans*, which increases the rate of reaction by more than 10^6 (Singer and Stumm, 1970).

$2FeS_2 + 7O_2 + 2H_2O \Rightarrow 2FeSO_4 + 2H_2SO_4$ [5]
pyrite

$4FeSO_4 + O_2 + 2H_2SO_4 \Rightarrow 2Fe_2(SO4)_3 + 2H_2O$ [6]
(bacteria)

$Fe_2(SO_4)_3 + FeS_2 \Rightarrow 3FeSO_4 + 2\ S$ [7]

$2S + 3O_2 + 2H_2O \Rightarrow 2H_2SO_4$ [8]
(bacteria)

(Leduc and Ferroni, 1994; Monticello and Finnerty, 1985)

Reactions [5], [6] and [8] are part of the direct leaching mechanism, which may require the physical attachment of the bacteria to pyrite particles (section 4.8). Reaction [7] comprises the indirect mechanism that can take place independently of the bacteria. The role of the bacteria is to reoxidise the ferrous ions to ferric ions and sulphur to sulphuric acid (Monticello and Finnerty, 1985). A cyclical system develops in which ferrous ions released from pyrite are oxidised by the bacteria to ferric ion, which can then oxidise

pyrite again, generating more ferrous ions. The biomass specific oxygen consumption rate is dependent on the ratio of ferric to ferrous irons in the culture (Boon *et al*., 1999). These ferric ions are known as the lixiviant because they carry electrons from the mineral to the bacterium's cell membrane (Leduc and Ferroni, 1994). The electrons are subsequently transported via an electron-transport chain to molecular oxygen in reaction [4]. Since iron is nearly always available in natural leaching environments, both the direct and indirect leaching mechanisms probably operate simultaneously in nature (Leduc and Ferroni, 1994; Monticello and Finnerty, 1985).

In the indirect reaction, bacterial activity is limited to the oxidation of pyrite (FeS_2) and ferrous iron. *Acidithiobacillus ferrooxidans* does not directly interact with the metal in the minerals. The role of the bacterium is to continuously provide a powerful oxidation agent, ferric sulphate [$Fe_2(SO_4)_3$] which is capable of dissolving a wide variety of metal sulphide minerals (Torma, 1991).

The following are examples of indirect leaching of minerals other than pyrites (Hutchins *et al*., 1986)

$CuFeS_2 + 2\ Fe_2(SO_4)_3^- \Rightarrow CuSO_4 + 5FeSO_4 + 2S^0$ [9]
Chalcopyrite

$Cu_2S + 2Fe_2(SO_4)_3^- \Rightarrow 2CuSO_4 + FeSO_4 + S^0$ [10]
Chalcocite

$CuS + 2\ Fe_2(SO_4)_3^- \Rightarrow CuSO_4 + 2FeSO_4 + S^0$ [11]
Covellite

$Cu_5FeS_4 + 6Fe_2(SO_4)_3^- \Rightarrow 5CuSO_4 + 13FeSO_4 + 4S^0$ [12]
Bornite

Ultimately, the indirect leaching mechanism depends on biological regeneration of ferric sulphate. Elemental sulphur (S^0) generated by the reactions above can be converted to sulphuric acid by *A. ferrooxidans*:

$2S^0 + 3O_2 + 2H_2O \Rightarrow 2H_2SO_4$ [13]

The sulphuric acid maintains the pH at levels favourable to the bacteria and effectively leaches a variety of copper oxide minerals, giving copper sulphate as an end product (Hutchins *et al*., 1986).

Uranium leaching proceeds in a similar fashion, again without the bacterium metabolising the uranium ion:

$UO_2 + Fe_2(SO_4)_3 + H_2SO_4 \Rightarrow UO_2(SO_4)_3^{4-} + 2FeSO_4 + 4H^{4+}$ [14]

or:

$UO_3 + 3H_2SO_4 \Rightarrow UO_2(SO_4)_3^{4-} + H_2O + 4H^+$ [15]

Acidithiobacillus ferrooxidans can be used to extract uranium from sulfidic ore bodies because the bacterium uses the iron and the sulfur in the ores, as energy sources (See reaction [5]). The oxidation of reduced sulfur results in the production of sulfuric acid, whereas the oxidation of reduced iron produces the oxidant Fe^{3+}. Under such acidic conditions, the insoluble tetravalent uranium is oxidised by ferric ions to the soluble hexavalent state as follows (Berthelot *et al*., 1993):

$$UO_2 + 2Fe^{3+} \Rightarrow UO_2\ 2+ + 2Fe^{2+} \quad [16]$$

Thus, the soluble, oxidised uranium is released from the mineral into the leaching liquid and can be easily recovered. For other minerals where the sulphate formed from the mineral is insoluble, as is the case with lead, gold and silver, the metal may be concentrated by leaching away other soluble metal sulphates, leaving a concentrated metallic product (Hutchins *et al*., 1986). Gold ores are often recalcitrant. The gold may be encased in a pyrite/arsenopyrite matrix that has to be decomposed before the gold is accessible to cyanide extraction (Rawlings and Woods, 1995).

Galvanic conversion

This is a lesser-known process in bioleaching where physical contact between two dissimilar metal sulphide phases immersed in an electrolyte such as dilute sulphuric acid or ferric sulphate solution creates a galvanic cell. In a mixture of pyrite and chalcopyrite, the former acts as a cathode while the chalcopyrite behaves as an anode and undergoes rapid dissolution. *Acidithiobacillus ferrooxidans* may accelerate the reaction by continuously oxidising the film of elemental sulphur that would obstruct the diffusion of copper and iron salts.

Acidithiobacillus thiooxidans

This species differs from *A. ferrooxidans* in its inability to oxidise iron (Fe^{2+}). Sulphur appears to provide the main source of energy for this bacterium (Sokolova and Karavaiko, 1968), and its ability to oxidise elemental sulphur allows it to take part in the indirect leaching of some minerals, particularly sulphides of cobalt, nickel and zinc (Hutchins *et al*., 1986; Norris, 1990). The sulphuric acid generated by *A. thiooxidans* through oxidation of sulphur (see reaction [8]), results in acid solubilisation of the metal. In mixed culture with iron-oxidising bacteria, *A. thiooxidans* oxidises the protective sulphur covering on the surface of minerals such as chalcopyrite. This allows bacteria such as *Leptospirillum ferrooxidans*, which cannot remove the sulphur, to attack the iron component beneath.

Acidithiobacillus thiooxidans can operate synergistically with *A. ferrooxidans*, or with *Leptospirillum ferrooxidans* which is able to use only ferrous iron. Either combination of bacteria can efficiently attack mineral sulphides and rapidly degrade a variety of ores (Paiment *et al*., 2001; Rawlings and Woods, 1995). *Acidithiobacillus thiooxidans* can augment the oxidation of pyrite in coal by *A. ferrooxidans* by oxidising elemental sulphur produced by the latter organism to sulphuric acid (see reaction [3]), which is then converted by *A. ferrooxidans* firstly to ferrous sulphate and then to ferric sulphate (Ford *et al*., 1977). Khalid and Naeveke (1991) have also observed that *A. thiooxidans* had the ability to solubilise heavy metals from carbonate-bearing complex sulphidic ore more efficiently than *A. ferrooxidans*. The reason may be that *A. ferrooxidans* alone could not produce sufficient acid to neutralise the carbonate contents and lower the pH, so that suitable conditions for growth of the bacterium could not be attained. A combination of both bacteria works efficiently since the sulphuric acid produced from sulphur by *A. thiooxidans* fulfils this condition.

I. Inhibition of bacterial oxidation and growth

A number of simple organic compounds have been found to inhibit bacterial oxidation of ferrous ion at low concentrations under laboratory conditions, including benzoate, sorbate and sodium dodecyl sulphate (SDS) (Onysko *et al*., 1984) and sodium tungsten (Sugio *et al*., 2001). The inhibitory effect of glucose, cellobiose, galacturonic acid and citric acid compared favourably with that of SDS (Frattini *et al*., 2000). Ferric and arsenite ions have a most detrimental effect on the growth of *A. ferrooxidans* and *A. thiooxidans* (Collinet and Morin, 1990). These compounds have been suggested for use in control of these species.

5. Characterisation of the Genomes (*e.g.* Presence of Large Plasmids, Insertion Sequences) and Stability of These Characteristics

A. Chromosome

Recently, Rawlings (2001) provided sequencing data of the chromosome of *A. ferrooxidans* and of other iron- or sulfur-oxidising bacteria. The *A. ferrooxidans* chromosome consists of 2.9 Mb and contains 61ats larger than 500bp (Tettelin *et al*., 2002). Harrison (1986) determined that *A. ferrooxidans* showed a wide range of genetic diversity encompassing seven DNA homology groups. This, with the discovery of high frequency mutations (Holmes *et al*., 1988; Schrader and Holmes, 1988; Yates and Holmes, 1987), may explain the frequently observed ability of *A. ferrooxidans* to adapt to specific laboratory culture conditions such as pH and resistance to metals. High frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al*., 1988). Recently, this phenomenon was correlated to the high frequency of the insertion and excision of ISAfe1 (1.3 kb), an ISL3 family insertion sequence in a gene *ResB* that encodes for a cytochrome c-type maturation protein (Cabrejos *et al*., 1999). In addition, Holmes *et al*., 2001 demonstrated recently that ISAfe1 or similar ISAfe-1 sequences exist in diverse strains of *A. ferrooxidans* and *A. thiooxidans.*

B. Genes

Several genes and their functions have been elucidated in the thiobacilli (Rawlings and Kusano, 1994; Rawlings and Woods, 1995; Tuovinen and Fry, 1993; Heinhorst *et al*., 2002). A list of some of the genes in *A. ferrooxidans* have been identified and cloned as shown in Table 4.13.

Table 4.13 Genes in *Acidithiobacillus ferrooxidans*

Gene	Gene Product	Function
glnA	Glutamine synthetase	Ammonia assimilation
nifH	Nitrogenase	Reduces N_2 to NH_3
ntrA	ntrA sigma factor	Promotes transcription
rbcL, rbcS,	RuBPCase	Fixes CO_2
recA	alanyl-tRNA synthetase	Recombination/ DNA repair
merA	Mercury reductase	Resistance to Hg
merC	unnamed	Mercury transport
merR	unnamed	merA regulator
iro	Fe(II) oxidase	Iron oxidation
Rusticyanin gene	Rusticyanin	Respiratory Electron Transport Chain[a]
ATP synthase genes	ATP-synthase	ATP synthesis
cysC, cysD	unnamed	Sulphur assimilation

[a] Exact function of the gene is unknown

C. Plasmids

Plasmids are found in a large number of *A. ferrooxidans* strains. With the exception of the arsenic and antibiotic resistance plasmids described in next section, most of the plasmids evaluated to date, are cryptic in that no phenotypic characteristic has been linked to their presence (Valenti *et al*., 1990). Rawlings *et al*., 1984 reported on the isolation of a 12,190 bp IncQ plasmid (pTF-FC2) from A. *ferooxidans* present in 12-15 copies per chromosome. Recently, another IncQ plasmid pTC-F14 was isolated from *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) consisting of 14 000 bp, 12-16 copies per chromosome (Gardner *et al*., 2001). A large review of these plasmids characteristics can be found in (Rawlings and Tietze, 2001).

Dominy *et al*. (1998) isolated and characterised a 19.8 kb plasmid from *A. ferrooxidans* ATCC33020. Fourteen complete open reading frames (ORFs) were identified, most of which were proteins involved in maintenance although three of the ORFs appeared to correspond to redox-active proteins and thus could constitute part or all of an electron transport chain.

None of the plasmids describe to date, have been correlated with metal resistance (Hutchins *et al*., 1986; Chisholm *et al*., 1998; Leduc and Ferroni, 1993), although circumstantial evidence suggests that genes for mercury and silver resistance may be located on an unnamed 19 x 10^6 daltons plasmid isolated from *A. ferrooxidans* (Visca *et al*., 1986). One isolate of *A. ferrooxidans* was found to possess mercuric reductase activity similar to that in heterotrophic bacteria containing the mer operon, but this could not be associated with a plasmid.

Valenti *et al*. (1990) discovered a 20-kb plasmid (pTFO) in eight out of twelve strains of *A. ferrooxidans* from Italy and Mexico, which was stably maintained for many bacterial generations. All of the strains resisted similar concentrations of metal ions in spite of overall differences in their plasmid pattern.

D. Genetic variation among leaching bacteria

Suzuki *et al*. (1989) demonstrated that naturally occurring strains of *A. ferrooxidans* vary widely in their ability to utilise iron and sulphur compounds, resistance to metal and mineral leaching activities. The abilities to adsorb onto solid surfaces and to oxidise sulphur with ferric ion are other variable properties. Natural variation and /or selection of mutants can be inferred from the ease with which new isolates are selected to enhance process efficiency in various industrial settings (see section 8).

E. Mutation

The occurrence of high-frequency mutation strains of *A. ferrooxidans* was previously mentioned (section 5.1). It was noted that high frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al*., 1988; Holmes *et al*., 2001). In addition, Holmes and Debus (1991) ascribed the high mutation rate of *A. ferrooxidans* to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise.

The high frequency of spontaneous mutations overshadows mutations derived by chemical mutagenesis and makes the latter difficult to detect.

An important implication for environmental regulation is that the inherent instability of these ‘naturally’ engineered strains makes it difficult to predict ecological behaviour thus complicating assessment of risk.

6. Genetic Transfer Capability

Classical genetic engineering techniques for the development of new microbial strains include mutation, conjugation, transformation, transduction and electroporation. Very little work has been done in these areas with bioleaching micro-organisms (Holmes and Debus, 1991). However, Young (1993) has presented a good review of attempts at genetic engineering of these micro-organisms, particularly *A. ferrooxidans*.

A. Transduction

The high mutation rate of *A. ferrooxidans* was ascribed by Holmes and Debus (1991) to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise (see also sections 5.1 and 5.5). One of these DNA sequences has been shown to be an insertion sequence. However, bacteriophages for transduction have not been reported for *Acidithiobacillus*, although these authors cited a report of bacteriophage-like particles in acidophilic heterotrophs, which may prove valuable for developing transduction systems.

B. Conjugation

Transfer of plasmid DNA from heterotrophic bacteria to chemolithotrophic colourless sulphur bacteria by conjugation was first achieved experimentally by Kulpa *et al*. (1983). *Halothiobacillus neapolitanus* was used in the study because of its ability to grow at the near neutral pH required by the heterotrophic donor, *Pseudomonas aeruginosa*. It was discovered that plasmid RP1, which governed resistance to three common antibiotics was accepted, replicated and expressed in the chemolithotrophic bacterium. Transfer from *Escherichia coli* into *A. thiooxidans* and back of four broad-range IncP plasmids with antibiotic resistance markers has also been achieved (Jin *et al*., 1992), and two of the three antibiotic resistance markers have been expressed in *A. thiooxidans*.

However, although plasmids have been demonstrated in many strains of *A. ferrooxidans*, most of them are cryptic in that no identifiable phenotype has been linked to their presence (Leduc and Ferroni, 1994), and there are few cases where plasmid transfer has been successful. In spite of being cryptic in *E. coli*, however, the plasmid pTF-FC2 has been found to have a broad host-range of replication, and the discovery of such plasmids does suggest that a conjugation system exists in *A. ferrooxidans* (Rawlings and Woods, 1995; Rawlings and Tietze, 2001). This plasmid has been deemed particularly suitable for use as a cloning vector for genetic manipulation of *A. ferrooxidans* (Rawlings *et al*., 1986). Four plasmids (pTF35, pTF-FC2, pTF3320-1, pTF3302-2) from three different strains of *A. ferrooxidans* have been successfully cloned into the plasmid pBR325 and two into the related plasmid pBR322 (Rawlings and Woods, 1995). However, the desirable properties of increased uranium and arsenic resistance, present in the *A. ferrooxidans* parents, were not expressed by the transformed *E. coli* mutants.

Two arsenic-resistant plasmids, pSDRA1 and pSDRA21 have been constructed and introduced into *A. ferrooxidans* by conjugation with *E. coli*, using Solid 2:2 Medium as a mating medium (Peng *et al*., 1994b, 1994c). Arsenic resistance was demonstrated in the progeny. Unfortunately most attempts at returning functioning genes from *E. coli* to *A. ferrooxidans* have been unsuccessful. This means that, in principle, the statement by Holmes and Yates (1990) that genes can be extracted from *A. ferrooxidans* and be genetically modified, yet cannot be returned to *A. ferrooxidans* to create an improved organism is still largely true. However, this position may change as techniques improve for introducing foreign genetic information into *A. ferrooxidans* and other thiobacilli.

C. Transformation

Transformation does not appear to take place naturally in *Acidithiobacillus* and other genera of colourless sulphur bacteria, but transformation has been accomplished experimentally through electroporation. Transport of naked DNA into bacterial cells by application of a high voltage electrical discharge (electroporation) has been successful in two cases. *Thiomonas intermedia* was transformed by this method using the plasmid pRK415Km (Jin *et al*., 1994), conferring kanamycin resistance. The transformation efficiency ranged from 10^3 to 10^4 transformants μg^{-1} plasmid DNA under optimal conditions. Kusano *et al*. (1992) transformed *A. ferrooxidans* with natural plasmids and an artificially constructed one, but the efficiency was much less. The reason why only one strain out of the thirty tested

was amenable to transformation by electroporation is still uncertain. The plasmids in the transformed cells were stable for at least 110 generations.

7. Behaviour in Simulated Natural Environments Such as Microcosms

Microcosms have been used to investigate the role of *A. thiooxidans* and other thiobacilli in the degradation of concrete (Sand, 1987; Sand and Bock, 1988). Samples of concrete were inoculated in a simulation apparatus with bacteria originally isolated from concrete and incubated for nine months at an relative humidity of 95% and a temperature of 30 ^{o}C. Three compounds were tested as sources of energy: hydrogen sulphide, thiosulphate, and methyl mercaptan. Hydrogen sulphide at a concentration of 15 mg/m^3 resulted in severe corrosion after nine months, and *A. thiooxidans* was the dominant species in the microflora. At the lesser concentration of 2 mg/m^3 moderate corrosion resulted and the dominant species were *Thiomonas intermedia*, *Starkeya novella* and *Halothiobacillus neapolitanus*. Similar results were obtained with thiosulphate but methyl mercaptan at concentrations of 22 and 2 mg/m^3 caused negligible corrosion and only heterotrophic bacteria and fungi thrived on the concrete blocks.

8. History of Use (Including Selection of Mutants and Examples of Environmental Applications of the Organism, and Information Derived from These):

A. Selection of industrially useful mutants

The rate of growth and mineral oxidation by a population of leaching bacteria can be improved simply by cultivating a population of bacteria in a continuous flow apparatus. If the flow rate through the apparatus is slowly increased, those bacteria that are capable of the most rapid growth will replace the others. Spontaneous mutants will be selected by their growth potential on the available substrate. The advantage of this method is that it does not require sophisticated procedures, but it may take a long time to improve the bacterial strain to any economically significant extent. This approach has nevertheless been used to improve the leaching rates of *A. ferrooxidans* to several fold over that of the original isolates. Vian *et al*. (1986) were able to progressively select mutants of *A. ferrooxidans* with high oxidative efficiency at low pH values (down to pH 1.5) and with high resistance to ferric ions, thus improving the leaching of metals from low-grade ore deposits and avoiding precipitation of oxidised iron compounds.

The improvement of biomining bacteria by mutation and selection has had a dramatic effect on the economics of biooxidation of gold-bearing arsenopyrite ores in particular (Rawlings and Woods, 1995). Natural selection in the laboratory and in pilot and full-scale plants over several years has produced *A. ferrooxidans* strains that are highly resistant to arsenic and are capable of rapid oxidation of gold-bearing arsenopyrite ores in a continuous industrial bioleaching process. Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates. Further improvement however, is likely to require the application of DNA recombinant technology to amplify genes or to enable the introduction of new genetic material. Similarly, resistance to nickel ions was enhanced in *A. ferrooxidans* by repeated culturing in a medium containing nickel and gradually increasing the nickel concentration (Kai *et al*., 1995) and the use of the modified strain, in turn, significantly increased the rate of industrial nickel extraction. No literature data was found on the selection of mutants of the three other species of *Acidithiobacillus*.

B. Main Industrial Uses

Historically there are nine main uses to which species of *Acidithiobacillus* have been applied. This is not meant to imply that their use should be restricted to these categories, but rather to illustrate the potential and diversity of organisms within this genus. The main uses of *Acidithiobacillus* are directly related to their bioleaching ability.

Removal of sulphides from industrial wastes

The toxicity, corrosive properties, and unpleasant odour dictate stringent control of release of sulphides into the environment. Reduced sulphur compounds can occur in industrial wastes of the oil and gas industries as a result of several processes. Whether directly as an end product of sulphate reduction (Buisman *et al*., 1989), or indirectly as a result of methanogenesis, the effluent from which the methane is generated may contain significant quantities of sulphate. The release of large amounts of sulphide into natural waters can result in oxygen depletion due to direct or biological oxidation (Kuenen, 1975) as well as corrosion of the concrete walls of reactors, sewer systems, and steel pipelines.

Acidithiobacillus ferrooxidans

Acidithiobacillus ferrooxidans has often been preferred for sour gas removal because the costs of neutralising sulphuric acid produced by the other bacteria can be avoided, since the sulphur is converted to ferric sulphate, and the bacterium is not inhibited by H_2S (Jensen and Webb, 1995; Shiratori and Sonta, 1993). The Bio-SR Process (Satoh *et al*., 1988) comprises the following steps. Sour gas is introduced into an absorber containing ferric sulphate solution where it is oxidised to elemental sulphur and the ferric sulphate is reduced to ferrous sulphate:

$$H_2S + Fe_2(SO_4)_3 \Rightarrow S^0 + 2FeSO_4 + H_2SO_4 \qquad [17]$$

Elemental sulphur is removed from the solution by a separator and the bacteria in the bioreactor then oxidise the ferrous sulphate in the solution back to ferric sulphate:

$$4FeSO_4 + 2H_2SO_4 + O_2 \Rightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad [18]$$

The oxidised solution is then recycled to the absorber to repeat the cycle. This procedure is environmentally sensible in that it is easy to operate, there are no waste products, no special chemicals are needed and the operating cost is low.

In earlier gas treatment processes (Onken *et al*., 1984; Sumitomo Jukai Envirotech, 1983) the hydrogen sulphide is first precipitated as CuS or FeS. These sulphides are then oxidised by the *A. ferrooxidans* to regenerate the precipitating agent. Cadenhead and Sublette (1990) have commented that the requirement of a low pH for these processes may induce corrosion, and these authors prefer *Thiobacillus denitrificans* with its higher pH tolerance as the microbiological conversion agent.

Acidithiobacillus thiooxidans

Berzaczy *et al*. (1990) have patented a microbiological conversion process for degradation of sulphur-containing pollutants such as H_2S, CS_2, COS, thioalcohols, thioethers and thiophenes in waste gas, especially from cellulose fibre manufacture. The gas makes contact with cells of the bacterium immobilised on packing material in a packed-bed reactor. The metabolic products (mainly H_2SO_4) draining from the reactor are neutralised by addition of lime and lime-water.

It has been suggested that *A. thiooxidans* could be used to convert hydrogen sulphide to sulphur or sulphate in industrial plants. This process has already been demonstrated experimentally, using a continuous column contacter (Lizama and Sankey, 1993). *Acidithiobacillus thiooxidans* was also shown experimentally to act as a bacterial deodorant in removing hydrogen sulphide and trimethylamine simultaneously from a mixture of these two compounds (Hirano *et al*., 1996). It has also been used as a deodoriser in a carrier-packed biological deodorisation reactor used in a sewage treatment plant (Shinabe *et al*., 1995). More than 99% of the hydrogen sulphide and 70-80% of the methanethiol were removed from

the raw gas in the early section of the packed bed. Lizama and Sankey (1993) pointed out that *A. thiooxidans* might have some advantages over *T. denitrificans*, since the energy requirement of *A. thiooxidans* for fixing carbon dioxide from the atmosphere is high. It must oxidise large quantities of sulphide for the production of relatively little biomass, and its tolerance to low pH makes it resistant to the sulphuric acid produced.

Removal of heavy metals from sludge and mine wastes

Extraction of heavy metals from sewage sludge

Application of sewage sludge to agricultural land is one of the most economical methods for final sludge disposal (Bruce and Davis, 1989), since it is a very good soil conditioner and sources of plant nutrients. However, the levels of toxic metals in sewage sludge make them unsuitable for agricultural land application because food plants for humans and animals take up these metals, causing them to accumulate in the food chain (Tyagi *et al*., 1993b).

Couillard and Mercier (1994) determined that bacterial leaching of metals from sludge using *A. ferrooxidans* was more economical than traditional methods of sludge management except in the case of a processing plant treating only 20,000 m^3 of wastewater per day. Furthermore, the use of biological leaching had less of an environmental impact and the product was acceptable for use on agricultural lands (Wong and Henry, 1984). Bacterial leaching was also more economical because acid consumption is reduced by more than 80% (Couillard and Mercier, 1991).

The presence of sulphur-oxidising microflora in sewage sludge is potentially useful for the removal of toxic metals found there (Blais *et al*., 1993). An acid medium is useful in inactivating many bacteria and viruses, though not all organisms are affected. The process is enhanced through the addition of elemental sulphur as an energy substrate, preferably in solid rather than powdered form since the sulphur is then easier to recover at the end of the operation (Ravishankar *et al*., 1994). Rapid decrease of sludge pH by a mixed culture through sulphur oxidation into sulphuric acid solubilised toxic metals to levels recommended for intensive use of residual sludge in agriculture (Tyagi *et al*., 1993b). The solubilised metals in the leachate could be separated from decontaminated sludge solids by centrifugation or filtration, precipitated by neutralising the leachate with lime and then safely disposed of. There exists the potential to recycle these metals for the metal industry. Decontaminated sludge solids must also be treated with lime to reduce acidity before application to agricultural land (Tyagi *et al*., 1993b).

Evidence seems to indicate that a combination of thiobacilli is more effective in the treatment of sewage sludge than the use of a single species. *Acidithiobacillus ferrooxidans* on its own, was not as capable of efficient solubilisation of metals as when compared to a mixture of indigenous sulphur-oxidising bacilli or with other *Acidithiobacillus* species (Couillard and Zhu, 1992; Tyagi and Couillard, 1987; Tyagi *et al*., 1993b). In Table 4.14, the efficiency of thiobacilli species to solubilise heavy metals is illustrated.

Table 4.14 Comparative % solubilisation of heavy metals in sludge

Heavy Metals	Indigenous bacterial microflora Wong and Henry (1984)	*A. ferro-oxidans* Tyagi *et al*. (1993a)	*A. ferro - oxidans* + *A. thiooxidans* Tyagi and Couillard (1987)	Mixture of thiobacilli* Tyagi *et al*. (1993b)	*T. thioparus* + *A. thio-oxidans* Blais *et al*. (1992)	*T. thioparus* + *A. thio-oxidans* Blais *et al*. (1993)
Cd	80-85%	55-98%	50%	51-93%	83-96%	83-90%
Cr	-------	0-32	-------	16-58	16-54	19-41
Cu	66-80	39-94	75	47-95	85-87	69-92
Mn	70-78	71-98	-------	------	91-94	88-99
Ni	37-98	37-98	-------	48-97	78-79	77-88
Pb	0	0-31	55	7-63	28-46	10-54
Zn	84-90	66-98	96	65-98	82-96	88-97

*Named species were *A. ferrooxidans* and *A. thiooxidans*. Other sulphur oxidising organisms were also present (*e.g. Sulfolobus acidocaldarius*)

Blais *et al*. (1992, 1993) showed that the bioleaching of metals from sewage sludge could be carried out by successive growth of moderately acidophilic bacteria (*H. neapolitanus, T. denitrificans, T. thioparus*) and the acidophilic *A. thiooxidans*. *Thiobacillus thioparus* VA-7 and *A. thiooxidans* VA-4 possess distinctive physiological characteristics that allow them to easily grow and solubilise heavy metals in municipal sludge (Blais *et al*., 1992). Strain VA-7 decreased the pH of the sludge initially from pH 7-8.5 to a value between pH 4.0 and 4.5. Strain VA-4 began to grow and further reduced the pH to values below 2.0.

Extraction of heavy metals from industrial wastes

Bosecker (1987) found that some products such as copper, chromium, zinc and vanadium were completely extracted from a variety of industrial waste products by the sulphuric acid produced by *A. thiooxidans*. In some cases bacterial leaching was as effective as chemical leaching with sulphuric acid. Heavy metals such as Cu, Pb, Zn, Fe, As and Cd could also be recovered from flue dust from a flash-smelting furnace (Shiratori and Sonta, 1993) using *A. ferrooxidans*. There were several advantages to bacterial oxidation, including low cost and clear separation of metals.

Aluminium could be recovered from red mud, a chemical waste produced by alkaline extraction of aluminium from bauxite (Bayer process). The mud usually contains about 25% Al_2O_3 (Vachon *et al*., 1994) and is still highly alkaline (pH 12-13). Traditionally, red mud has either been disposed of in the sea or allowed to settle in 'red lakes' for further processing; neither process is environmentally innocuous. Bioleaching, after the addition of sewage sludge to a concentration of 30% V/V to red mud, solubilised up to 47% of the aluminium and brought down the pH to 3.5-2.2. The bacteria responsible were not identified but were probably *T. thioparus* and *A. thiooxidans*.

Silver recovery from waste photographic processing solutions has been accomplished using *A. ferrooxidans*, *A. thiooxidans, Starkeya novella, T. denitrificans* and *T. thioparus* (Kitajima and Abe, 1979).

Biomining and acid production

Bacterial leaching is used in the recovery of metals from ores that are often too poor for conventional metallurgical extraction methods (Robertson and Kuenen, 1992; Paiment *et al*., 2001). The main metals that have been recovered on a commercial scale by microbial leaching are copper and uranium. In the U.S.A., 10-15% of all copper is obtained in this way (Cripps, 1980). The potential of this technique, however, is not limited to copper and uranium, since it can, in principle, be extended to all sulphide and

some oxide ores. Other metals that have been extracted using processes that involve bacteria include zinc, cobalt, lead, gold and molybdenum (Robertson and Kuenen, 1992).

Biological copper leaching is practised in many countries, including the U.S.A., Russia, Chile, Peru, Australia, Spain, Canada and Mexico. Typically, copper ore mined from open pits is segregated, higher-grade material being concentrated for smelting and the lower-grade ore subjected to leaching. The latter is piled to form a 'dump' up to 40 m high and several hectares wide. After the top is levelled, a leaching solution containing ferric sulphate and *A. thiooxidans* is flooded or sprayed onto the dump (Merson, 1992). Bacterial colonisation occurs mainly in the top metre. Leachates enriched with copper exit at the base of the dump and are conveyed to a central recovery facility. The copper in the solution is recovered by mixing it with iron scraps in large container units according to the following reaction:

$$CuSO_4 + Fe^0 \Rightarrow FeSO_4 + Cu^0 \qquad [19]$$

The finely divided "cement copper" is periodically recovered and refined for sale, while the barren solution is recycled to the leachate dumps. A typical large dump may have an operating life of over ten years (Hutchins *et al*., 1986). Total copper recoveries of 80% were attained by the Chilean company *Sociedad Minera Pudahuel* (SMP) after leaching times ranging from 150 to 230 days, and about 90% recovery was attained after 7 to 11 months, more than double the quantities that would have been obtained without bacteria (Acevedo and Gentina, 1993).

Acidithiobacillus thiooxidans has also been used to obtain a high degree of copper extraction from covellite (Curutchet *et al*., 1995) by oxidising the layer of sulphur covering the sulphide surface and allowing sulphide oxidation by ferric ion. Both *A. thiooxidans* and *A. ferrooxidans* are effective in leaching covellite, although at different rates (Donati *et al*., 1996).

Acidithiobacillus ferrooxidans was used to extract uranium from low-grade ores, as for example in the Denison Mine project in Elliott Lake, Ontario, Canada (Brierley, 1990; McCready, 1988). The operation is similar to copper leaching in that it employs a bacterially assisted, flood-leaching process, but it is performed underground on mine waste rubble. About 12-13% of the uranium production is attributed to bioleaching at present, which could increase to 25% after refinement. After the uranium-bearing ore is leached, a uranium-bearing solution drains to lower portions of the mine and accumulates in sumps, after which it can be pumped to the surface for uranium recovery (Hutchins *et al*., 1986). The addition of elemental sulphur or sulphur slag as an external energy source enhanced the leaching process (Bhatti *et al*., 1991).

Acidithiobacillus ferrooxidans and *A. thiooxidans* were used together to extract cobalt from an ore containing 40.3% iron and 1.4% cobalt (Battaglia *et al*., 1994). It was found that for the system to operate at the highest efficiency, the acidity had to be maintained at a pH of between 1.1 and 2. Furthermore, the dissolution of pyrite was depressed when the concentration of ferric ions reached a level of 35 g/L.

According to Brierley (1995), an estimated one-third of the world's total gold production is now from refractory deposits such as gold-arsenic concentrates. In these concentrates, gold and silver are finely disseminated in sulphide minerals, mainly arsenopyrite as well as pyrite, and partly antimonite (Karavaiko *et al*., 1986). Bio-oxidation, in which chemolithotrophic bacteria such as *A. ferrooxidans* have been used to decompose the ore, is a low-energy alternative to conventional methods which involve roasting and pressure leaching.

Lindstrom *et al*. (1992) and Morin (1995) have reviewed the process of microbial leaching for recovery of gold. *Acidithiobacillus ferrooxidans* was found to rapidly and selectively oxidise arsenopyrite and other sulphide minerals in the concentrates. Bio-oxidation increased gold recovery from low levels up

to 95-99% (Maturana *et al*., 1993; Morin, 1995) in comparison with traditional acid leaching. Compared to roasting, bio-oxidation with thiobacilli and their relatives could generally reduce capital costs by 12-20%, operating costs by 10% in some cases and construction time by 25% (Brierley, 1995). At the same time, the process was less polluting to the environment and had lower energy requirements since it operated at relatively low temperatures (Cripps, 1980). Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates.

Acidithiobacillus ferrooxidans accelerated the leaching of silver and other metals present in a mixed sulphide ore from Idaho (Ehrlich, 1986; 1988). Continuous leaching where iron in the solution was supplied to the reactor from a reservoir resulted in selective leaching of the silver.

Zinc has been effectively recovered from a zinc sulphide concentrate by continuous microbiological leaching with *A. ferrooxidans* using a two-stage reactor sequence (Sanmugasundaram et. al., 1986). Direct leaching by *A. ferrooxidans* together with *A. thiooxidans* has been found to be effective (Pistorio *et al*., 1994).

Sub-marginal mercury/antimony sulphidic ores were separated under experimental conditions into their components using a culture of *A. ferrooxidans* isolated from a coal field in the Moscow region (Lyalikova and Lyubavina, 1986).

Oil recovery and purification of oil shale

The position with oil reserves is similar to that of metallic ores outlined above: some 30,000 billion barrels of oil are present in shales, of which only about 2% are available because the recovery of the rest is uneconomical. Conventional extraction methods involve crushing and heating the shale to high temperatures to release the oil from the inorganic matrix. In this way, vast quantities of energy are consumed, only 75% of the organic material is liberated and large quantities of expended shale must be disposed of (Dalton, 1979). A biological process has been invented (Yen *et al*., 1976) to extract oil at ambient temperatures, giving good yields and avoiding the production of vast quantities of insoluble residue. Organisms mentioned in the patent included *A. ferrooxidans, A. thiooxidans, H. neapolitanus, T. thioparus,* other species of *Thiobacillus* since relocated, and various species of *Desulfovibrio.* These bacteria remove most of the organically bonded disulphides and polysulphides in the inorganic matrix of the shale oil, leaving an organic structure that can be used as fuel or can be converted into other materials such as petroleum or synthetic natural gas.

Bioleaching of pyrite from the Aleksinac oil shale in Yugoslavia was successfully carried out by using *A. ferrooxidans* as an alternative to chemical removal; chemical removal formerly led to undesirable changes in the oil substrate (Vrvic *et al*., 1988).

Desulphurisation of coal

To some extent coal desulphurisation is similar to the process above, in that in both cases sulphidic ores are oxidised; however both the aim and the end products are different. Coal, being of fossil origin, is not a homogeneous substance, containing a variable quantity of fixed carbon, hydrogen, oxygen, sulphur, nitrogen and trace minerals (Mannivannan *et al*., 1994). The aim of coal desulphurisation is to produce coal which is as free of sulphur and its derivatives as possible and it is necessary therefore to convert reduced sulphur compounds to soluble forms (Robertson and Kuenen, 1992).

Microbial desulphurisation by *A. ferrooxidans, A. thiooxidans* and *Sulfolobus brierleyi* can remove 90% or more of the inorganic sulphur from coal within a few days (Khalid and Aleem, 1991; Kilbane, 1989). The pyrite-oxidising capacity of *A. ferrooxidans* and related organisms has also been successfully

exploited in the desulphurisation of coal (Bos *et al*., 1988; Bos and Kuenen, 1990; Tuovinen and Fry, 1993) with the production of sulphuric acid instead of sulphur dioxide.

Sulphur is bound in inorganic and organic form in coal. Sulphur dioxide emissions arising by coal burning represent an important ecological problem, which can be solved by the conversion of the sulphur, compounds in the coal into different end products (Beck *et al*., 1988). These workers found that *A. ferrooxidans* was the most useful species to use, but other species such as *A. acidophilum, (T. acidiphilus*), *Thiomonas perometabolis* (*T. perometabolis)* and *T. plumbophilus* could also be used. Beyer *et al*. (1988) and Bos *et al*. (1988) found that it was possible to remove 90% of the pyrite from coal within 8 to 10 days, using a mesophilic pyrite-oxidising microbial system for which a plant design involving a cascade of Pachuca tank reactors was devised.

Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* have also been used to remove sulphur from lignite, the lowest rank of coal intermediate between peat and anthracite (Raman *et al*., 1994).

Desulphurisation of rubber

Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* satisfactorily removed sulphur inclusions in rubber materials that could be recycled from urban wastes (Torma and Raghaven, 1990). The two bacteria together were more efficient than the individual bacteria alone.

Detection of sulphur impurities in wine

A rapid and accurate sensor system was developed to determine free sulphite in wine (Nakamura *et al*., 1989), using immobilised cells of *A. thiooxidans* S3. The concentration of free sulphite could then be controlled so as to protect wine from oxidation processes and microbial spoilage. The same strain was used to detect sulphur dioxide in wine and various foodstuffs. The bacterium is converted into a microbial sensor by setting a piece of microbial membrane onto an O_2 electrode soaked in 0.1 M citrate buffer and covered with a gas-permeable Teflon membrane (Kawamura *et al*., 1992; Kurosawa *et al*., 1990, 1994). A similar biosensor using strain JCM7814 was developed to detect concentrations of sulphur dioxide in wine up to 50 mg/l, with a limit of detection of 5 mg/l and a response time of 20 minutes (Nakamura *et al*., 1993).

Agricultural fertilisation

Since thiobacilli are involved in the sulphur cycle, the presence in the soil can be used to assess fertility. In Australia, for example, thiobacilli are scarce in sulphur-deficient areas (Kuenen *et al*., 1992).

Under warm climatic conditions rock phosphate pelleted with sulphur and seeded with thiobacilli has been shown to be a useful slow release source of phosphate and sulphate for soil fertilisation (Swaby, 1975). However, the addition of *A. thiooxidans* to a mixture of rock phosphate and sulphur granules, called 'Biosuper', has given variable results; in some cases the mixture increased the level of phosphorus in the soil and gave plant yields equivalent to those produced by the more expensive super-phosphate (Muchovej *et al*., 1989; Schofield *et al*., 1981), whereas in others, addition of *A. thiooxidans* did not improve performance of the fertiliser (Alvarez *et al*., 1981; Rajan, 1982). In pot experiments with guavas and ryegrass, the presence of *A. thiooxidans* in the soil enhanced the uptake of Fe, Zn and Mn (Azzazy *et al*., 1994; Schnug and Eckardt, 1981).

Acidithiobacillus thiooxidans is perhaps more useful in low-cost production of a supply of sulphuric acid for the dissolution of apatite in the production of phosphate fertilisers (Donati and Curutchet, 1995).

Soil Reclamation

Incorporating certain species of *Thiobacillus* with sulphur enrichment can reclaim alkali soils, since these soils are naturally poor in sulphur-oxidising bacteria. *Thiobacillus thioparus* has been inoculated with crushed sulphur into a calcareous solonetzic soil in virgin Alberta prairie land in order to promote acidification. This, combined with ripping the soil at a 60 cm depth and weekly irrigation, released several soluble salts, particularly those of sodium, calcium and magnesium (Bole, 1986). *Acidithiobacillus thiooxidans* was used to reclaim a saline alkaline soil by inoculating it together with elemental sulphur, thus lowering the pH and increasing the quantity of soluble salts (Bardiya *et al*., 1972).

III. Human Health Considerations

1. Diseases Caused and Mechanism of Pathogenicity, Including Invasiveness and Virulence

No species of *Acidithiobacillus* are known to be pathogenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health and Current Contents.

2. Toxigenicity

There is no evidence to indicate that any species of *Acidithiobacillus* are toxigenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health,Toxnet and Current Contents.

3. Allergenicity

No literature was found on the allergenicity of thiobacilli. However, the organisms are Gram negative and would therefore be expected to exhibit some of the characteristics associated with endotoxin. Nevertheless, no allergens of significance to humans have as yet been traced to this group of bacteria.

IV. Environmental and Agricultural Considerations

1. Natural Habitat and Geographic Distribution: Climatic Characteristics of Original Habitats

A. General overview

Although *Acidithiobacillus* is probably widely distributed, this distribution is usually related to the presence of sulphur as for example, coastal ecosystems such as salt marshes, sediments, mine dumps and sulphur-rich products of human industrial activity such as metal pipelines and concrete (Kelly and Harrison, 1989; Smith and Strohl, 1991).

As well, some of the products of human industrial activity such as metal pipelines and concrete have become a new ecological niche for some species. This has potentially destructive consequences (see below).

B. Correlation of natural incidence with usage and environmental impacts

While it can be assumed that *Acidithiobacillus* plays a role in the sulphur cycle, the extent of their involvement is largely unknown. Vitolins and Swaby (1969) showed that most thiobacilli in Australian soils rich in sulphur were autotrophic. *Acidithiobacillus* species' natural habitats, usage and environmental impacts are presented in Table 4.15.

Table 4.15 Usage and environmental impacts of *Acidithiobacillus*

Species	Physiological status	Natural habitats	Usage	Impacts
Acidithiobacillus albertensis	AA	Acid sulphurised soil (Bryant *et al*., 1983)	None at present	Not known
Acidithiobacillus caldus	AF	Coal spoil (Hallberg and Lindstrom, 1994)	None at present	Not known
Acidithiobacillus ferrooxidans	AA	Sulphurised soil and rock, in nature, *e.g.* pyrite (FeS_2). Iron and sulphur springs, sulphur iron-rich acidic waters, mines with various ores (Berthelot *et al*., 1993; Blowes *et al*., 1995; De Kimpe and Miles, 1992; Harrison, 1982; Johnson, 1995b; Valenti *et al*., 1990; Vitolins and Swaby, 1969; Zagury *et al*., 1994).	•Removal of heavy metals, •Bioleaching of ores, Desulphurisation of coal and rubber.	Pyrite-oxidising bacterium involved in Acid Mine Drainage (AMD) (Evangelou, 1995)
Acidithiobacillus thiooxidans	AA	Sulphurised soil and deposits fresh water, mines + various ores, corroded concrete (Cho and Mori, 1995; Emde *et al*., 1992; Evangelou and Zang, 1995; Fliermans and Brock, 1972; Harrison, 1982; Parker, 1945; Robertson and Kuene, 1992; Sokolova and Karavaiko, 1968).	•Oxidation and removal of sulphidic pollutants in gas •Recovery of heavy metals •Recovery of silver in photoprocessing, •Recovery of certain ores •Sulphur transformation •Desulphurisation of coal and rubber •Detection of sulphur impurities in wines •Enhancement of phosphorus fertiliser •Soil Amelioration	•Potential threat to buildings, drains, •Deterioration of rubber, •Pyrite-oxidising bacterium involved in AMD (Evangelou, 1995), •Development of acid soils (Arkesteyn, 1980).

AA = Acidophiles, strictly chemolithotrophic and autotrophic
AF = Acidophiles, facultatively chemolithotrophic or mixotrophic
It is assumed that all thiobacilli play some role in the sulphur cycle.

2. Significant Involvement in Environmental Processes, Including Biogeochemical Cycles and Potential for Production of Toxic Metabolites

A. Utilisation of sulphur

The sulphur cycle

Colourless sulphur bacteria, which include the thiobacilli, play an important role in the sulphur cycle by oxidising sulphur and sulphides to sulphates so that they can be utilised by plants (Weir, 1975). Sulphide, which originates from anaerobic sulphate reduction and from decaying organic matter, is

oxidised to sulphate under both aerobic and anaerobic conditions and by both chemical and biological means. Sulphate is assimilated by plants and micro-organisms and reduced to sulphides by other micro-organisms when these die.

In nature, a variety of reduced inorganic sulphur compounds occur as intermediates between sulphide and sulphate, which normally react very slowly with oxygen. Biological oxidation by the colourless sulphur bacteria plays an important role in the recycling of reduced sulphur compounds under aerobic conditions (Kuenen, 1975). The thiobacilli have received more attention than the other main groups of sulphur-oxidising micro-organisms. These comprise several genera of heterotrophic and facultative autotrophic bacteria and yeasts and are far more numerous than the thiobacilli. The thiobacilli, however, were deemed to be more efficient when conditions suited them (Vitolins and Swaby, 1969).

Role of thiobacilli in geologic sulphur deposits

Evidence shows that thiobacilli play a fundamental role in the development and weathering of sulphur deposits. In a review of sulphur deposits and waters with high sulphidic content in the former USSR, Sokolova and Karavaiko (1968) found that *T. thioparus* and *A. thiooxidans* were often associated. However, slight differences in distribution, according to the redox potential and the acidity of the environment, roughly correlating to the pH ranges cited in Table 4.12 were observed. Two examples show that these bacteria may play a role in the build-up and breakdown of sulphur deposits:

Formation of sulphur deposits

In the Shor-Su sulphur mines, *A. thiooxidans* occurred in the upper horizons of the deposit where an oxidative environment prevailed and the rocks were highly acidic (Sokolova and Karavaiko, 1968). In the aquifers throughout the lower horizon where the pH was neutral or weakly alkaline due to the proximity of limestone, *T. thioparus* was widespread and *A. thiooxidans* was absent. The presence of hydrogen sulphide prevented oxidation of the ore bed in the main deposit. Hydrogen sulphide was produced daily up to a rate of 0.2 mg/l by numerous sulphur-reducing bacteria in the groundwater and in the rocks, and ascended towards the surface waters where it was oxidised by *T. thioparus* to sulphur and water.

$$2H_2S + O_2 \Rightarrow 2H_2O + 2S^0 \qquad [20]$$

In several parts of the sulphur mines, sedimentation of molecular sulphur still continued by oxidation of the hydrogen sulphide of the underground waters, as shown by the presence of bacterial cells and small, freshly-deposited sulphur crystals retrieved on culture slides.

Degradation of sulphur deposits in the soil

In the early 1970s, millions of tonnes of elemental sulphur were extracted from sour natural gas and stored in blocks in Alberta, Canada (Maynard *et al*., 1986). Since 1979, large quantities of sulphur have been deposited in adjacent forest systems due to mechanical break-up and weathering of these blocks, causing considerable damage to the understory vegetation. Three sites at distances of 50, 250 and 750 m from a sulphur block were studied. The pH was 2.6, 3.7 and 4.4 respectively for each site. This increasing acidification with the declining proximity to the sulphur block, was attributed to *A. thiooxidans*, which was the main soil micro-organism responsible for elemental sulphur oxidation at all three sites. *Thiobacillus thioparus* was also present, but at a significantly lower population level at the first site (that with the lowest pH) than at the other two. The nutrient concentration of the soil as measured by recoverable calcium, magnesium and potassium also decreased sharply towards the sulphur block.

Reaction on pyrite (FeS_2) in nature

Pyrite is the most prevalent form of iron disulphide and is usually associated with coalfields in the U.S. and elsewhere in the world. It is associated with many ores, including zinc, copper, uranium, gold and silver. Pyrite is formed in a reducing environment with a continuous supply of sulphates and iron in the presence of easily decomposable organic matter (Evangelou and Zhang, 1995).

Oxidation of pyrite deposits due to the combined action of *A. ferrooxidans* and *A. thiooxidans* in empoldered or flooded land has caused a pronounced acidification of the soil (Arkesteyn, 1980; Kuenen, 1975). When soils rich in pyrite are brought into agricultural production, "cat clay" is often formed where clay particles are cemented together by jarosite formed during the oxidation of pyrite, reducing agricultural production (Kuenen, 1975; Pronk and Johnson, 1992). Jarosite [$KFe_3(SO_4)_2(OH)_6$] is a basic ferric sulphate also found in deposits associated with pyrite (Ivarson, 1973). *Acidithiobacillus ferrooxidans* has been shown to play a part in the weathering of sulphide minerals with jarosite formation under humid conditions in metamorphic and igneous rocks in Ontario (De Kimpe and Miles, 1992). Other acidophilic species, such as *T. prosperus,* are able to perform the same reactions (Johnson, 1995b) but strains of *A. ferrooxidans* tend to grow more rapidly on ferrous iron than do other iron-oxidising acidophiles, thus causing them to dominate mixed populations (Pronk and Johnson, 1992).

Acid mine drainage (AMD)

Acid and metal pollution can be the result of the activities of thiobacilli in mine wastes (Tuovinen and Kelly, 1972). The natural oxidation of sulphide to sulphates, including sulphuric acid, as part of the sulphur cycle has become greatly enhanced by the world's increasing demand for metals and fossil fuels. Acid mine drainage (AMD) result from land disturbances due to mining and ore processing, and has become an economic and environmental burden (Evangelou and Zhang, 1995). Acid mine drainage may be enriched with soluble iron, manganese, aluminium, sulphate and heavy metals, and the pH may be as low as 2. One of the effects of AMD is to kill established vegetation associated with mine sites and spoil tips. The typical situation found on a reclaimed spoil tip is a slow fall in pH over most of the site. Severe acid generation tends to neutralise any added lime or buffering systems in the soil quickly, and further accelerates the rate of oxidation of pyrite (Backes *et al*., 1986). The large quantities of sulphuric acid that are produced make the environment in which *A. ferrooxidans* grows, and to which it is well adapted, inhospitable to most other organisms (Rawlings and Woods, 1995; Léveillé *et al*., 2001; Leduc *et al*., 2002).

Mine spoils that were alkaline in nature (pH 9), with low sulphur content and a high concentration of chlorides tended to be free of *A. ferrooxidans* (Twardowska, 1986). The limiting pH value for growth of *A. ferrooxidans* in rock material and drainage was found to be about 7.2 (Twardowska, 1987).

B. Corrosion

Because many of the colourless sulphur bacteria produce sulphuric acid or ferric ion, they are often associated with the oxidative corrosion of concrete and pipes, and have been implicated in the corrosion of buildings and ancient monuments.

Corrosion of concrete by Acidithiobacillus thiooxidans (Thiobacillus concretivorus)

Bacterial involvement in concrete corrosion has been known since the pioneering work of Parker (1945). *Acidithiobacillus thiooxidans* has been found mainly responsible for the deterioration of concrete in sewage pipelines (Cho and Mori, 1995; Jozsa *et al*., 1995; Mori *et al*., 1991, 1992; Parker, 1945; Sand and Bock, 1988, 1991). The bacterium is able to use hydrogen sulphide released from the sewage and oxidise it to sulphuric acid which then attacks the concrete. It was found to coexist with an acid-resistant

fungus which could oxidise H_2S to thiosulphate, and it is thought that the bacterium used the latter as an energy source, producing sulphuric acid that was responsible for the corrosion.

$$CaCO_3 + H_2SO_4 \Rightarrow CaSO_4 + H_2O + CO_2 \quad \text{(Sand and Bock, 1988)} \qquad [21]$$

The calcium loss results in a reduction in the stability of the structure.

The pH of fresh concrete sewer pipes is about 13, so that little microbial activity would normally occur in them. The pH would be lowered by carbonation of the concrete due to exposure to air, as well as by exposure to H_2S and then by the activities of the bacterium itself. Other bacteria, including *H. neapolitanus, T. intermedia* and *S. novella,* have been found to accompany *A. thiooxidans* but played a minor role in corrosion (Sand and Bock, 1991).

Acidithiobacillus thiooxidans was shown experimentally to be able to degrade Sulphlex, a mixture of elemental sulphur and plasticisers used as a paving material and as a substitute for asphalt in road construction with a simultaneous production of sulphuric acid (Ferenbaugh *et al*., 1992). In concurrent studies, plants grown in soils amended with Sulphlex exhibited higher sulphur content and reduced growth consistent with poisoning. Indications are, therefore, that *A. thiooxidans* has the potential for adverse effects on sulphur-containing construction materials as well as on the local environment.

Corrosion of steel

Transportation of low-grade coal in railway carriages has been linked with accelerated corrosion of the steel framework (Brozel *et al*., 1995), involving scaling, pitting and cracking. Corrosion of 3CR12 steel coupons embedded in coal occurred under experimental conditions after the pH of the coal had been lowered to 2.5. The extent of the damage increased when the fungus *Hormoconis resinae,* another dominant member of the natural flora, was present in the substrate.

C. Deterioration of rubber

Thaysen *et al*. (1945) reported that the deterioration of rubber hoses was the result of the microbial oxidation of elemental sulphur present in the rubber by *A. thiooxidans*. This was confirmed by Raghaven *et al*. (1990) who also note similar effects on polyethylene.

3. Interactions with and Effects on Other Organisms in the Environment

Acidithiobacillus plays an important role in making sulphur available to plants. *Acidithiobacillus thiooxidans* may also make phosphorus, iron, zinc and manganese more available for plant growth. However, plants can be adversely affected by ~~too~~ high concentrations of sulphate and increased acidity in the soil as a result of activity due to *A. thiooxidans*. Very little is known about antagonists of these bacteria.

4. Routes of Dissemination: Biological or Physical

A. Biological

All species of *Acidithiobacillus* are motile (Kelly and Wood, 2000) so that they are able to disseminate within their immediate environment.

B. Physical

Water appears to be the major means of passive dissemination, and some dispersal must also be due to the spread of particles of soil or rock to which the bacteria have become attached.

V. Application of the Organism in Industry

1. Containment and Decontamination

A. Chemical Methods

The traditional method for controlling acidity in coal spoil and the deposition of pyrite in field drains and soils was to add high levels of lime in order to maintain the pH well above 4 to limit the activity of ferric ions and of *A. ferrooxidans*, so restricting the oxidation of pyrite to a process involving oxygen alone (Backes *et al*., 1986; Poissant, 1986; Trafford *et al*., 1973). Alkaline chemicals, such as limestone, sodium carbonate or sodium hydroxide, have been applied or pumped into active mines to neutralise acid soils. Limestone could hydrolyse most heavy metals, precipitating them as metal hydroxides (Evangelou and Zhang, 1995). This method depended on maintenance of the pH at a consistent value, and very often reacidification occurred as the lime was neutralised or washed out of the surface layers. Limestone is usually readily available but massive over liming of the site may result (Pulford *et al*, 1986) and its effectiveness is reduced because a coating of ferric hydroxide precipitates develops to shield it from further dissolution (Evangelou and Zhang, 1995). The use of soluble neutralising agents such as sodium hydroxide avoids this problem but can be costly and not very practical.

McCready (1987) successfully controlled *A. ferrooxidans* in pyritic shale in the laboratory by adding sodium chloride to reach a concentration of 1.5%. He has suggested that the incorporation of a salt layer would prevent AMD in pyritic sites. Another promising method of controlling pyrite oxidation is considered to be the application of phosphate, which can precipitate Fe^{3+} in an insoluble form as $FePO_4$ or $FePO_4.2H_2O$ (strengite). Coating of the pyrite with iron is prevented by leaching the pyrite with low but critical concentrations of H_2O_2 and a pH buffer, with or without KH_2PO_4. In the first case, iron phosphate precipitates as a coating on the pyrite surface; in the second, it precipitates as an iron oxide (Evangelou and Zhang, 1995). Treatments with phosphate, silicate, citrate and EDDDHA (ethylenediamine di-orthohydroxyphenylacetic acid) inhibited the release of acid and iron from pyritic mine waste (Pulford *et al*., 1986).

Growth of *A. thiooxidans* strain NB1-3 was also greatly reduced in the laboratory by 5 mM of $NiSO_4$, the nickel binding to the cells and inhibiting the enzymes involved in sulphur oxidation (Maeda *et al*., 1996).

Inhibition of iron-oxidising bacteria may be achieved through the use of anionic surfactants (including common cleaning detergents), organic acids and food preservatives (Kleinman, 1989). Acid production may be reduced by 60 to 95%. However, wide use of surfactants is limited by the necessity for frequent treatments, since they are very soluble and motile, and they may also be adsorbed onto the surfaces of minerals without reaching the pyrite-bacterial interface (Evangelou and Zhang, 1995). The Witco product Microwet II™ incorporating various surfactants, when continuously applied to refuse leaving a coal mine satisfactorily controlled *A. ferrooxidans* and reduced acid production (Stancel, 1982). Sodium lauryl sulphate was shown to be inhibitory at a concentration of about 10^{-6} M in culture media and in mine spoils at higher concentration (Fox and Rastogi, 1983). It also has the additional benefit of being low in mammalian toxicity and quite biodegradable, so subsequent environmental problems are unlikely. For restoration of a good ground cover over reclaimed overburden, these authors proposed that a controlled release system be set up to promote a good ground cover, which, over a period of years, would eventually deprive the mine spoil of oxygen and water needed to generate acidity. 0.25% Sodium dodecyl sulphate at about 5,000 litre ha^{-1} resulted in dramatic reductions in acidity, sulphate, and dissolved-iron concentrations of discharge water for 3-6 months (Monticello and Finnerty, 1985).

Application of ProMac, a bactericide that can be applied both as a spray and in controlled-release monolithic pellets has been successful in controlling *A. ferrooxidans* by destroying its protective outer coating, making it susceptible to the acid it produces (Sanda, 1989). Controlled release bactericides including ProMac inhibited *A. ferrooxidans* and promoted regeneration of a mining site (Sobek, 1987; Sobek *et al*., 1990).

B. Biological Control

Inundation of pyritic material and acid sulphate soils or of disused mine shafts has been suggested (Backes *et al*., 1986); Evangelou and Zhang, 1995), since no significant growth of *A. ferrooxidans* has been demonstrated to occur in water-saturated environments (Kleinman and Crerar, 1979). A similar concept has been applied to the construction of wetlands to receive AMD in an anaerobic environment (Evangelou and Zhang, 1995). This environment encourages the activity of sulphate-reducing bacteria and so reduces acidity. Most of the hydrogen sulphide produced by these bacteria react with heavy metals to yield insoluble precipitates. Typical wetlands however, may not have sufficient permeability to take full advantage of this process.

Canada's Natural Resources Department 'Centre for Mineral and Energy Technology' (CANMET) proposed to develop an anaerobic "sulphuretum" for mitigation of acidic mine drainage by envisioning construction of a system of drainage ditches to control the flow of effluent through a bed of straw (McCready, 1991). As aerobic degradation of the straw proceeds, the sugars released will be fermented to organic acids by acidophilic heterotrophs. The organic acids will then be utilised by anaerobic sulphate reducers to reduce the sulphate in the effluent to hydrogen sulphide. Hydrogen sulphide percolating through a water column will precipitate dissolved metal ions as metal sulphides. Microbially produced CO_2 assists in buffering the system and provides a carbon source that may be combined with excess hydrogen by the methanogens to produce methane. In a laboratory study, the pH of the incoming liquid was 3.5 and after passage through the treatment zone it rose to pH 8.1. This process achieved a 65% reduction in the sulphate concentration and metals were not detected in the effluent.

Christison *et al*. (1985) reported that an unidentified zooflagellate was an effective predator, reducing the population of *A. ferrooxidans* from 10^8 cells/ml to 10^2 cells/ml within 18 hours at pH 2.3. Rotifers, as well as flagellated and ciliated protozoa were recorded as significant predators of *A. ferrooxidans* (McCready, 1987) but were incapable of eradicating them in liquid culture; moreover their large size makes it difficult for them to pursue their prey in interstitial spaces of mine tailings.

Padival *et al*., (1995) found that an unidentified strain of yeast introduced into continuously stirred tank reactors with *H. neapolitanus* or *A. thiooxidans* resulted in a 99% decrease in the population of the latter. The effect on these thiobacilli was enhanced by limitation of nitrogen. The results suggest that strategies based on the competitive displacement of thiobacilli to inhibit corrosion of concrete sewers may be feasible.

2. Description of Detection and Monitoring Techniques, Including Specificity, Sensitivity and Reliability

A. Thallous sulphide test

The ability to oxidise thiosulphates to sulphates with the production of elemental sulphur can be utilised to distinguish the sulphur-producing bacteria including *Acidithiobacillus*, from natural samples (Galizzi and Ferrari, 1976). Thallous sulphide paper moistened with pyridine is pressed onto agar plates with the colonies to be tested and then placed in dilute (0.12 N) nitric acid. The black thallous sulphide paper is bleached except in the presence of free sulphur, due to the presence of thallous polysulphides. If

sulphur is present, a brown spot is left at the site of the replicated colony. This test specifically enabled quantification of *T. thioparus* and *A. thiooxidans* in natural samples.

B. Molecular probes

There are some specific problems associated with the identification and quantitation of micro-organisms in biohydrometallurgical operations (Yates and Holmes, 1986). The numerous species of autotrophic and heterotrophic bacteria may be morphologically similar and are often difficult to purify since they grow poorly or not at all on solid media. Analysis may be frequently aggravated by the presence of small rock particles and by the production of ferric precipitates. Molecular probes using cloned DNA sequences in Southern Blots and Dot blots, could distinguish between *A. ferrooxidans* and other species (*Acidiphilium acidophilum*, *Starkeya novella* and *T. thioparus*) as well as recognising several strains of *A. ferrooxidans*. This technique could, moreover, detect as few as 10^5 bacterial cells of a given species. The reverse sample genome probing (RSGP) technique was used by Léveillé *et al*. (2001) to monitor the presence of *Acidithiobacillus* species in AMD environments. Another genomic tool such as fluorescent *in situ* hybridisation (FISH) was successfully used in the laboratory to detect strains of *A. ferrooxidans* in an ADM environment (Leduc, personal communication).

C. Polymerase-chain-reaction (PCR) and related methods

Recently PCR has been used in the detection and identification of *Acidithiobacillus* and other sulphur bacteria. Strains of *A. ferrooxidans* were differentiated by use of RAPD (random primer amplified polymorphic DNA) (Novo *et al*. 1996). Extending this observation, Selenska-Pobell *et al*. (1998) used genomic fingerprinting in the form of RAPD, rep-APD (repetitive primer amplified polymorphic DNA) and ARDREA (amplified ribosomal DNA restriction enzyme analysis) to distinguish four strains of *A. ferrooxidans,* and one strain each of *A. thiooxidans, E. coli, Burkholderia cepacia, T. thioparus* and *Thiomonas cuprinus.* The procedures not only discriminated between the different species but also suggested that one of the strains of *A. ferrooxidans* was only distantly related to the three others. This variable sequence homology was attributed by the authors to the greater ability of the variant strain to accumulate uranium, although all strains were isolated for some ability to do this. Such variability suggests that more than one method should be used to identify or distinguish different strains or species of thiobacilli.

The differentiation of one strain from the others tested may not be surprising since the strains were isolated from different strata and Novo *et al*. (1996) suggested that strains of *A. ferrooxidans* isolated from different micro-environmental sources could give varying patterns on RAPD.

D. Isolation Media

Most sulphur bacteria can be isolated from natural habitats by the use of mineral media containing elemental sulphur or thiosulphate as an energy substrate. Use of media of different pH will assist differential selection of the neutrophilic and acidophilic species, whereas use of acid ferrous sulphate medium will frequently select for *A. ferrooxidans* (Kelly and Harrison, 1989).

A procedure has been described for the enrichment of facultatively autotrophic, mixotrophic bacteria, using a continuous flow chemostat provided with both organic and inorganic substrates (Gottschal and Kuenen, 1980). This provides a means of avoiding the predomination of heterotrophs in standard batch enrichment media containing supplements such as thiosulfate and glucose or acetate. In the latter, a mixture of obligatory chemolithotrophic thiobacilli and chemoorganotrophs normally develops. Harrison (1984) has described a general medium for cultivation of acidophilic bacteria comprising a basal mineral salts solution (MS) with the following (%w/v):

Media composition (per litre)	
$(NH_4)_2SO_4$	0.200
KCl	0.010
K_2HPO_4	0.025
$MgSO_4.7H_2O$	0.025
$Ca(NO_3)_2$	0.001

The pH is adjusted to pH 2-4 with 1N H_2SO_4.

Johnson (1995a) has since reviewed various solid media formulations for acidophilic bacteria published in the literature. The use of agarose, a purified derivative of agar, was recommended to overcome the fastidiousness of thiobacilli in this and other media, as well as the use of a double-layered medium, the underlayer incorporating an acidophilic heterotroph. The latter significantly lowered the proportion of monosaccharides and resulted in a dramatic increase in plating efficiency of most strains of *A. ferrooxidans*.

The basic medium recommended comprised ferrous sulphate and tryptone soya broth and potassium tetrathionate, and enabled differentiation and identification of isolates of several iron-oxidising bacteria based on colony characteristics.

Acidithiobacillus albertensis

This species can be grown on the media suitable for *A. thiooxidans* (Kuenen *et al*., 1992) (see below). The pH should not be lower than pH 2.0.

Acidithiobacillus caldus

Hallberg and Lindstrom (1994) used tetrathionate as a main growth substrate adjusted to pH 2 and held at 32 ^{O}C or 52 ^{O}C according to the strain.

Acidithiobacillus ferrooxidans

This species does not readily form colonies on standard agar media because it is inhibited by some of the organic compounds found in unpurified agar (Holmes and Yates, 1990). The ability to form colonies on a solid medium is a necessary precondition for growth studies and for strain development. This problem has been overcome by substituting pure agarose for the routine agar medium or by growing the bacterium on membrane filters placed on the solid agar medium (Tuovinen and Kelly, 1973).

Growth of this species on solid media can be enhanced by the addition of small quantities of a surfactant such as Tween 80 (Garcia *et al*., 1992). Use of ferrous sulphate ($FeSO_4.7H_2O$) with other basal salts has also been successful in enhancing the growth of the bacterium (Kuenen *et al*., 1992; Harrison, 1984; Visca *et al*., 1989). One of the *Thiobacillus* solid media, TSM-1, developed by Visca *et al*., (1989), produced discrete and easily countable colonies and could be used for the isolation of single clones.

The ferrous sulphate medium is given by Kuenen *et al*. (1992) as follows:

Solution I (per litre)

K_2HPO_4	0.5 g.
$(NH4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.5 g
H_2SO_4	5 ml of a 15N solution

Solution II (per litre)

$FeSO_4.7H_2O$	0.5 g
H_2SO_4	150 ml of a 15N solution

Four parts of solution I are mixed with 1 part of solution II to give a medium containing 120 mMFe^{2+}. Formation of iron precipitates can be avoided by lowering the pH through successive subcultures to a value of 1.3 with H_2SO_4.

Solid 2:2 medium for genetic manipulation of *A. ferrooxidans*

This medium was developed by Peng *et al*. (1994a) for the isolation of mutants. The medium, prepared in four parts, contains ferrous sulphate and sodium thiosulphate as energy sources for the growth of the bacterium at pH 4.6-4.8. Strains resistant to kanamycin and streptomycin could be obtained by incorporating increasing concentrations of these antibiotics in the medium and selecting out those colonies which developed. The medium encouraged the growth of a wide morphological range of colonies. It is also possible to introduce plasmids into bacterial cells using this medium and to develop strains resistant to heavy metals.

Acidithiobacillus thiooxidans

Kuenen et. al. (1992) list the isolation and growth media for this species as follows:

Media (per Litre)

K_2HPO_4	3.5 g
$(NH_4)_2SO_4$	0.3 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.018 g
CaCl	0.25 g
Flowers of Sulphur	5.0 g
Adjust to a pH of 4.5	

Both *A. ferrooxidans* and *A. thiooxidans* may be cultivated in minimal salts (MS) containing 1% powdered sulphur. Sulphur melts at ~113 ^{O}C, so the MS-sulphur slurry is sterilised by heating at 105 ^{O}C for one half-hour on two successive days (Harrison, 1984). These acidophilic thiobacilli have also been isolated on a mineral basal salts medium supplemented with ferrous sulphate and substituting 0.4% Gelrite, a bacterial polysaccharide, for agar (Khalid *et al*., 1993). Dark brown circular colonies have been observed to develop on this medium within 72-96 hours.

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Lead Country and Published Year of Each Consensus Document

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