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Genetic improvement of trees and shrubs, pest/disease control, exchange, evaluation and joint testing for energy purposes

Agricultural University of Norway – Advisory Service, Ås, Norway

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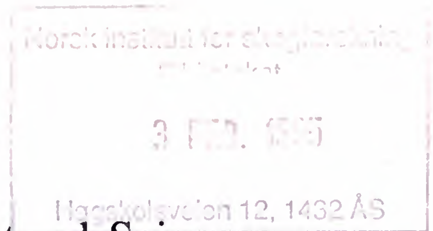
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Proceeding from
The International Energy Agency (IEA), The Bioenergy Agreement

Task VIII

Joint meeting for the activity groups on genetic improvement, pest/disease control and exchange, evaluation and joint testing, Biri, Norway, Sept. 4-9, 1994

Activity leaders
Genetic improvement:
Dr. Louis Zsuffa, Faculty of Forestry, Forest Genetics Laboratory, University of Toronto, 33 Willcocks Street, Toronto, Ontario, Canada M5S 3B3

Pest/disease control:
Dr. David J. Royle, Department of Agricultural Sciences, University of Bristol, Long Ashton Research Station, Long Ashton, Bristol, UK BS18 9AF

Joint testing:
Dr. Vic Steenackers, Faculty og Agronomy, Catholic University of Leuven, Voskensstraat 28, B 9500 Gerhardsbergen, Belgium

Editor
Dr. Jon Dietrichson, Agricultural University of Norway, Department of Forestry, Section of Silviculture, P.O.Box 5044, 1430 Ås, Norway

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Agricultural University of Norway –Advisory Service, Ås, Norway

Preface

This volume of the *Norwegian Journal of Agricultural Sciences* addresses some of the important aspects of finding the best species and varieties, related to trees and shrubs as bioenergy producers. Three activity groups within IEA, Task VIII, from 11 countries joined to discuss the problem at Honne Conference Center, Biri, Norway, 4-9 September 1994. From the about 30 participants the 14 contributions in the proceeding represent some of the work that has been done in the project period ending in 1994. On behalf of the Department of Forestry, Agricultural University of Norway, I express my appreciation to each contributing author making this proceeding possible.

Jon Dietrichson

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Wood as an energy resource in Norway

OLAV GISLERUD
The Research Council of Norway, Energy Office, Ås, Norway

Gislerud, O. 1994. Wood as an energy resource in Norway. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 5-7. ISSN 0802-1600.

In the period 1920-1990 the growing stock has increased by 70%. The annual harvest for sale and industrial production is 8-10 million solid cu.m. In comparison the annual growth is approximately 18 million cu.m. The present use of bioenergy in Norway represents approximately 10 TWh or 5% of Norway's energy use.

Key words: Bioenergy, biomass, energy, forestry, wood.

Olav Gislerud, The Research Council of Norway, Energy Office, P.O. Box 234, N-1430 Ås, Norway.

GENERAL BACKGROUND INFORMATION

Norway, excluding Svalbard, covers an area of 30.8 million hectars, of which 60% is above the timber line. The population is about 4.3 million, of which 70% live in towns and densely populated areas.

GENERAL FORESTRY AND FOREST INDUSTRY DESCRIPTION

The productive forest area is about 7 million hectars; only 11% is publicly owned. 79% of the productive forest area is owned by individuals. There are approximately 125,000 forest holdings in Norway with an area of more than 2.5 hectars. Norwegian forestry is characterized by many small units and a relatively strong association with agriculture. The average size of forest land owned by the Norwegian Forest Owners' Association members is about 65 hectars.

In recent years felling has constituted 9-11 million solid cu.m, of which 8-10 million cu.m has been for sale and industrial production. The annual growth on produtive forest land is about 18 million cu.m. In the period 1920-1990 the growing stock increased by 70% and in 1990 the standing wood volume including bark was 620 million cu.m.

The following figures give an estimate of the 1990 value of forest production in Norway:

	Million USD
- Production/sale of wood	470
- Accumulation of wood for later sale	70
- Hunting	25
- Binding of carbon dioxide	270
- Negative environmental impacts	21

Forestry accounts for approximately 7,000 "man-years" (full-time positions per year). The actual number of persons having income and work from forestry is of course considerably higher.

FOREST INDUSTRY

Forest industry employs approximately 30,000 persons and has an annual export of about USD 1.5 billion. 10% of the Norwegian export has its origin in forestry. The pulp and paper industry consists of about 20 mills. The Norwegian production accounts for only 0.75% of the production on the world market. Norwegian specialities are newsprint and magazine paper, with market shares of 3 and 10%, respectively.

Sawmills, planing mills and wood-working industry have together an annual production value of approximately USD 2 billion, of which 15-20% is export. Particularly the saw timber market and the market for solid wood products play and will play an important role in the income from forestry.

ENERGY SITUATION

The domestic energy consumption has increased eightfold since the turn of the century. The current domestic energy use is 188 TWh/year, or close to 44,000 kWh per capita. Petroleum exploration started in 1971 and Norway was a net energy exporter from 1975. The energy production/exploitation in 1993 was 1850 TWh, almost 90% being exported. Norway has traditionally based its electricity production on hydropower. The normal production capacity is approximately 110 TWh. The possibilities for further exploitation of waterfalls is limited due to conservation of waterfalls and the relatively high construction costs of utilizing remaining waterfalls. Based on resources, gas will become more important than oil. Natural gas plays presently a minor role in the domestic energy supply. Generally bioenergy has to compete on a commercial basis with other energy sources/carriers.

BIOENERGY RESOURCES AND USE

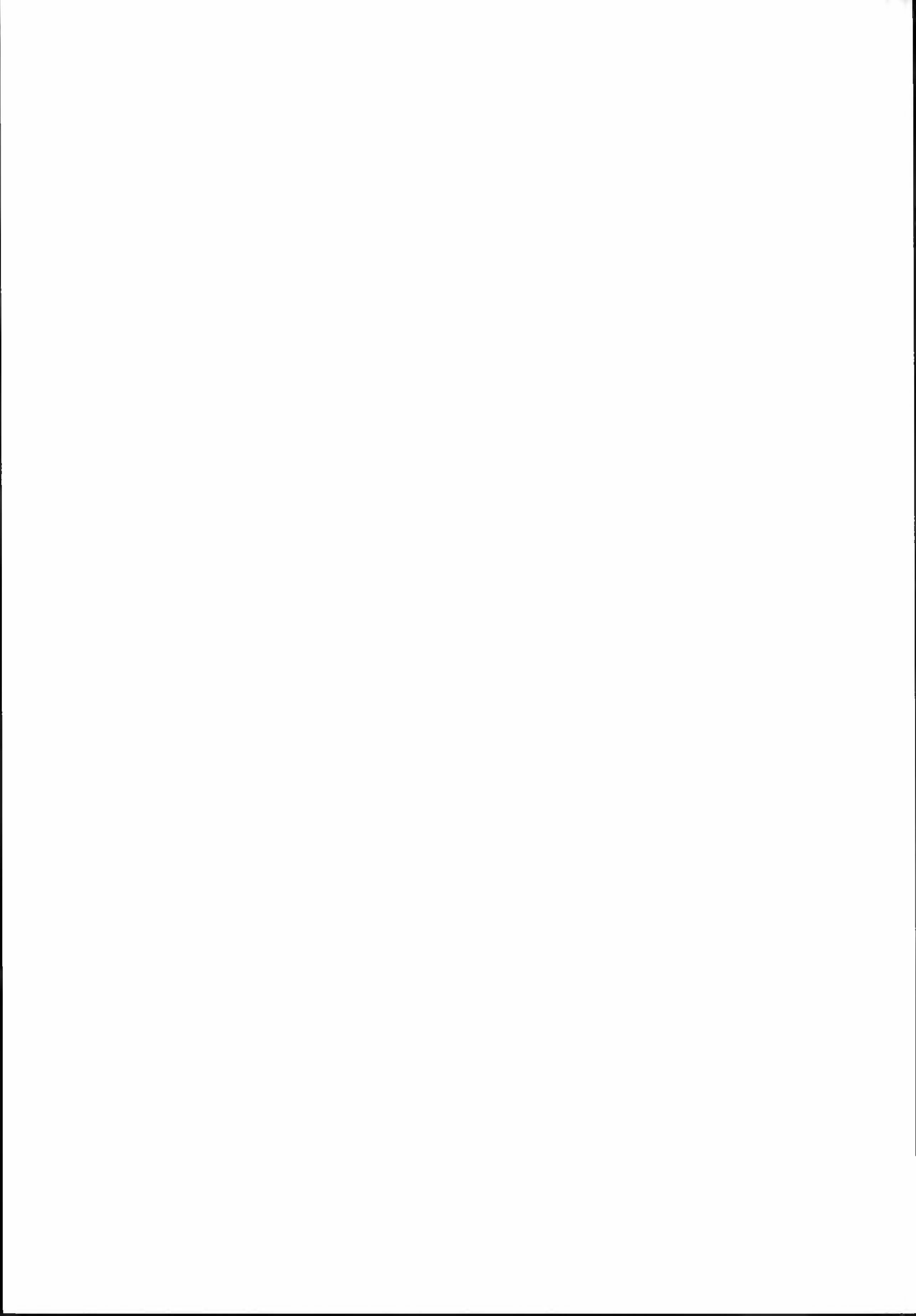
The annual production of biomass in Norway has an energy content of 250 TWh. If needed, probably 30 TWh of bioenergy could be available as an energy source; the biomass presently used for energy purposes has an energy content of 10 TWh. Forest-based biomass

dominates the potential for and use of bioenergy in Norway. The two dominating biomass use patterns are:
- residental heating with firewood in stoves and fireplaces
- use of byproducts and residues by the forest and wood-working industry

Norway has limited district heating and there is presently almost no domestic commercial market outside the wood-based industry for wood chips or upgraded wood fuels. There is also little interest in Norway in intensive biomass forestry or energy forestry. So far only a few small demonstration plots have been established.

BIOENERGY R&D

In 1979 Norway started a Bioenergy R&D programme which lasted until 1994 with a total public expense of approximately USD 15 million. In 1994 the previously separate programmes on new renewable energy were merged into one programme with an annual public funding of USD 1.5 million. The aim of this programme is primarily product/-business development.



Genetic variation in Icelandic birch

KESARA ANAMTHAWAT-JÓNSSON
Agricultural Research Institute, Reykjavik, Iceland

Anamthawat-Jónsson, K. 1994. Genetic variation in Icelandic birch. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 9-14. ISSN 0802-1600.

The genus *Betula* in Iceland comprises of two northern European species: tetraploid pubescent tree birch (*B. pubescens* Ehrh.) and diploid dwarf birch (*B. nana* L.). The two species can hybridize where they occur sympatrically, not only in Iceland but throughout their distribution range. Our genetic and cytogenetic studies have confirmed the interspecific hybridization and described the hybrid introgression that leads to genetic variation in the birch. The mechanism by which gene introgression occurs is predominantly through back-crossing of the triploid interspecific hybrid to the tetraploid tree birch, and the selection is presumably driven by climatic and biotic pressures such as animal grazing. As introgression and genetic variation ensure the adaptability of the species, components of the variation have yet to be characterized. Here we use major ribosomal genes to detect molecular variation in the birch genome. The current results show extensive variation in *B. pubescens*. This type of variation will reflect evolutionary and possibly ecological relationships within the species, and help identifying the extent of gene flow between the species. The understanding of introgression, genetic relationship, population structure and evolution will be important to the breeding programmes steered towards environmental conservation and forestry.

Key words: Cytogenetics, hybrid introgression, selection.

Kesara Anamthawat-Jónsson, Agricultural Research Institute, IS-112 Reykjavik, Iceland.

GENETIC VARIATION AND ADAPTABILITY

Birch, particularly *Betula pubescens* Ehrh or pubescent tree birch, is an extremely variable species and has a very wide climatic and topographical tolerance (Anamthawat-Jónsson et al. 1993). Its main distribution in Eurasia extends northerly to above 70°N of Fennoscandia, southerly to below 45°N of Spain, eastwards through Siberia to about 127°E and westwards to the British Isles, Iceland and the south of Greenland (Atkinson 1992; Fredskild 1991; Kallio et al. 1983). The taxonomy of tree birch in Europe has long been in dispute but there are generally acknowledged to be two major species; the *B. pubescens* complex (tetraploid, 2n=56) and *B. pendula* Roth (diploid, 2n=28), both belonging to the subsection *Albae*. *Betula pendula,* silver birch, has its distribution further to the south than that of *B. pubescens* and it is commercially grown for wood and paper industries in several countries. *Betula nana* L. (diploid, 2n=28) or dwarf birch, a circumpolar species and a member of subsection *Nanae*, is a small and prostrate shrub occupying more northern

latitudes and higher altitudes than the tree birch. Some of the morphological differences among these species are shown here and this is an important basis of the present review aiming to describe the genetic variation and the effect of hybrid introgression on the diversity.

Two birch species co-exist in Iceland; *B. pubescens* and *B. nana*, the latter being more wide spread in the north and the central highland regions (Kristinsson 1989). Whilst *B. nana is* relatively stable, *B. pubescens* is highly polymorphic. *Betula pubescens* is the major indigenous tree species in Iceland forming birch forest and woodland in various climatic and soil conditions. The trees in an inland forest may grow up to 12 metres tall with single or many stems, but most often in shrub woodland they may be only one to two metres tall with extensive branching close to the ground. Other characteristics are also variable, especially the parameters of leaf shape and dimension (see Fig.) that are least influenced by the environment. This Icelandic woodland birch is similar to the variety *B. pubescens* Ehrh. spp. *tortuosa* (Ledeb.) Nyman, often known as mountain birch in the alpine zone of Fennoscandia.

Fig. 1. A drawing of birch twigs showing some morphological differences among northern European birch species and their natural interspecific hybrids. The critical characters include leaf shape (ovate, orbicular), leaf apex (acute, rounded), crenation of leaf margin (high, low number), petiole length (sessile, elongate) and stem surface (hairy, glandular). (1) *Betula pubescens* Ehrh. (3) *B. nana L.* (5) *B. pendula* Roth (2) *B. nana x B. pubescens* (4) *B. nana x B. pendula*. The samples were collected at Ivalo (68°40'N, 27°E), Finland, by Matti Sulkinoja

Such morphological and physiological variation often reflect the adaptability of plants. To survive sheep grazing for example, the ability of plant to regenerate from basal or lateral buds would be essential. Most successful land conservation activities in Iceland during the last decades have been to allow natural regeneration of birch in areas where sheep grazing has been removed. Physiological evolution may occur in birch under grazing pressure, for example the study in Finland by Bryant et al. (1989) showed that Finnish and Siberian birches had chemical defence against browsing of mammals such as hares, while birch of Icelandic origin which was least exposed to such pressure was most frequently attacked. Many physical pressures also influence growth and habit: cold climate, short growing season with low thermal sum, long daylength at high latitude, oceanic wind, low fertile volcanic soils and snow-ice packing in winter. Similar mountain birch zone in Scandinavia has been associated with climatic conditions, especially oceanic-continental gradient (Kallio et al. 1983). Many of these adaptive characters also occur in the dwarf birch, suggesting that there may be gene transfer between species via hybrid introgression.

HYBRID INTROGRESSION

The first report suggesting hybrid introgression to be the source of genetic variation in *B. pubescens* is probably that of Elkington et al. (1968), who examined several morphological characters of birch from Icelandic populations (66°N) and from Scotland (53°N), and found a significant variation in Icelandic *B. pubescens* that extended close to *B. nana*. Although interspecific hybridization was long known to be common in the genus *Betula* and the possibility of introgression existed (Woodworth 1929), it was not until recently that we were able to describe the hybrid introgression process using cytogenetic evidence (Anamthawat-Jónsson & Tómasson 1990). The morphological observation has, however, continued with further birch populations and hybrids, mainly in Scotland (Kenworthy et al. 1972), Finland (Sulkinoja et al. 1981), Greenland (Sulkinoja 1990), Norway (Jetlund 1994) and Iceland (Tómasson 1994).

The cytogenetic studies of Icelandic birch from natural populations and controlled crosses (Anamthawat-Jónsson et al. 1986; Anamthawat-Jónsson & Tómasson 1990) suggested that the gene flow from the diploid *B. nana* to the tetraploid *B. pubescens* occurred through interspecific hybridization, followed by back-crossing of the partially fertile triploid hybrid with the tetraploid *B. pubescens*. The back-crossed progeny showed a wide range of morphological variation resembled that in the natural populations, and surprisingly there was no aneuploid variation of chromosome number. Most had the tetraploid number of *B. pubescens* and some had the triploid number. The results could only be explained that there is a strong selection pressure at meiosis of the triploid hybrid giving rise only to viable gametes with full chromosome complement of the haploid set of 14 or 28 chromosomes. Chromosome stabilizaton at the tetraploid level, hence restored fertility, could then be achieved in one step, which is the most efficient mechanism of introgression.

Further work is now focussed on using molecular cytogenetic techniques to examine the hybrid introgression. The introgression is likely to have occurred by intergenomic chromosome substitution or rearrangement, and some may be detectable using the method

of *in situ* hybridization with species-specific DNA probes or total genomic DNA (Anamthawat-Jónsson & Heslop-Harrison 1992; 1993). Since the chromosomes of birch are extremely small, among the smallest plant chromosomes, the progress in birch cytogenetics has been slow. We have localized highly repetitive ribosomal genes on chromosomes of B. *pubescens* and *B. nana* (Anamthawat-Jónsson & Heslop-Harrison 1994), and are now isolating DNA sequences that are essentially specifiic to *B. pubescens* chromosomes.

MOLECULAR ECOLOGY

Genetic variation in birch can be measured with molecular DNA markers in the nuclear genome, for example using restriction fragment length polymorphism (RFLP) of the ribosomal genes or random amplified polymorphic DNA (RAPD) markers (Anamthawat-Jónsson et al. 1993). Hybridization of the ribosomal genes can reveal molecular variation within the species, essentially in the intergenic spacer regions where DNA undergoes evolutionaty changes. The analysis of ribosomal genes has shown an extensive amount of variation in *B. pubescens,* much more than in its closely related diploid species. The extent of variation between populations also corresponds to the morphological observation, and this may be associated to certain ecological factors. However, some RFLP markers are detected in many different populations in Iceland, suggesting that the birch is likely to have evolved from the same gene pool. The understanding of genetic variation and population structure will be important to the breeding programmes of this highly adaptable birch species, especially in the future forestry of northern Europe where natural regeneration, mixed forest management and utilization of surplus farmland are common practices.

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Introgressive hybridization between the birch species (*Betula pubescens spp. tortuosa)* and *Betula nana* in the mountains in "Gudbrandsdalen", Norway

A summary of a thesis fullfilling the requirement for the master degree in forestry in 1994

SVEIN JETLUND
Agricultural University of Norway, Department of Forest Science, Section of silviculture, Ås, Norway

Jetlund, J. 1994. Introgressive hybridization between the birch species (*Betula pubescens spp. tortuosa)* and *Betula nana* in the mountains in "Gudbrandsdalen", Norway. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 15-18. ISSN 0802-1600.

Key words. Birch, introgressive hybridization.

Svein Jetlund, Agricultural University of Norway, Department of Forest Science, Section of silviculture, P.O. Box 5044, N-1432 Ås, Norway.

Downy birch (*Betula pubescens spp. tortuosa*) covers approximately 16000 km^2 of the Norwegian land area. Compared to the productive forest, which accounts for approximately 68000 km^2, it is clear that this species represents a large resource. The species is found in the mountain regions up to approximately 1200 m a.s.l.

An important part of the mapping of the birch-resources is to identify what kind of birch it is. A large number of hybrids between different birch species are observed many places in the world, and it is also likely that hybridization takes place in Norway.

The basis for the study has been observations of abnormal birch-forms in the central mountain region of South Norway, not always possible to identify as any of the particular species. Morphologically they seem to be intermediates between downy birch (chromosome number $2n = 56$) and dwarf birch (*Betula nana)* (chromosome number $2n = 28$).

The most characteristic appearance of the intermediate forms is the leaf size being smaller than downy and larger than the dwarf birch (Fig. 1). The leaves also appear to have a darker green colour than the downy birch. In the autumn the intermediate forms get colours which are more like the reddish dwarf birch, than the downy birch, which turn yellow. Furthermore the intermediates are bushy and have shorter, stouter twigs compared to the downy birch. The intermediates are most often found in an altitudinal belt between the two assumed pure birch species. The female catkins seem to sit on the intermediate trees for a longer period through the winter than on the downy birch. The intermediates are most often seen in open areas above the productive forest area, and where the dwarf birch

often will be the dominant species. The distance to the nearest downy birch trees might be several hundred meters.

Simple morphological measurements of the leaves and trees have been an important basis for the study (Fig. 2a,b): leaf length (L), leaf width (B), distance from the widest part of the leaf to the top edge in proportion to the total length (AB/L), the angle of the leaf edge (SV), the angle to the second vein from the base (NV), the number of veins in proportion to leaf length (N/L) and tree height (H). These characteristics proved useful and statistical significance for separation of the intermediates from either of the pure species, and the two pure species from each other. The width of the tree crown in proportion to the tree height (V/H) as well as the starting point for the forking of the branches in proportion to the tree height (F/H) also appeared to distinguish the intermediates from downy birch.

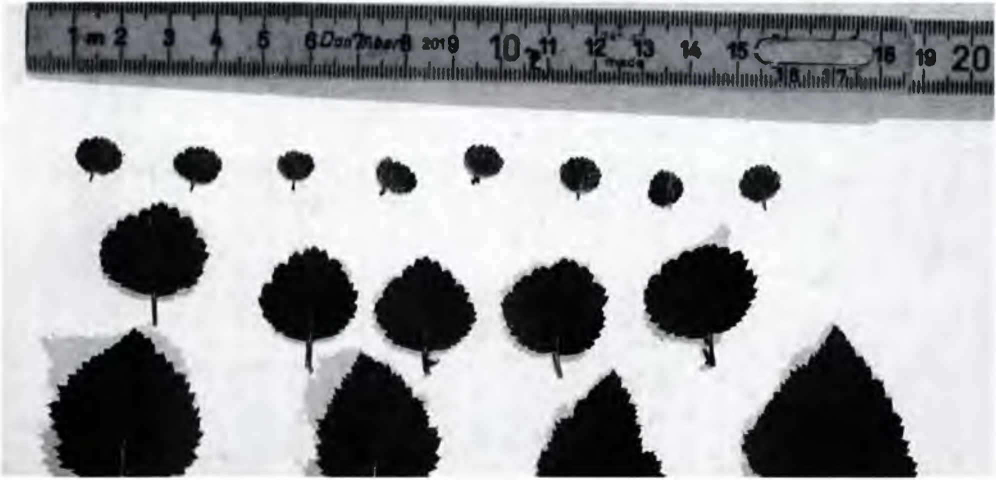

Fig. 1. The most characteristic appearance of the intermediate forms is the leaf size being smaller than downy and larger than the dwarf birch

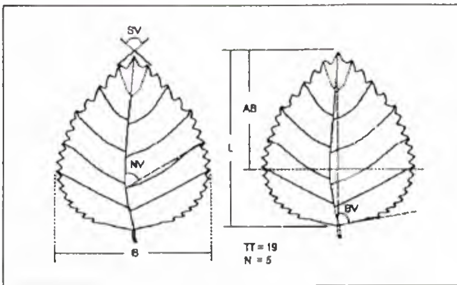

Fig. 2a. Measurements made on leaves

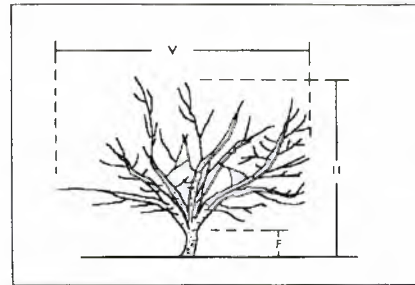

Fig. 2b. Measurements made on trees

Pollen-diameters were measured in some of the trees. Downy birch had larger pollen diameters than the others (Fig. 3). The mean diameter for three downy birch trees was 31m. The variation in diameter within the intermediates were from 24 to 27m (mean

25.5m). The pollen diameter for the two dwarf birch plants was 27.1m. Smaller pollendiameter for the intermediates compared to the dwarf birch is strong evidence for abnormality.

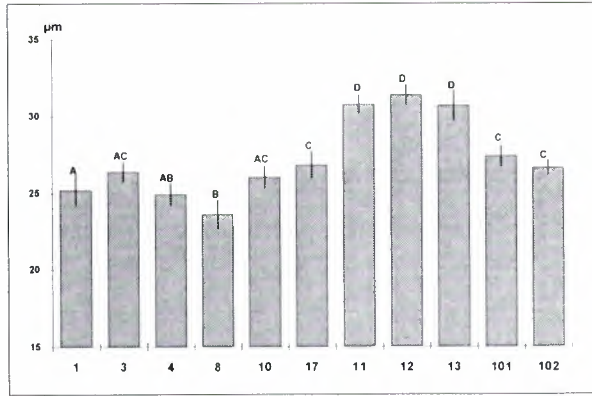

Fig. 3. Mean value, standard deviation and Tukey's test at 5% level for pollendiameter
Tree no 1, 3, 4, 8, 10, 17 = intermediates
Tree no. 11, 12, 13 = *B. pubescens ssp. tortuosa*
Tree no. 101, 102 = *B. nana*

The different characteristics showed strong correlations, and a principal component analysis was used. Here the different leaf-characteristics came into the same index. The first component (Fig. 4) explained 72% of the variation in the data set. The three different "species-groups" segregated based on this first component. The mean of the three groups (marked x) showed that the intermediates were somewhat closer to downy birch than the dwarf birch. Assuming that the intermediates are hybrids, they have a higher proportion of the downy birch genes than dwarf birch genes.

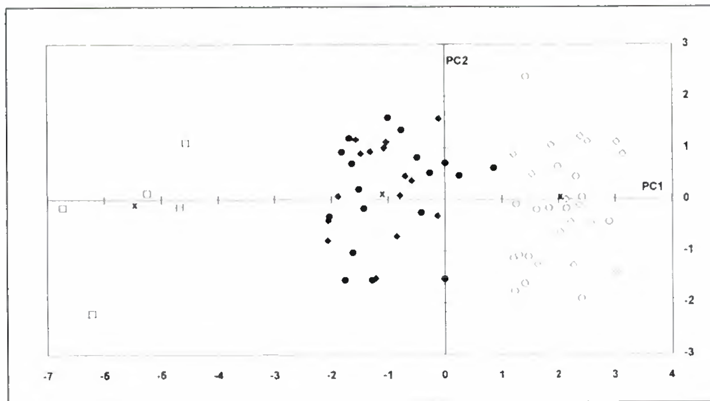

Fig. 4. First and second principal component of 7 leafcharacters of 68 birches.
●, ◆ = intermediates, ○, ◇ = *B. pubescens ssp. tortuosa*, □ = *B. nana*, x = mean

Differences in the measured characteristics between the pure species and the intermediates are primarely due to hybridization, but environmental conditions may also contribute to this variation. The intermediate forms grow in environments with more severe temperature and humidity conditions than the downy birch, and have accordingly found a niche.

Introgressive hybridization between birch species with different chromosome numbers raises an interesting discussion regarding the degree to which gene-modified birch material should or could be used in forestry in the future.

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Genetic variation and breeding strategy of birch in Finland

ANNELI VIHERÄ-AARNIO
The Finnish Forest Research Institute, Department of Forest Ecology, Vantaa, Finland

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Birch is an inspiring model object in tree breeding due to its favourable biological characteristics. The native birch species in Finland, silver birch (*Betula pendula* Roth) and pubescent birch (*Betula pubescens* Ehrh.) are important raw material for both the mechanical and chemical forest industry in Finland. The main emphasis in birch cultivation and breeding has been given to silver birch. Plus tree selection of birch was started in the late 1940's, the main selection criteria being fast growth and good stem quality. Breeding activities of birch were extended considerably from the 1960's by carrying out various crossing schemes and testing the progeny in field trials. Considerable variation was reported among families in growth, stem quality, wood quality and resistance against herbivores. The best part of plus tree material was selected for practical seed production. Genetically improved seed is mass-produced in polythene greenhouse seed orchards. Clonal propagation of birch by tissue culture can be done using both juvenile and mature trees as starting material. It was expanded to commercial scale in the late 1980's, and is governed by regulations. The most severe problems in cultivation of birch have been related to the planting of silver birch on unsuitable sites, and damage caused by browsing, especially voles and elks. Studies into the physiological and genetic basis of herbivore resistance are being carried out. In 1988, a new long-term breeding program for silver birch was proposed for the southern and central parts of the country.

Key words: *Betula*, clonal propagation, herbivore resistance, phenotypic selection, progeny testing, seed orchards.

Anneli Viherä-Aarnio, The Finnish Forest Research Institute, Box 18, FIN-01301 Vantaa, Finland

Shifting cultivation and burning of land, which was commonly carried out until the early 1900's, has been of great importance to the wide distribution of birch forests in Finland. There was an over-supply of birch in the country to the 1950's and birch was disfavoured by forestry. The plywood industry anticipated, however, the threatening shortage of raw material. With the considerable financial donation from the Finnish Plywood Association research and breeding of birch was extended in the Forest Research Institute in the 1960's (Raulo and Koski 1977). Since then birch has been an object of continuous interest in

forestry. Cultivation of birch has inreased in recent years. In 1993, 25 million birch seedlings were planted on 15000 ha, which is about 20 % of the annual area of artificial regeneration in Finland (Aarne 1993). Birch is an important raw material for both the mechanical and chemical forest industry in Finland.

The native birch species in Finland are the silver birch (*Betula pendula* Roth), the pubescent birch (*B. pubescens* Ehrh.) and the dwarf birch (*B. nana* L.). Silver birch has been favoured in cultivation and breeding activities due to its higher growth potential on fertile mineral soils and better stem quality compared to pubescent birch (Heiskanen 1957, Raulo 1977). Pubescent birch, on the other hand, is better adapted to peatlands.

The genus *Betula* can be regarded as a favourable object for tree breeding. Besides normal genetic variability within species, different levels of polyploidy have been reported (Löve and Löve 1961). In evolutionary terms *Betula* is a young genus, and its evolution is still continuing (Elkington 1968, Vaarama and Valanne 1973). Interspecific hybridization also provides more options to breeders (Johnsson 1949, 1967). Fast juvenile growth of the seedlings and early flowering in intensive cultivation allows accelerated breeding cycles (Longman and Wareing 1959; Lepistö 1973; Kärki 1976; Holopainen and Pirttilä 1978). It is possible to organize effective mass-production of genetically improved seed in polythene greenhouse seed orchards (Lepistö 1973; Hagqvist 1991) and vegetative propagation further widens the possibilities for breeding (Ryynänen and Ryynänen 1986; Viherä-Aarnio and Ryynänen 1994).

The main strategy of birch breeding in Finland has been conventional plus tree selection, hybridization, progeny testing and recurrent selection based on additive gene effects. The outlines of birch breeding in Finland are described in this article.

SELECTION OF PLUS TREES AND PROGENY TESTING

The selection of birch breeding material was started in Finland in the late 1940's. At that time the main emphasis in forest tree breeding was, however, on conifers, and the breeding of birch was largely ignored. The practical breeding of silver birch expanded strongly at the Finnish Forest Research Institute in the early 1960's. In a two-phased systematic stand selection of birch plus trees, whole birch stands were selected as seed production stands, and the best dominant trees in these stands were chosen as plus trees (Raulo and Koski 1977).

The most important criteria for selection of plus trees, in addition to their state of health, were fast growth and good stem quality (Raulo 1969). At present there are 66 silver birch seed collection stands and 47 pubescent birch seed collection stands in Finland (Pajamäki and Karvinen 1994). The majority of plus trees have been selected in the southern half of the country. The current number of plus trees of silver birch and pubescent birch is about 1800 and 600, respectively. Plus tree selection is still being continued in order to improve the geographical coverage of the breeding material (Pitkäntähtäyksen... 1988). Recurrent selection in the offspring of phenotypic plus trees is practised as combined family-individual selection, with growth and stem quality as selection criteria.

The first comprehensive controlled crossings with the selected plus trees were carried out in the early 1960's, both within and between selected stands. Extensive progeny tests

with both families from controlled crosses and open-pollination were established in southern and central parts of the country in the late 1960's in order to test the breeding value of the selected plus trees (Raulo and Koski 1977). Thereafter, crossings and progeny testing have been continued both by the Forest Research Institute and the Foundation for Forest Tree Breeding. At present, the total area of progeny trials of birch in Finland is some 350 hectares (Pajamäki and Karvinen 1994).

GENETIC VARIATION IN DIFFERENT TRAITS

The early progeny tests established in the 1960's, which have been reported by Raulo and Koski (1975, 1977) and Raulo (1979) have provided the basic guidelines for breeding and seed production of silver birch. Seed transfers within southern and central Finland of less than 200 km do not have an effect on growth or stem straightness and branchiness of silver birch. Transfers greater than 150 km in a south - north direction are not, however, recommended because of the risk of lower survival. Distant crosses (distance between parent trees > 100 km), do not result in a heterosis effect, i.e., improve the growth or stem quality. Velling (1979a) showed birch of Baltic origin to be poorly suited to Finnish conditions by comparing the phenology, survival and growth of Finnish and Baltic origins in southern and central Finland.

Available genetic variability exists among individual trees rather than between stands or between localities and there is usually large variation in growth and stem quality between progenies from different plus trees within one stand (Raulo and Koski 1977; Raulo 1979). Raulo and Koski (1977) reported that many progenies attained a stem volume that exceeded the mean value for the test by more than 40%. Progenies from open-pollination and controlled crosses do not differ from each other on average. Good families can be found among both open-pollinated and control-pollinated families, indicating that the possibility of improving the growth rate and stem quality are good. Estimates of 20-30 % genetic gain in growth have been given (Koski 1991). The total production of the best families has been over 300 m^3/ha in 30 years (Koski 1991). Good growth and excellent stem quality are, however, quite seldom combined within one progeny (Raulo 1979). The genetic differences and the genetic gain in, for example the supply of veneer at the final cutting, will not be known, however, until the eldest progeny tests reach an adequate size.

Variation in wood density, is generally smaller than that in growth and stem quality characteristics (Velling 1979b). Significant differences can, however, be found between progenies as regards wood density. Seed transfers within southern and central Finland and distant crosses do not have an effect on wood density. Lepistö (1980) showed that there is considerable variation in the fiber length of silver birch. The inheritance of wood characteristics, for example basic density and shrinkage, is fairly strong, as shown by Nepveu and Velling (1983). Volume growth and pulp yield, in contrast, have low heritability (Nepveu and Velling 1983).

LONG-TERM BREEDING PROGRAM

In 1988 a new long-term breeding programme for silver birch was proposed for the southern and central parts of the country in order to widen the basis of breeding, and to ensure continuous genetic gains in the future generations (Pitkäntähtäyksen... 1988). The southern half of the country was divided into three breeding zones according to geographic variation in climatic conditions, and the first generation breeding populations will be formed with 600 - 700 phenotypically selected plus trees per zone.

PRODUCTION OF GENETICALLY IMPROVED MATERIAL

The birch seed used for seedling production has been obtained from polythene greenhouse seed orchards or collected from seed production stands. At present the seed production capacity of existing seed orchards covers almost all the demand for nursery birch seed in Finland (Hagqvist 1991). By means of polythene greenhouse seed orchards the results of birch breeding can be rapidly transferred into practical forestry and the mass-production of genetically improved seed can be arranged effectively.This method has become a well-established practice in the production of genetically improved seed since the 1970's (Lepistö 1973). Flowering and seed yield of birch is abundant in the polythene greenhouses, and the risk of pollen contamination is eliminated.

Most of the birch seed orchards are multiclonal orchards. Biclonal seed orchards have also been established in order to produce desired full-sib families for practical cultivation (Hagqvist 1991) The polythene greenhouse seed orchards are producing seed of silver birch, pubescent birch and curly birch (*Betula pendula* var. *carelica*).

Clonal propagation of birch by tissue culture can be done using both juvenile and mature trees as starting material (Simola 1985; Ryynänen and Ryynänen 1986). The application of tissue culture in practical forestry started in 1987 when a joint project to micropropagate birches on a larger scale was set up by three companies. The first clonally propagated birch plantlets were sold for practical forestry application in spring 1989. The use and trade of clonally propagated material is governed by regulations. Preliminary results from clone tests in the field emphasize the importance of careful selection of clones and testing of the clonal material before use on a large scale (Viherä-Aarnio 1994).

OTHER EFFORTS IN GENETIC IMPROVEMENT

In the early years of tree breeding, mutations, including polyploidy were expected to offer wide possibilities. A large effort to apply induced mutations in the genetic improvement of birch was initiated by the University of Turku in 1960 (Valanne 1972). No substancial genetic gain was obtained directly from polyploids, though (Särkilahti 1990).

A small crossing scheme with inbred lines of silver birch is being carried out by the Foundation for Forest Tree Breeding. The aim is to perform repeated selfing for several generations, and then cross the inbred lines with each other. Birch is typically out-crossing, but with controlled selfings a few germinable seeds can be obtained. Inbreeding depression

in the lines is severe, resulting in seedlings of low general vigour and having various morphological anomalies.

Severe damage in young birch plantations is often caused by mammal herbivores, namely elk, hare and voles. Different birch species vary in their resistance to grazing (Rousi et al. 1989, 1990). Large differences in hare and vole resistance have been observed among birch families in the breeding populations and even among individuals within the families (Rousi et al. 1989). The resistance of silver birch is strongly and positively correlated to the number of the resin droplets on the surface of the bark (Rousi et al. 1991). Resin droplets contain triperpens, mainly papyriferic acid, which has a strong effect on the food choice of voles. By means of hybridization it might be possible to combine good growth and high resistance in certain birch species. The mechanism of defense varies among birch species and it may be possible to combine the defensive systems of two species through hybridization or transfer of resistance genes (Rousi 1990).

Different techniques for gene transfers of silver birch, including both *Agrobacterium*-mediated and biolistic gun methods, are being studied in the Finnish Forest Research Institute (Häggman and Aronen 1993). The Technical Research Centre of Finland (VTT, Biotechnology and Food Research) is trying to work out a genetic marker map for silver birch. Microsatellite and AFLP markers for wood and stem quality characteristics are being looked into in order to accelerate selection of breeding and planting material (Lapinjoki et al. 1992, Åkerman et al. 1994).

CONCLUSIONS

In spite of the expectations of more sophisticated methods of genetic manipulation, the best results in practice have been achieved using a simple mass selection procedure. The offspring of phenotypically selected plus trees are, in general, fairly good. Progeny tests still reveal considerable variation among families, and provide material for recurrent selection. 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. The superior genotype does not, however, alone guarantee success in the cultivation of birch. Only the selection of proper site and intensive maintenance, together with good genotype, can result in a good stand in a short time.

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Budburst in birch cuttings (*Betula pendula* Roth) of different varieties winter-stored in darkness at three different temperatures

ROAR SKUTERUD & JON DIETRICHSON
Agricultural University of Norway, Department of Forest Science, Section of Silviculture, Ås, Norway

Skuterud, R. & J. Dietrichson 1994. Budburst in birch cuttings (*Betula pendula* Roth) of different varieties winter-stored in darkness at three different temperatures, Norwegian Journal of Sgricultural Sciences. Supplement No. 18: 27-38. ISSN 0802-1600.

Budburst timing and the relationship to storage temperature and duration were investigated in four varieties of 1-2 meter tall silver birch (*Betula pendula* Roth) trees. A total of 2160 cuttings were sampled, and the material stored in darkness at 0, 3 or 6°C from November 29, 1993. The varieties were representative of the wide range of genetically controlled budburst performers found within an ecotype. When the cuttings were placed in storage they had been through a period of 29 days with temperatures below 0°C (since October 15). By that time the autumn dormancy was assumed already broken, and the trees were expected to respond to increased temperature by bud development. On January 4, 1994, and four subsequent dates, cuttings were taken out of storage and set in growth chambers at 9, 12 or 15°C. The time to budburst was recorded. Duration of storage, storage temperatures and varieties were all highly significant for budburst. The interaction terms were of less statistical importance. Based on the contrasts between the three different growth chamber environments, three different methods were used to calculate the threshold temperatures for each variety. In spite of the pre-selection of variable budburst performers, the threshold values, varying between -1 to -2°C, could not be shown to be statistically different between varieties. According to the results, the time to budburst changes in accordance with both winter and spring temperatures, being extremely early after a mild winter and warm spring, given sufficient autumn chilling. The similarities in the threshold temperatures indicate that the ranking in earliness between varieties will most likely be the same from year to year without regard to climate change. This is good news for the tree breeder.

Key words: *Betula pendula,* genetics, budburst, threshold temperatures, degree days, winter climate.

Roar Skuterud & Jon Dietrichson, Agricultural University of Norway, Department of Forest Scienc, Section of Silviculture, P.O. Box 5044, N-1432 Ås, Norway.

Many studies on northern forest trees have shown that the characteristic "budburst" is highly heritable. Spring phenology varies between different provenances, progenies from different trees, different genotypes within the same category of trees, and even between

individuals or clones within the same stand or family. By means of selection and tree breeding it is possible to change the phenology, either to the very early or the very late budbursting type. Reports on genetics and budburst phenology are numerous, including Norway spruce (*Picea abies*) (Stern 1966) and the recent report on silver birch (*Betula pendula*) by Wang & Tigerstedt (1993). The timing of budburst is critical for spring frost-resistance of trees, and could also be of importance for frequently observed late winter damage. This has been well documented in Norway spruce (Oksbjerg 1954; Langlet 1960 and Dietrichson 1992, 93). But environment also plays an important role in regulating budburst timing.

Review

The longest running phenological study of budburst at the same location was carried out in England (Williams 1953). In a nearly 200 years observation serie from 1751 to 1947, the leafing out of ash (*Fraxinus excelsior*) and oak (*Quercus robur*) varied as much as 63 and 54 days respectively. Such large differences between years must primarily be explained by thermal effects.

In the case of birch in Eastern Canada and U.S.A., Braathe (1957) postulated a connection between the birch dieback and the March thaw of 1936. According to newer studies of Braathe (pers. com.), too early budburst was the likely cause of the birch dieback.

Several researchers working with different species have studied both genetic and environmental components of budburst-phenology. (Nienstaedt 1966; Campbell & Sugano 1975; Campbell 1978; Cannell & Smith 1983; Worrall 1983; Cannell 1984; Murray et al. 1989; Heide 1993). Provenance trials with wide seed tranfers in forestry have given good evidence for genotype-environment interaction for budburst timing. (Kriebel & Wang 1962; Hermann & Lavender 1968; Dietrichson 1969; Mc Gee 1974; Krutszch 1975).

Sarvas (1974) subdivided the annual cycle of forest trees into the following three parts:

1. The active growth through the summer.
2. The chilling period and autumn dormancy.
3. The winter dormancy.

The autumn dormancy is considered complete when the buds in the trees are able to respond by bud development and eventual budburst if the temperature rises above a certain threshold. The autumn dormancy might be very short. Sugar maple (*Acer saccharum*) in U.S.A. has been reported to complete autumn dormancy as early as the beginning of December (Kriebel & Wang 1962). Similar results have been shown in silver birch in Norway (Heide 1993). Based on the study of Murray et al. (1989) it is evident that trees in the early stage after autumn dormancy need a high number of degree days to reach budburst. But they found that as winter progresses, the total required temperature sum is reduced as a function of the increased number of chill days. However, exact temperature requirements for chilling remain unknown. Traditionally, chill days have been counted as days when the temperature is below the threshold value, but chill days defined this way can only be counted if the threshold value is known.

According to Worrall (1983, 93), the threshold values in forest trees show genetic variation. In Pacific silver fir (*Abies amabilis*), subalpine fir (*Abies lasiocarpa*) and subalpine larch (*Larix lyallii*), the lowest threshold values were found in the alpine provenances having early budburst. Worrall (1983) reworked the data of Oberarzbacher (1977). Here early flushing clones of Norway spruce had a threshold value of approximately 2.6°C, and late flushing 5.7°C. But since the material was collected along a transect from low to high elevation in Tirol the result might be masked with a provenance effect. Sarvas (1972) stated that progress of the active period of *Populus tremula* begins at temperatures below zero.

The following study was conducted in order to elucidate some of the many problems related to the winter ecology of forest trees, tree breeding and the threat of a climate change leading to a rise in temperatures. In Northern Europe the long term rise is predicted to be 2°C in the summer, and 4°C in the winter (IPCC 1990).

MATERIAL AND METHODS

Four varieties of silver birch were used in these experiments, two clones from South Finland, and two openly pollinated families from South Norway (Table 1.).

Table 1. Material used in the experiment located at "Skansgården", Norway 60°12' N. Latitude, 12°04' E. Longitude, 170 m.a.s.l.

Variety	Origin	Lat.	Long.	m.a.s.l.
421 Clone 802	Pälkäne, Finland	61°30'	24°20'	100
423 Clone 806	Pälkäne, Finland	61°30'	24°20'	100
538 Progeny No. 57	Ådal, Norway	60°30'	10°10'	150
541 Progeny No. 1	Ås, Norway	59°40'	10°80'	100

The four varieties were planted in early May 1991 in one tree plots randomly distributed within each of 20 blocks. By the end of the 1993 growing season the plant heights varied from 1 - 2 meters.

On November 25, 1993, after a cool autumn (Fig. 1.), 12 trees from each variety were selected for sampling of cuttings. The selection was made blockwise from Block I to XII. One tree of each variety was sampled within each block. In the cases where a tree of a particular variety was too small to give 50 cuttings, trees of the same variety were selected from Blocks XIII to XIX.

The 50 cuttings taken from each tree, 12-15 cm long, were put into plastic bags, marked and transported frozen to Ås. The following day, all open cuts on each cutting were sealed with wax, and the cuttings were subdived into three equal groups and placed into bags with a small portion of wet spaghnum moss. The fungicide "Bravo" was sprayed lightly inside each bag, and the material was stored at 0°C until November 29. The three groups of material were then put into cool-storage, under complete darkness at 0, 3 or 6°C.

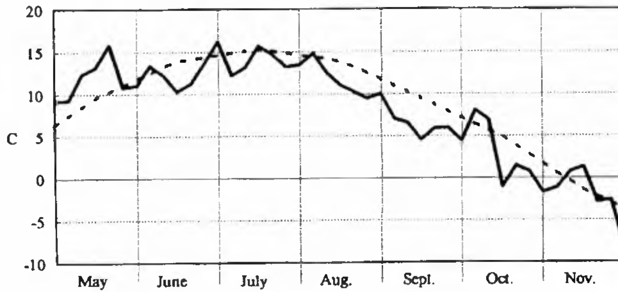

Fig. 1. Pentad means some months 1993: —— Normal mean: -------

On January 4, January 19, February 1, March 4 and March 17, 1994, material was taken out of storage and placed in growth chambers at three different temperatures: 9, 12 and 15°C. For each variety and treatment combination there were 12 replicates. The total number of cuttings was 2160: 4 varieties 12 replications 3 storage temperatures 3 growth temperatures 5 placement dates.

Before being placed in the growth chambers, the cuttings were glued to tape in strips of 12 (Fig. 2.), in a completely randomized design, and placed into 100 ml glasses half filled with distilled water. Four strips from each winter storage temperature were put together in each of the growth chambers, making the experimental set 144 cuttings (48 cuttings 3 storage treatments) for each growth chamber on each placement date.

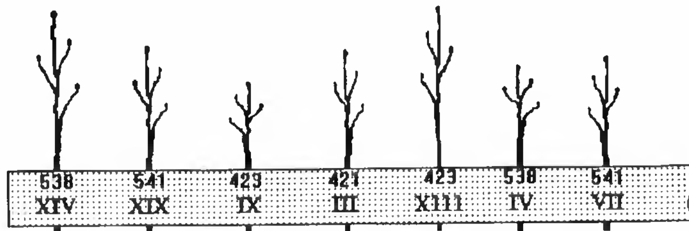

Fig. 2. The cuttings were randomly glued on tape in strips of 12

The growth chambers had eight hours of natural daylight suplemented with 125 μmol m^{-2} s^{-1} (Lightline HPI 400 W). Sixteen hours of night light was also provided by incandescent lamps which gave approximately 5 mol m^{-2} s^{-1}. All the chambers had a water vapour pressure deficit of 530 Pa.

Daily observation and recording of budburst was carried out from the time the first buds began to develop on the cuttings. Budburst was estimated to be the first day when the first bud on each cutting broke the budscale and the leaf edge became visible.

Calculations

The calculations, all concerning budburst, concentrated upon three important topics:

1. The effect of storage temperature and duration of storage on the number of days each variety needed to reach budburst at a given growth chamber temperature.

2. The effect of growth chamber temperatures in days, and the effect of temperature without regard to storage conditions, for calculation of the threshold values.

3. Degree days to budburst once the threshold values are known.

Step 1: The analyse of variance have followeda three-factor model (Snedecor & Cocheran 1972). The mean values were set up in histograms.

Step 2: Threshold values can be calculated in several ways, and in this experiment we used three different methods:

A. The equation method

$T = (t - x)\, n$

T	=	The heat sum to reach budburst
t	=	The growth chamber temperature
n	=	Number of days in the growth chamber
x	=	The threshold temperature

This equation assumes that the heat sum requirement is the same whether the variety is grown at a mean temperature of 9, 12 or 15°C, but this is only true if the correct threshold temperature (x) is being used (Bliss 1967, 1970). By using the experimental data from the different growth chambers, a total of 39 equations for each of the four varieties was set up as follows:

$$(t_1 - x)\, n_1 = (t_2 - x)\, n_2$$

x	=	The threshold temperature
t_1	=	The growth chamber temperature 1
t_2	=	The growth chamber temperature 2
n_1	=	Number of days in the growth chamber 1
n_2	=	Number of days in the growth chamber 2

The mean of the threshold values (x) found for each variety on the basis of each equation was calculated, and the variance tested. Because the budburst observations on each variety were made only on 12 small cuttings in each chamber, and only once a day, the experimental errors of the n-values were high. To reduce the error of the threshold values for each variety the calculations were made on the basis of the total means of the budburst in the three different growth chamber environments, 9, 12 and 15°C.

Complete linearity in the heat response up to 15°C has been assumed, and might not be entirely true. However, development rates of Norway spruce shoots (Mork 1941) and pollen-catkins in birch (Sarvas 1972), fitted to temperature response equations, have been shown to be approximately linear up to temperatures of 12 - 15°C.

B. The standard deviation method
According to Arnold (1959), threshold temperatures can also be calculated in other ways. His method to find the threshold temperature which gives the least standard deviation in days has also been used.

C. The slope of the equation method
The third method uses regression, testing the different values for the threshold temperatures in the equations. The value that gives a regression coefficent value equal to zero should also be the correct threshold temperature (Wittink 1988).

All three calculation methods were used and compared.

Step 3: The number of degree days the different varieties needed to reach budburst was calculated for each storage treatment, using threshold values calculated in Step 2.

RESULTS

The effect of storage temperatures and duration of storage upon budburst in the growth chambers

No significant difference was found in standard deviation between the two groupings of varieties: the clones (421 and 423) and the families (538 and 541), in days to budburst. The data from the four varieties was therefore combined into one analysis of variance, presented only for the 9°C growth chamber experiment in Table 2. The analysis shows that all the main effects are highly significant, and the interactions are of minor importance.

Duration in storage was the most important component regulating time to budburst, but budburst was also affected by storage temperature and somewhat less by variety. There were significant interactions between variety and storage temperature, and between storage temperature and duration of storage. Figure 3 illustrates the mean effects of storage temperature and the four growth chamber temperatures for the different varieties.

Figure 4 shows that the difference in budburst between 0 and 3°C storage temperature is slight in the beginning, but increases as duration of storage increases. The cuttings stored at 6°C had already started to leaf out by March 4, and were therefore excluded from the data analysis from that point. The material stored at 0 and 3°C until March 17 developed their buds in a few days, and approximately twice as fast in the 3°C storage as in the 0°C storage. If the material had been stored a few weeks longer, both storage temperatures would have likely led to full budburst in spite of the complete darkness. The results show that birch reacts to rather low winter temperatures, and that light is not a necessity for leafing out.

Table 2. Analysis of variance of 3-factor birch experiment with a randomized block design. Data in days to budburst in a growth chamber at 9°C. Experiments started January 4, January 19, and February 1, 1994

Source of variation	Degrees of freedom	MS
Replications (R)	11	8.76
Variety (V)	3	543.56***
Storage temperatures (ST)	2	982.15***
Duration of storage (DS)	2	1249.88***
V ST	6	9.81*
V DS	6	8.07
ST DS	4	9.88*
V ST DS	12	3.62
Error	385	3.97

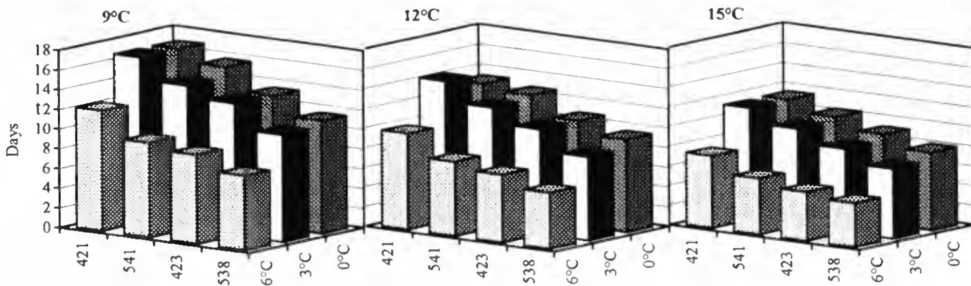

Fig. 3. The number of days the different varieties needed to reach budburst in three different storage temperatures (0, 3 and 6°C) and three different growth chamber temperatures (9, 12 and 15°C). The material is the mean of the experiments started January 4, January 19 and February 1, 1994. The three different analyses of variance as in Table 2 showed the following variety within treatment mean errors in days: 9°C (0.58), 12°C (0.49) & 15°C (0.41)

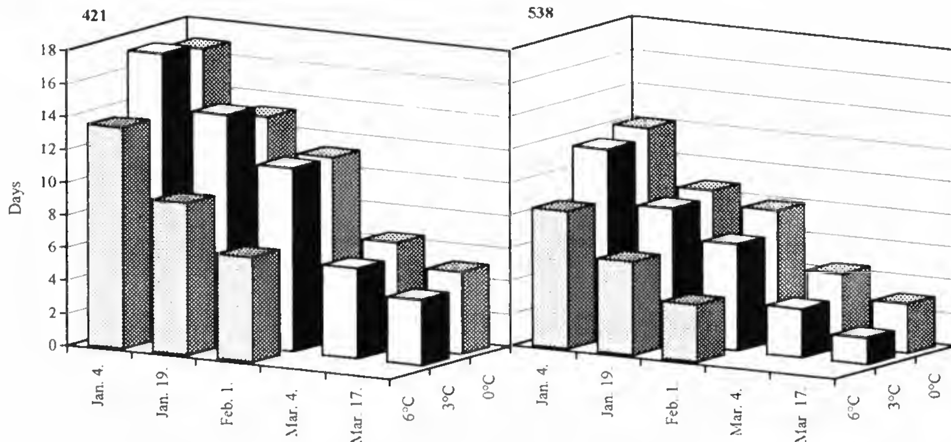

Fig. 4. The mean number of days the latest (421) and the earliest budbursting variety (538) needed to reach budburst with five different placement dates in the growth chambers at 9, 12 and 15°C, after having been stored at 0, 3 or 6°C. Material from the storage at 6°C was excluded from March 4 because some of the cuttings had started to leaf out

The threshold temperatures

Results from the three calculation methods are presented in Table 3. The three methods led to approximately the same results. A test of the separate threshold values showed that threshold temperatures were not affected by either storage temperature or duration of storage (Skuterud 1994). The threshold temperatures could not be found to be significantly different for the different varieties.

Table 3. Results from the three calculation methods

Variety	Method A	Method B	Method C
421	-2,08	-2,15	-2,13
423	-0,89	-0,96	-0,94
538	-1,75	-1,59	-1,63
541	-1,42	-1,57	-1,52

Degree days and budburst for the four different varieties

Based on the estimated mean values for the thresholds for the different varieties, the degree days needed to reach budburst have been calculated for each variety, and set up as a function of storage duration and temperatures (Fig. 5.). The requirement for degree days to reach budburst is drastically reduced by increased winter temperature and its duration.

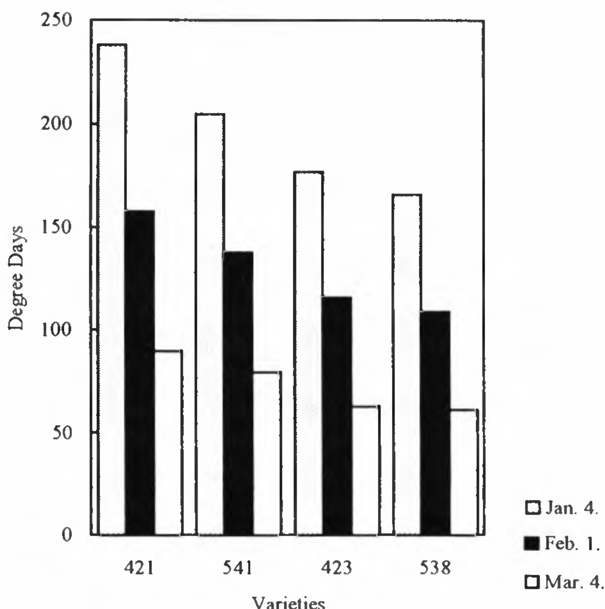

Fig. 5. The mean number of degree days the different varieties stored at 0 and 3°C needed to reach budburst by different placement dates in growth chambers, January 4, February 1 and March 4, 1994. The calculation is based on estimated threshold values for

DISCUSSION

The budburst-timing in silver birch is highly influenced both by genetics and the winter environment. Within the same provenance, different families have large heritable differences in budburst timing. (Wang & Tigerstedt 1993). The largest variance component for budburst, if the latitudinal range of the material is not very large, is nearly always found for the families within the same ecotype.

The four varieties in this study, in order to make them representative, were orginally selected out of larger populations representing different budburst performers. The material covers a latitudinal range of two degrees north-south, and an altitudinal range of 50 m.a.s.l. (Table 1). Latitudinally and altitudinally the ecotypes are very similar, though the east-west distance from central South-East Norway to South Finland is rather wide. The temperature climate for the two areas is, however, much the same (See also Dietrichson 1964).

In spite of the large differences in budburst performance, the different varieties respond in much the same linear way to duration of storage, storage temperature and different growth chamber temperatures. In this study, the early performers were always the earliest, regardless of treatment. The geneticist selecting early or late performing trees with the goal to change the population mean in a certain direction should be able to do so without risks of future interactions, if the climate should change in either a cooler or a warmer direction. This is good news for the tree breeder.

In this study the threshold temperatures were very low compared to the thresholds for other tree species, as mentioned in the introductory review. The values varied from approximately -2 to -1°C, and could not be found to be significantly different in spite of the rather large differences in budburst between varieties. The silver birch used in this study is of continental origin, adapted to relatively warm summers and cool winters. This might explain the low threshold values. If the winters become warmer, or if this birch is grown in coastal areas, then the low threshold values might become a problem. If winter temperatures are higher after this birch has passed autumn dormancy (in this material only 29 days with temperatures just below 0°C, see Fig. 1.), the trees might respond by early budburst, and the earliest performers be subject to late winter or spring frost. This in turn may lead to additional problems, such as the birch dieback observed in the U.S. and Canada (Braathe 1957, and pers. com.).

This study also raises many other questions which need answers. If the chilling effect is efficient only when temperatures are below the threshold values, no chilling differences should have occurred in this study. The small interactions found in this experiment indicate that chilling has been of small importance during the experimental period. New studies, using storage temperatures below the assumed threshold, might throw further light upon the importance of chilling. Different ecotypes, from south to the north, or from low to high altitudes need additional study for a basic understanding of the adaptation problem. But if threshold temperatures within ecotypes are more stable than between ecotypes, it is likely that tree breeders will make the most progress in their selection goals by within ecotype selection.

For nursery growers of silver birch the low threshold values must be taken into account. In order to avoid plant damage, storage temperatures must be kept below the threshold.

ACKNOWLEDGEMENT

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Breeding strategy for poplar in Sweden

BRUNO ILSTEDT
Swedish University of Agricultural Sciences, Dept of Forest Genetics, Uppsala, Sweden

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Results from cultivation of selected clones from Central Europe indicate that they are not hardy in Sweden. To get productive and hardy clones with a good resistance to pathogens and a stem quality that allows production of e.g. veneer and sawnwood, we have to breed especially for Scandinavia. The breeding has been concentrated on *Populus trichocarpa* Hook. Until now two types of crosses have been carried out; an 8 x 8 factorial with phenotypically classified parents, and a 20 x 35 factorial, resulting in 130 families, with more randomly selected parents from the IUFRO collection 1973-75. The preliminary results of the former crosses suggest a large variation in growth. An aim with the latter crosses was to study differences between and within groups of northern, southern, maritime and continental origin. This is also an aim for future crosses. In both the crosses the northern material is insufficient, but still there are several progenies that had no frost damage last winter, in spite of the occurrence of severe frost. Knowledge on which to base a long-term breeding program for poplar in Sweden is lacking today, but the promising result we have already seen suggests that much can be done even in a simple short-term program.

Key words: Breeding, breeding strategy, *Populus trichocarpa*.

Bruno Ilstedt, Swedish University of Agricultural Sciences, Dept of Forest Genetics, Uppsala, Sweden.

CONDITIONS FOR POPLAR CULTIVATION AND BREEDING

It is uncertain today whether poplars will be economically competitive in Scandinavia. The advantage of fast growth and short rotation may be canceled by high costs for establishment, less valuable products such as pulpwood and fuelwood and the risks for damage by game.

The possibility of getting funds for poplar breeding is dependent on the surplus of farm land in the future. A public investigation (SOU 1992:90) calculates the surplus of farmland at 800 000 hectars. There is a tendency towards less intensive farming at present, which means that the area potentially available for poplar cultivation might be considerably lower than was thought some years ago.

A necessary condition for profitable poplar cultivation is that it is not directed to unsuitable soils, e.g. compact clays and very light soils. We also must have clones with a better growth capacity than the old ones we have in the Swedish genetic archives.

Efforts to import bred clones from Central Europe indicates that they are not hardy in Sweden. The poplars from Geraardsbergen, Belgium (*Populus trichocarpa*, and hybrids), that we got as a part of the IEA exchange 1989, grew very well the first three or four years (Figure 1). After three years six of the clones had a mean height above 5 meters. We selected 30 of the clones for the establishment of three new clonal trials, where they were compared with six old, known poplar clones. During the last two winters, however, the frost damage has been severe. The winters, and following springs, were normal, exept for -13.7°C the 13th of October 1992. Such a low temperature has not previously occured in the last 30 years. On September 18th 1993 there was an early frost of -4.5°C, which is also a rare event. Severe frost damage, however, was not visible until early spring. Lammas shoots and poor bud-set indicates that these poplars are unprepared for sudden frost exposure during the autumn. Poor bud-set is also probably the main reason for the frost damage during early spring.

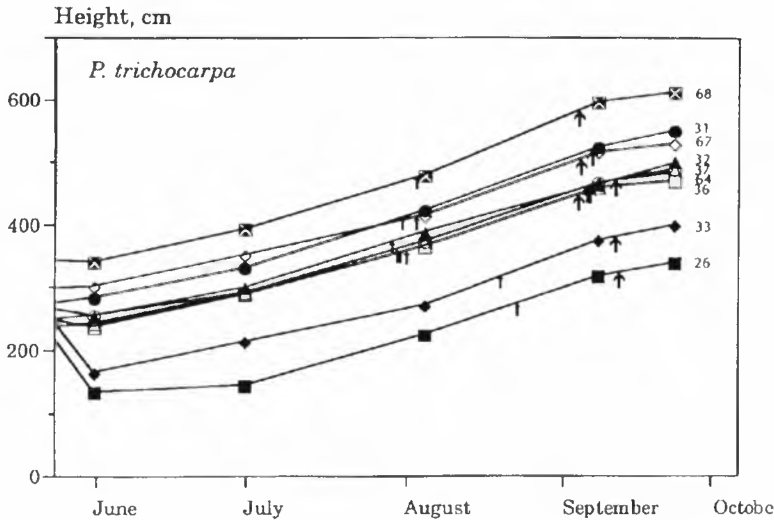

Figure 1. Monthly growth of the *Populus trichacarpa* family (V.26 x V.23) x V.24 the growth season 1991 (third growth period. ↑ and ↟ indicates 50% and 90% of growth 1991 resp.

The frost damage on various clones in the winter 1992-93 are illustrated in figure 2. In the winter 1993-94 every tree, except for one of the control clones originating from and called Kenai Island, were severely damaged by frost, with 30-70 cm of the stems destroyed.

To get good material suited to our latitudes and economically interesting enough to be an alternative on surplus farm land, we have to breed especially for the Scandinavian countries.

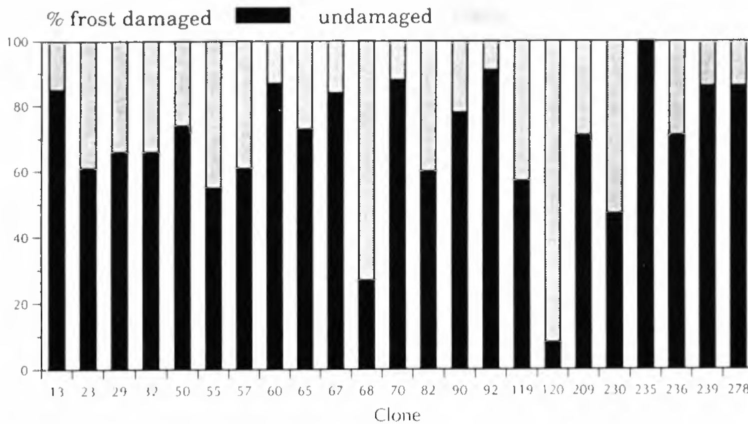

Figure 2. Per cent frost damaged apical buds and stems at Vittinge, winter 1992-93

SPECIES AND BREEDING TRAITS

Because of limited resources we have until now found it advisable to concentrate on one species. *Populus trichocarpa* has been judged to be the most promising and suitable for our climate.

Selection for more than three traits theoretically means that the need for basic population size will be unreasonably large. Still we have to consider four traits; growth capacity, hardiness, stem quality and disease resistance.

Growth capacity has great genetic variance and is the poplars' most distinctive competitive advantage (Heilman et al. 1985). We may look upon growth capacity as a composite of growth rhythm, growth rate, hardiness and disease resistance. Hardiness depends on growth rhythm and disease resistance. The lack of hardiness is the biggest problem in our climate regions. An improvement in this trait will increase the stem quality considerably. A rule of thumb is that quality pays. Thus it is advisable to breed for high quality products, such as veneer and sawnwood. The stem quality also depends on apical dominance, which is often positively correlated with the growth rate. In spite of the fact that pathogens until now have not been a problem, we ought to breed for disease resistance. Preferably the test would be done on 2-3 year old seedlings according to Ridé (Steenackers 1984).

BUILDING UP A BASE POPULATION

The first task in the breeding plan has been to build up a breeding population. Because of the sanitary aspects it seems unrealistic to import clones from North America.

Most of the poplar material in our collection originates from the coastal areas, partly

within lat 54-60° and partly within lat 44-50°, with around 50 clones from each. Around 30 clones come from "Northern interior", the eastern part of British Columbia and a few from "Southern interior", Idaho and Montana. Most of the clones, however, are not of a flowering size. Besides, half of the flowering clones are phenotypically intermediate or inferior (Table 1).

Table 1. Potential candidates for a base population or for generating of useful progenies

	Flowering size				Non flowering size
	Phenotypically superior			Phenotypically intermediate	Selected in clonal trials
	Growth	Quality	Growth and quality		
Northern, coastal	1	1	3	5	40
Southern, coastal	1	1	8	10	30
Northern, interior			1		30
Southern, interior			2	3	

Among the sources of the breeding material described above, the IUFRO collection 1973-75 is the most extensive. 79 provenances were collected all over the native area from California to Alaska and were distribuated to 27 countries (Schulzke 1985). In Finland there are around 100 clones from 15 of the most northern provenances, unfortunately not of flowering size. Most of the clones from the IUFRO collection in Central Europe are too southern for Scandinavian conditions.

POSSIBLE WAYS OF DESIGNING A BREEDING MATERIAL

Thus there are too few phenotypically superior clones for a base population, especially in the material originating from Scandinavian latitudes. Such border provenances may have a useful genetic constitution for some traits, simultaneously with a lack of fitness-promoting genes for other traits.

Populus trichocarpa from areas south of British Columbia has, according to what we know today, insufficient variation in growth cessation, bud-set and hardiness to make it advisable to build up the base population exclusively on this material (Figure 3). However, it may have genetic qualities, e.g. disease resistence, that may be difficult to find in material originating from northern populations.

Progenies from wide crosses must be examined especially carefully with respect to adaptive traits.

An extension of the breeding population can be done by using known and good clones we are able to find in Europe, especially in the Scandinavian countries. Because of the danger of infection, the only way to get material from North America is by seed or pollen. From seed it will take 10-15 years be-fore we are able to use the plants in crossings. As regard pollen the method does not allow us to test the donor's performance in Sweden. In around ten years, when the clones now too small to flower will give sufficient flowering, it will be possible to get a valuable contribution from them. Until then we have to use material from Europe, if necessary supplemented with pollen from North America.

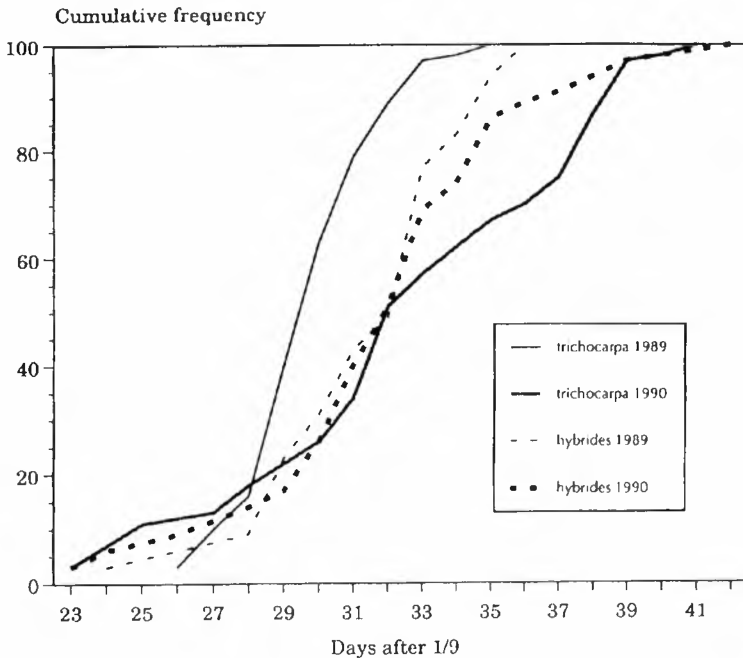

Figure 3. Cumulative percentage showing time of growth cessation 1989 (first growth period) and 1990 (second growth period of 62 *Populus trichocarpa* and 35 *Populus* hibrids from Belgium. The difference in growth cessation is significant on the 0.1 % level both within and between families

LONG-TERM BREEDING

Kang (1982) defines long-term in breeding as covering at least three generations. The base population used for long-term breeding has to be closed at an early stage. At that time each member should be well known, carefully selected and genetically valuable. Few of the clones in our collection today meet these demands.

To-day we do not have a good starting-point for the long-term breeding of poplar. The existing base population is insufficient, and to select a base population from wide crosses may take a long time.

BREEDING RESEARCH GOING ON AT THE DEPT. OF FOREST GENETICS

There are two types of crosses going on at the Dept. of Forest Genetics, Uppsala;

a) with phenotypically classified parents from the preliminary base population, and
b) with parents from the more randomly selected IUFRO material.

We intend to estimate the genetic parameters, the variance and combining ability, the last as a selection criterion for the parents.

a) Progenies after an 8 x 8 factorial crosses with phenotypically classified parents are now growing in the nursery and cloning will be carried out as soon as we can obtain a sufficient number of cuttings. Preliminary recordings of the progenies indicate that some of them have a remarkable growth capacity, some families seem to have a higher within-family variation and some parents a distinct positive general effect on growth.

In order to study differences between and within groups, characterized by northern, southern, mari-time and continental origins, we made crosses in the IUFRO collection from 1973-75. The lack of parents has until now only allowed us to evaluate data from crosses among three groups; northern maritime, southern maritime and continental.

b) The best preserved material from the IUFRO collection is kept in Hannovers-Münden. In spring 1991 we went there and collected branches with fertile buds. The resulting crosses, a 20 x 35 factor-ial that gave 130 families, was aimed at studies according to the above guidelines. However the northern material is insufficient. The progenies have now grown for the second season in a trial with four blocks and five individuals in each.

From both the crossings above there are several progenies that had no frost damage last winter. We hope to be able to extend the crosses between and within groups to the preliminary base population, and especially with additional northern clones, a northern continental group and one from the west coast of British Columbia, around lat 54-55°.

SOME CICUMSTANCES THAT ENCOURAGE THE USE OF POPLARS

There are several circumstances that speak in favour of poplars:

* Results from abroad, the great variation in important traits (Weber et al., 1985), and the promising results we have already seen in Sweden, make us believe that there is a substantial potential for breeding improvements for conditions in southern Scandinavia.
* The surplus of agricultural land and the use of broad leaved trees to counteract acidification, creates a need for alternative species.
* Poplar has possibilities to be used as a soil conditioning catch crop. If accepted for a grant-permit-ted permanent fallow according to the rules within EU the profitability should rise markedly.
* The poplars, moreover, may be of interest in the long run. Breeding is time consuming and we ought be prepared next time when there is a need for broad leaved trees.

CONCLUSIONS

Much is left to be done before we are able to close the base population and continue the long-term breeding. There is not only a lack of breeding material. There is also the shortage of knowledge even about the material we have. We not only have to calculate the

genetic structure: genetic parameters, variance and combining ability. If we are not able to more or less get what we need for the base population from one breeding area, there have to be very time-consuming studies of the progenies.

The uncertainty as to whether the poplar is going to be economically competitive in Scandinavia mak-es it probable that funds for poplar breeding will remain low for a long time. The results from our crosses suggest however, that improvements may be obtained even in a simple short term programs. The most essential point now is to improve our breeding population.

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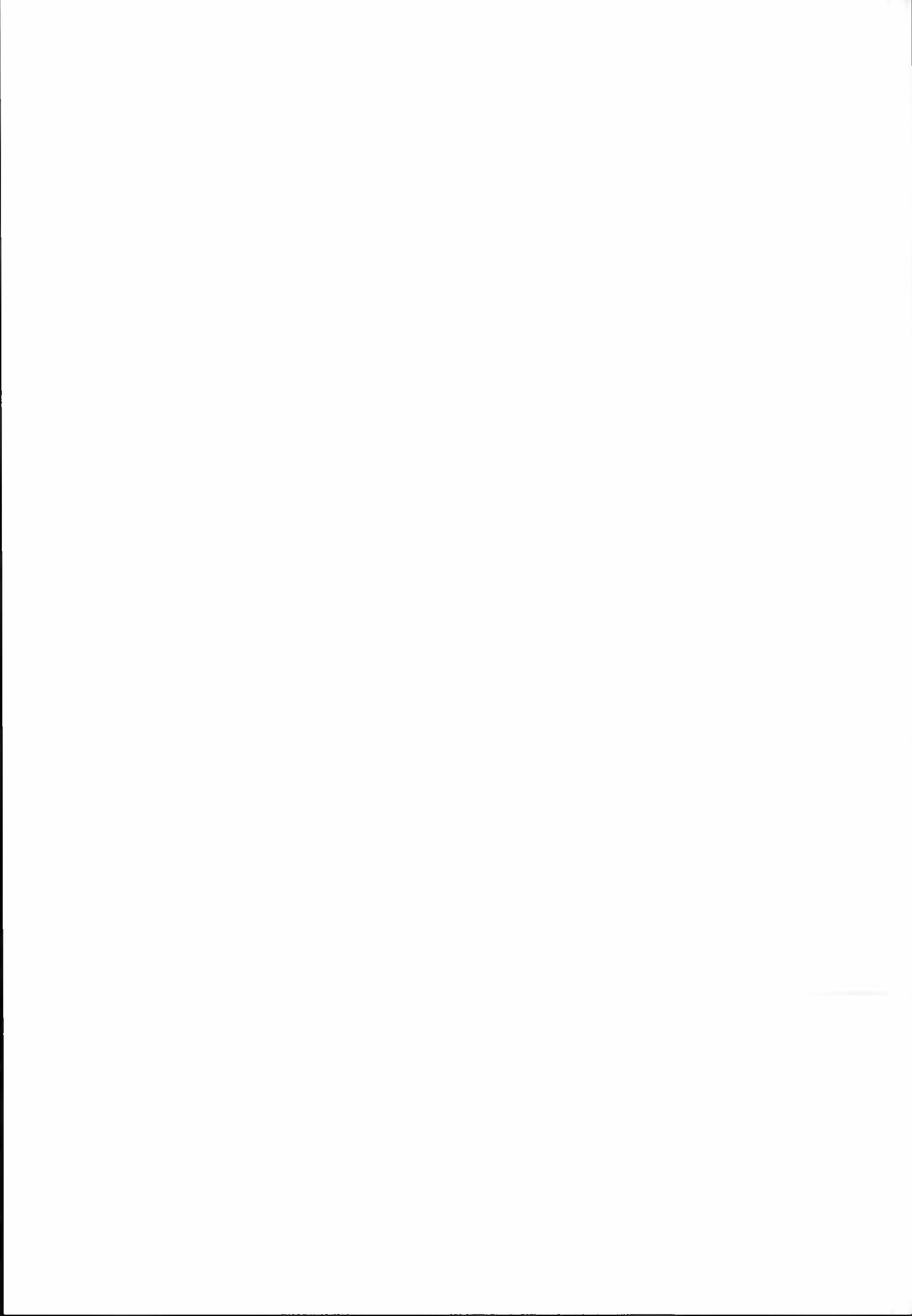

Genetic improvement of willow (*Salix*) as a source of bioenergy

INGER ÅHMAN & STIG LARSSON
Svalöf Weibull AB, Svalöv, Sweden

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The acreage of willow for bioenergy is increasing in Sweden. Clones of *Salix viminalis* originally propagated from wild or naturalized stands dominate in the plantations. In 1987 a breeding programme started at the company Svalöf Weibull AB aiming at willow varieties for a reliable and mechanized production of biomass on agricultural land. The breeding material is primarily based on collections made in Sweden, Central Europe and Russia. Both inter- and intra-specific crosses are being made. The number of cross combinations have increased successively. Selections are being made among seedling plants, which were more than 18 000 from 185 cross combinations in 1994. Selected plants are cloned and reevaluated in observation trials. The most promising clones among these are tested again in yield trials in southern and central Sweden. Selection criteria are: high yield, erect and straight shoot growth, leaf rust resistance, frost tolerance and resistance to certain insect pests. In 1994 the first two, bred varieties of willow were released for production of biomass. Now there is an increasing international interest in testing clones from this programme. Trials have been established in Denmark, Finland, UK, Germany and France.

Key words: Biomass, breeding, coppice, resistance. *Salix*, willow.

Stig Larsson, Svalöf Weibull AB, S-268 81 Svalöv, Sweden.

BACKGROUND

The cultivation of coppiced willow to produce biomass for energy extraction is expanding in Sweden. At present it covers about 12.000 hectares (Fig. 1). Within a few years it is anticipated that the acreage of willows will exceed the acreage of traditional crops like potato, rye and spring wheat.

The plant material dominating in biomass plantations today consists of clones propagated from wild or naturalized stands, of the species *Salix viminalis* L. in particular. Even though these cultivated clones have been found superior to others of similar origin they do have some weaknesses. Through plant breeding, crop traits like biomass yield, plant shape and resistance to pests, diseases and frost can be improved, thereby increasing the economic gain from cultivation.

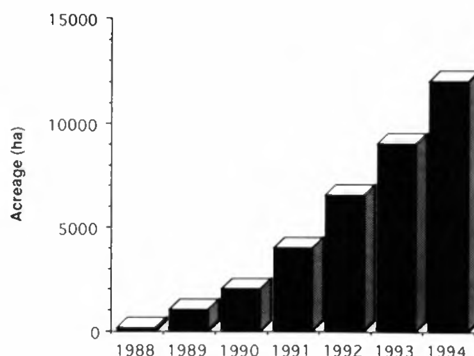

Figure 1. Acreage of willow coppice for bioenergy purposes in Sweden

Willow is not new as a crop, *S. viminalis* and some other *Salix* species have been cultivated for hundreds of years to produce raw material for wicker baskets and furnitures. Such a crop, however, has been selected to produce many, slender and straight rods, not maximum amount of above-ground biomass in a highly mechanized cultivation system. Willow is thus a novel crop when it comes to plant breeding aiming at a reliable and efficient production of biomass on agricultural land. Rapid improvements in these aspects can therefore be expected. A breeding programme was initiated at the plant breeding company Svalöf Weibull AB in 1987.

ORIGIN, TAXONOMY, COLLECTIONS AND GENE POOL

There are indigenous willows on all continents except Australia. Species interbreeding and great intraspecific variation in morphology of willows makes it difficult to distinguish between species. The taxonomists' view on species numbers has varied a lot over the years (reviewed by Pohjonen 1991). About a hundred years ago, more than 1600 species were acknowledged to occur in Europe only. Nowadays figures vary between 30 and 60. The growth form of willow species varies between dwarfed bushes to tall trees. Bushy types with erect stems, rapid growth and good rooting ability have turned out to be the most suitable plants for biomass coppice. *S. viminalis* is the most widely used species so far, but also *Salix dasyclados* Wimm. shows some promise.

Since the starting material in the Swedish willow breeding programme was dominated by Swedish and Central European plant material, collections have been made in Russia to broaden the genetic base for breeding. During 1989 willow material was gathered in the Kirov region, followed in 1990 by collections in Siberia, from Novosibirsk to the river Amur close to the Chinese border. Many of the collected clones are of *S. viminalis* type including one closely related species, *Salix schwerinii*. E. Wolf. Also clones of *S. dasyclados* type have been collected. Altogether about 700 new clones representing 10-15 Salix species have been amassed.

PLANT BREEDING IN WILLOW COMPARED TO OTHER AGRICULTURAL CROPS

A striking difference compared to other crops is the great variation that exists in various plant characters of willows. This is partly due to the fact that other crops have become more homogenous over the years of domestication and breeding, and partly to the previously mentioned broad natural variation that exists among willows. In breeding material of *S. viminalis* it is relatively easy to find clones which show progress in several characters such as an increased yield combined with improved disease and insect resistance.

Part of the interspecific variation is also accessible to the breeder. Interspecific crosses are useful not only to introduce specific, desirable traits in the breeding material but also heterosis may occur. However, the success with attempts to make interspecific crossings varies. Certain combinations of parents may not result in any offspring, others just a few. Those few hybrids produced may, nevertheless, be valuable.

Since propagation from willow seedling plants is made by cuttings there is no problem with loss of heterosis and variation due to segregation as there is in successive generations of crops propagated by seeds. Furthermore, such cloning speeds up selection work since accessions of genetically homogenous material can be evaluated in plantations already the second season after hybridization.

Another practical thing about willows is that they are dioecious, i.e. plants are either females or males. Thus there is no need for emasculation to prevent self pollination in controlled crossings, as there is in many monoecious crops.

CROSSINGS

Since it is difficult to predict the outcome of a specific cross, the breeding strategy at Svalöf Weibull AB has been, firstly, to make many different crosses (Tab. 1). Secondly, the main part of the combinations includes one parent known to perform well in biomass plantations and another parent from the collections of Central European or Russian origin. Thereby it is supposed that genetic variability will not become too low at the same time keeping the traits necessary for a biomass willow in the breeding material. Furthermore, heterosis can be expected, and has been found in offspring after interspecific crossings between Swedish and Russian willows.

Table 1. Development of the crossing programme at Svalöf Weibull AB during 1989-1994

Year	No. of females	No. of males	No. of combinations	No. of hybrid plants	No. of selected plants
1989	6	3	9	763	299
1990	8	6	38	2500	199
1991	5	10	31	3222	167
1992	7	14	31	7888	968
1993	17	18	95	13392	1671
1994	42	21	185	18266	900

Since 1989, when the first crosses were made at Svalöf Weibull AB, routines for crossings and subsequent management of the seedlings have been developed. Twigs with catkins can be collected in the field during December to March. It is possible to store such twigs at about - 3°C, but normally the twigs are moved directly from the field to the greenhouse. There they are kept in beakers with water for the flowers to start developing. To secure supply of pollen when females are receptive, male twigs are brought inside a couple of weeks earlier than female twigs. The pollen is harvested and stored in small glass vials in a refrigerator until needed. Female twigs are enclosed in perforated plastic bags during flower development to protect them from uncontrolled pollination. The pollen is transferred to the stigmata with a brush. Seed development lasts for about two weeks. The mature seeds are sown in bulks on the surface of a sand/peat mixture in pots covered with plastic lids. Already a few hours after sowing, plant development is visible. At a plant height of about 3 cm the plants are transplanted singly to cubes of mineral wool. After further plant growth, to a height of about 10 cm, the cubes with plants are transferred to soil containing slow-release fertilizer (Osmocote) in plastic trays, with 18 or 24 plants each depending on tray size (30 x 50 x 9 cm or 40 x 60 x 7 cm). The trays are moved to the field where they are placed in double rows. The plants are irrigated, when necessary, through tubes on the ground. Extra fluid fertilizer is given 3-4 times during the season.

Tray cultivation in the field was started in 1992. Earlier, plants of about half a meter height were planted in the field with a 2-plants-per-m^2-spacing without the possibility of regular irrigation. The tray cultivation has many advantages: more seedlings per m^2, more cuttings per plant (2.5 m long shoots are not unusual), a weed- and rabbit-free culture and a microclimate promoting Melampsora rust infection.

METHODS OF FIELD TESTING AND PROPAGATION

In the breeding programme, plants are selected for further evaluation in 5 steps: selection of 1) parents from collections or breeding material, 2) seedling plants in the tray culture, 3) clones from field observation trial no. 1, 4) clones from observation trial no. 2 and 5) clones to become new varieties from yield trials.

Seedlings and clones in observation trial no. 1 are evaluated in Svalöv, Scania, Southern Sweden (Fig. 2). To prevent a selection biased for clones adapted only to more southern conditions, observation trial no. 2 is planted at two locations further north. Furthermore, yield trials are planted on 4 or 5 locations from southern up to central Sweden (Fig. 2), where willow coppice is presently cultivated. According to results from scorings of various plant characters, approximately 10% of the most promising plants/clones are selected in each of step 2, 3 and 4. If conditions for selection are favourable, e.g. if there is a high infection rate by diseases and pests a greater proportion of the material can be discarded. This was the case in the seedling trial in 1994 where only about 5% of the plants were retained for observation trial no. 1 in 1995 (Tab. 1).

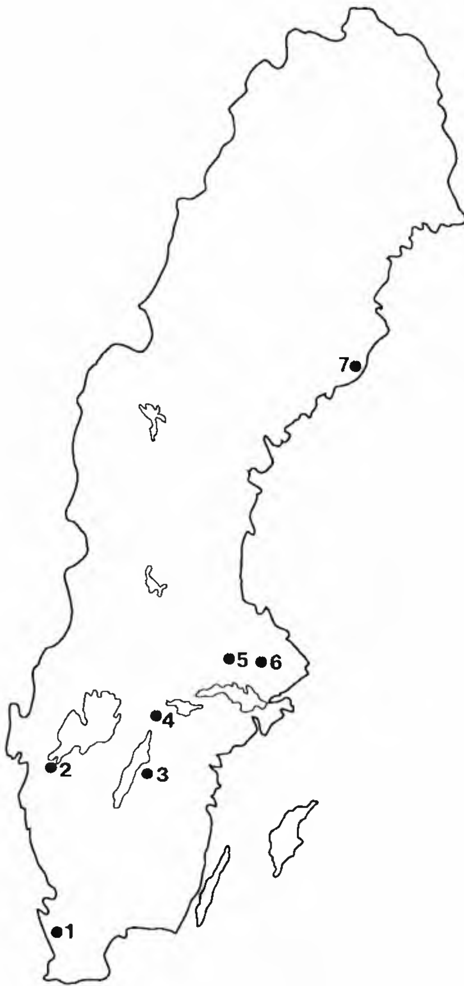

Figure 2. Sites in Sweden where willow trials are performed. 1) Svalöv: centre of breeding with seedling, observation and yield trials 2) Nygård: yield trials, now moved to Bjertorp further east 3) Bränna: yield trials 4) Mårsta: yield trials 5) Bennebo: frost trials 6) Ultuna: yield trials, now moved to Haga further west 7) Röbäcksdalen: frost trials

As the number of genotypes decreases in each selection step, number of plants per genotype is increased in the successive plantations. The observation trials consist of non-replicated rows with 9-18 plants per row. Yield trials consist of 52-plant plots (4x10 m) in 3 replicates. Those trials also include reference clones and the number of clones tested each year has varied between 16 and 46 since the yield trials started in 1989.

The routines outlined above are essentially the same as those used when the breeding programme began at Svalöf Weibull in 1987, with clones produced at the Swedish University of Agricultural Sciences. Slight modifications mainly have to do with the difficulties to get enough planting material for next years' plantations. Another difficulty related to this is to decide when to start propagation of potentially new varieties. In this early phase of breeding biomass willows, propagation must start before there are any results from harvests of the yield trials. Otherwise it takes too long for the improved material to reach the farmers.

SELECTION CRITERIA

Without no doubt a high yield level is very important for this new crop. However, resistance to biotic and abiotic stress is also essential, since a plantation is supposed to last for a 25-year period, i.e. much longer than other agricultural crops. A lot of emphasis is therefore placed on selection for resistance to diseases, frost and insects. Furthermore, the crop has to be adapted to the machinery for planting, weeding, fertilization and harvesting.

Yield

Visual scorings of stem biomass are the basis for selections of seedling plants and clones in observation trials. Scorings are normally made on current year shoots on 1 or 2 year old roots in the observation trials. This selection promotes quickly established clones, a character which should be favourable in terms of competition with weeds.

In order to receive a plantation which is as even as possible, shoots are always cut at ground level in the winter the year after planting. The first yield estimates are taken from plants with 2 seasons of stem growth on 3 year old roots. A second yield estimate is taken after 2 years of regrowth.

Clone L 78183 is the most widespread clone in the plantations established so far. Our results confirm that this clone is the most reliable among the old clones tested (Tab. 2 and 3). However, the first clones bred for biomass; Orm, Rapp, Jorr and Jorunn; generally show better growth. Orm and Rapp have been available for biomass plantations in 1994 and Jorr and Jorunn will be released in 1995.

Table 2. Relative yields of old clones and new varieties in biomass trials in southern and central Sweden. Two year old stems on three year old roots were harvested. (Reference clone: L 78183)

Clone	Combination	No. of trials	Yield
L 78183			100
L 78021		10	83
L 78101		12	98
Orm	L 79195 x L 78101	13	105
Rapp	L 78195 x L 78101	13	114
Jorr	L 820332 x L 81102	10	129
Jorunn	L 830201 x L 81102	10	106

An important question is whether yield estimates from very young plants are representative for a more long-term period with repeated harvests. So far we have only got data from 2 plantations with 2 rotations (Tab. 3) which show that there are no dramatic changes in rankings of the *S. viminalis* clones between the first and the second rotation. However, the second yield estimate from a reference clone of another species, *S. dasyclados* clone L 81090, was much more impressive than the first.

Table 3. Relative yields of old clones and new varieties in two trials (Fig. 2) harvested twice after two years of growth on three and five year old roots. (Reference clone: L 78183)

	1st harvest		2nd harvest	
Clone	Bränna	Nygård	Bränna	Nygård
L 78183	100	100	100	100
L 78112	83	89	97	111
L 77683	107	75	93	91
L 78101	72	79	88	98
L 78102	86	90	93	87
L 78115	92	79	85	82
L 79069*	56	43	62	59
L 81090**	78	87	105	139
Orm	112	114	102	120
Rapp	99	108	99	122
Tons dry matter from reference per ha and year	10.5	7.3	11.0	12.5

* = Salix schwerinii
** = Salix dasyclados

Plant morphology

Biomass willows are planted as cuttings in double rows at a density of about 18 000 per hectare. For harvesters to cut these rows efficiently it is important that the plants have erect basal shoots. Erect growth also facilitates mechanical weeding between rows.

For cutting production it is essential that the shoots grow straightly. The 20 cm long cuttings must be able to pass a 22 mm diameter tube to be approven in quality tests. Otherwise the cuttings may get stuck in the planting machines, where they have to pass through cylinders as they are pushed into the ground. Another planting technique, which becomes more and more common, uses 1-2.5 m long rods which are cut into 20 cm pieces and planted successively. In this case rods which are not straight have a tendency to get entangled as they are stored in bundles on the machine.

A conflict may come up between selection for high biomass yields and demands on cutting characteristics. Some of the promising clones in the breeding material of S. dasyclados type and the *S. viminalis* x *S. schwer*inii hybrids have a relatively large stem diameter. However, the solution for these clones may be to have denser and less fertilized plantations than normal for cutting productions. Another option is to use only the upper, and thinner, part of the shoots. Further development of the planting machines is another way to solve this problem.

Rust

Among all the organisms that live and feed on willows, *Melampsora* rust is considered to be the most serious threat to the cultivation. The rust infests the leaves and cause them to wither prematurely. If infection is severe the shoots do not inwinter as normal, but become sensitive to frost and weak pathogens. Eventually the whole plant may die. In less severe cases biomass production is reduced.

Basic studies have shown that there is consistency between years and between sites in Sweden in how seriously the clones are attacked by rust (Åhman 1992). Thus repeated selections for rust resistance, as in this breeding programme, should result in clones with lower degrees of rust infestation, as long as there is no change in virulence patterns of the rust. One must try to lower such a risk for unintentional selection for clone-specialized rust races by introducing new varieties with new resistance genes to be mixed with the previous ones in the plantations. That is one of the reasons why much efforts have been placed on introducing new plant material from Russia into the Swedish breeding programme.

Scorings for rust are made in the autumn, normally September, when the rust has had time to spread in the plantation, but leaf fall, due to rust or inwintering, is limited. A score is given which combines proportion of infested leaves and density of rust uredinia on infested leaves. By systematic selections for rust resistance in all the 4 or 5 steps of seedling and clonal selections new varieties released or about to be released show a consistent decrease in rust score (Tab. 4).

Table 4. Scores of Melampsora rust and leaf roll gall midge, *Dasineura marginemtorquens*, for varieties released or about to be released.(Reference clone: L 78183)

Clone	Rust		Gall midge
	No. of trials	Rel. score (%)	score (0-2)
L78183	16	100	2
Orm	16	51	1
Rapp	14	42	1
Jorr	12	30	2
Jorunn	12	14	2
Björn*	2	0	0.5
Tora*	2	3	0.5

*=hybrids between Salix viminalis and Salix schwerinii

Frost

Clones used in biomass plantations today cannot be grown successfully in northern Sweden due to their sensitivity to frost. Even in central Sweden frost damage can be severe in certain places and years. In order to improve the frost tolerance in the breeding material, observation trials are placed not only at sites with normal climatic conditions but also at a site in central Sweden (Fig. 2), which prior to being cultivated was a bog, with generally much lower temperature than the surroundings. Some of the clones are also sent to Röbäcksdalen in the north of Sweden for tests under even more severe conditions (Fig. 2).

In general, the *S. dasyclados* clones seem to have a better tolerance to frost than clones of S. viminalis. However, a promising source for frost resistance in S. viminalis has been received from Iceland. This clone can obviously stand the severe winter conditions both on Iceland and in northern Sweden.

At present no selection is being made with regards to stem-infecting fungi. However, improved frost and rust resistance should secondarily lower the risk for stem infections by weak parasites like *Cryptodiaporthe salicella*.

Insects

Although there is a great number of insects feeding on willows, at present, only two types are selected against in breeding material; those that cause ramification by feeding on shoot tips and a gall midge, *Dasineura marginemtorquens*, forming pocket galls on the leaf margins.

Ramification cause extra work at cutting production since ramified shoots need to be sorted out from the bundles of normally unramified annual shoots. The ramified shoots are either discarded or used after the side shoots have been cut away. Various lepidopterans and a recently identified gall midge, *Dasineura novo* sp., induce such ramification, as their larvae feed on terminal leaf buds. Selection against this type of damage is made in all the steps of the breeding.

The extent of damage due to other types of insect attack such as sucking of plant juices by aphids and leaf hoppers or leaf browsing by beetles, sawflies or lepidopterans is not known well enough to motivate breeding efforts. As leaf galls by *D. marginemtorquens* are very common in the *S. viminalis* plantations and there are indications of biomass losses due to this midge when infestations are severe, clones with resistance are preferred if there is variation for the trait. The released varieties Orm and Rapp are partially resistant to this midge and the coming Björn and Tora are even more resistant (Tab. 4).

BREEDING FOR EUROPE

Attempts to breed willows for biomass coppice have earlier been made also in other countries like Canada (Zsuffa 1988), Finland (Viherä-Aarnio 1988; Pohjonen 1991) and New Zealand (Hathaway 1988). However, the Swedish programme is, so far, the most extensive and longlasting. In addition to the breeding activities at Svalöf Weibull AB described in the present paper there are longterm breeding activities at The Swedish University of Agricultural Sciences in Uppsala (Gullberg 1988). Now there is an increasing interest in testing clones from the Swedish programme in other European countries. Plantations of the most promising clones have been established in Denmark, Finland, UK, Germany and France. Furthermore, a proposal has been sent to the EC which suggests an international cooperation on willow breeding. The aim is to produce clones adapted to a wide range of biotic and abiotic conditions, using molecular markers as one of the breeding tools.

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Gender determination in *Populus*

D. NICHOLAS MCLETCHIE [1] & GERALD A. TUSKAN [2]
[1] Biological Sciences, University of Kentucky, Lexington, KY 40506, USA
[2] Biofuels Feedstock Development Program, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

McLetchie, D.N. & G.A. Tuskan 1994. Gender determination in *Populus*. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 57-66. ISSN 0802-1600.

Gender, the expression of maleness or femaleness, in dioecious plants has been associated with changes in morphology, physiology, ecological position, and commercial importance of several species, including members of the Salicaceae family. Various mechanisms have been proposed to explain the expression of gender in Salicaceae, including sex chromosomes, simple Mendelian genes, quantitative genes, environment, and genotype-by-environment interactions. Published reports would favor a genetic basis for gender. The objective of this study was to identify molecular markers associated with gender in a segregating family of hybrid poplars. Bulked segregant analysis and chi-squared analysis were used to test for the occurrence of sex chromosomes, individual loci, and chromosome ratios (i.e., ploidy levels) as the mechanisms for gender determination. Examination of 2488 PCR based RAPD markers from 1219 primers revealed nine polymorphic bands between male and female bulked samples. However, linkage analysis indicated that none of these markers were significantly associated with gender. Chi-squared results for difference in male-to-female ratios between diploid and triploid genotypes also revealed no significant differences. These findings suggest gender is not controlled via sex chromosomes, simple Mendelian loci or ratios of autosome to gender-determining loci. It is possible that gender is 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. It is also possible that gender is determined environmentally at an early zygote stage, canalizing gender expression.

Key words: Bulked segregant analysis, chi-squared analysis, gender determination, polyploidy, *Populus*, RAPD markers.

D. Nicholas McLetchie, Biological Sciences, University of Kentucky, Lexington, KY 40506, USA

Genetic selection for desired traits (e.g. biomass production, disease resistance, or drought tolerance) in economically important crop species is an integral part of agricultural research and development. Plant species with a dioecious reproductive habit differ from other crops in that male and female reproductive structures occur on separate individuals. Traits may vary between the sexes in dioecious systems partially because males and females are under different reproductive constraints; i.e., males are typically involved in high gamete output

and effective gene transfer, while females are typically committed to maximizing the success of individual zygotes (Stebbins 1950). Consequently, morphological and/or physiological differences may exist between the sexes, differences which often have a genetic basis (Dellaporta & Calderon-Urrea 1993).

In plant breeding programs, attempts to select for a trait of interest can be complicated by gender since maleness or femaleness can only be determined at the onset of flowering in most dioecious plants species. If the trait of interest is gender ratio adjustment in different ecological settings or if the trait of interest is physiologically or morphologically associated with gender then trait evaluations can only be completed at reproductive maturity. The ability to identify gender in dioecious species at the zygote or seedling stage, would increase genetic gains per unit time within a breeding program can be increased.

Examples of gender associated traits in Salicaceae (the willow family - which contains two genera, *Salix* and *Populus*) have been identified where male clones have been shown to maintain higher turgor pressures at lower water contents (Dawson & Bliss 1989, Tschaplinski et al. 1994; Tschaplinski & Tuskan 1994). Female clones of intraamericana *Populus* hybrids tend to produce greater numbers of lateral branches (Tschaplinski et al. 1994). In *S. arctica*, Dawson & Bliss (1993) found that females have higher carbon assimilation rates than males. Grant & Mitton (1979), working with *P. tremuloides*, found that female clones produce higher mean annual growth than male clones. Female clones also tend to maintain higher basal area in vegetatively propagated stands (Sakai and Burris 1985). Alternatively, Farmer (1964) reported that male clones of *P. deltoides* were significantly taller than female clones, though there was no apparent male numerical advantage as evidenced by a 1:1 ratio of male to females in the examined native stands. In *P. trichocarpa*, the frequency of male clones was higher on more xeric sites (Reed 1994) and male clones tend to be more common in *P. tremuloides* (Pauley & Mennel 1957) and in *P. tremula* (Falinski 1980). Independent of gender ratio, male clones of *P. tremuloides* are the preferred food source for ruffed grouse (Schladweiler 1968; Gullion & Svoboda 1972). And, Dannell et al. 1985 and Elmqvist et al. 1988 reported that male clones of *S. myrsinifolia x phylicifolia* are the preferred browse of voles. Insect damage has also been reported to be greater on male clones of *S. cinerea* (Alliende 1989). The occurrence of phenolic glucosides is thought to confer resistance to herbivory. Although no gender-based differences were detected in *S. sericea* (Nichols-Brians et al. 1993), Boecklen et al. (1990) and Elmqvist et al. (1991) were able to detect higher concentrations of phenolic glucosides in female clones of *S. lasiolepis* and *S. rigida*, respectively. Such differences have fueled interest in gender-determining mechanisms.

The gender-determining mechanism within Salicaceae is as yet unknown. Lester (1963) reported that 38% of the sampled *P. tremuloides* individuals (5.3% of the sampled flowers) expressed deviations from dioecy across environmental gradients. Heslop-Harrison (1924) noted that mite infestation causes female flower structures to arise on male flowers. Both of these reports suggest an environmental factor controlling gender in Salicaceae. Alternatively, Heslop-Harrison (1924) reported the identification of sex chromosomes in several *Salix* species and in *P. tremula*, though later researchers have not been able to confirm their existence (Mitton & Grant 1980 and ref. therein). Mosseler & Zsuffa (1989) reported female bias among progeny of intra- and interspecific crosses of numerous *Salix* species. Gender ratio was found to be genetically determined in *P. tremuloides* L.

(Valentine 1975). Furthermore, Heslop-Harrison (1924) noted that in *Salix*, diploid species tend to have a 1:1 gender ratio while polyploids tend to be female biased. Several studies report no gender reversals, including Dawson & Bliss (1989) working with *S. arctica* over a four year period, Alliende & Harper (1989) working with *S. cinerea*, and Mitton & Grant (1980) working with *P. tremuloides*. These reports suggest that gender is genetically and not environmentally determined in this taxonomic family.

In addition to the applications noted above, basic research dealing with gender differentiation will be facilitated through the use of a gender-related marker. Gender identification using molecular techniques has been met with some success at the level of isozymes (Maestri et al. 1991; Schnabel & Hamrick 1990) and DNA probes (Mulcahy et al. 1992). The primary objective of this study is to use DNA markers to test for the presence of genetic markers associated with gender; and secondly, to draw inferences regarding the importance of genetic vs. environmental factors controlling gender.

MATERIALS AND METHODS

An F_1 hybrid pedigree consisting of six male and six female progeny, from a cross between a black cottonwood (*Populus trichocarpa* Torr. & Gray '93-968') female clone and eastern cottonwood (*P. deltoides* Bartr. 'ILL-129') male clone at the University of Washington, was used to obtain DNA for the male and female bulked samples. DNA characterization relied on a Polymerase Chain Reaction (PCR) procedure for generating randomly amplified polymorphic DNA (RAPD) markers (Welsh & McClelland 1990 and Williams et al. 1990). The RAPD reactions were conducted in a MJ Research PTC-100 thermocycler (MJ Research Inc., Chatham, NJ) with a 35 cycle protocol involving 5 s at 94°C denaturation, 30 s at 36°C annealing, and 1 min at 72°C polymerization steps within each cycle, followed by a 5 min final polymerization step. Each reaction contained 50mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 1.0 ng Bovine Serum Albumin, 0.5 units *Taq* polymerase, 200 *u*M each dNTP, 10 ng primer, and 1.0 ng DNA template, to a final volume of 10 *u*l. Bulked segregant analysis was used to identify polymorphic DNA markers (Michelmore et al. 1991). Similar techniques have been used to identify genetic markers associated with 1) differences between and within cultivars (Hu & Quiros 1991), 2) disease resistance (Martin et al. 1991; Paran et al. 1991), and 3) the Y chromosome in *Silene latifolia* (Mulcahy et al. 1992). This analysis involved screening male and female bulked samples of DNA from individuals originating from the above pedigree with approximately 1219 different primers (539 primers from Operon Technologies inc., Alameda, CA and 680 primers from the University of British Columbia, Vancouver, B.C., Canada). DNA markers that were found to be polymorphic between the bulks were used to confirm the presence of gender-determining loci by separating individual plants that constituted the bulks and then testing for consistency within gender by means of linkage analysis. Logarithm of the odds ratio for linkage (LOD) scores and linkage estimates were provided Dr. Toby Bradshaw, University of Washington, through the use of MAPMAKER-QTL version 1.1 (Lander & Botstein 1989).

Chi-squared analysis was used to test for the possibility that the ratio of gender-determining loci to autosomal loci controls gender expression in *Populus*. Pedigree

information regarding gender and ploidy level from the *Populus* breeding effort at the University of Washington (Stettler 1991) was used in this analysis. First, observed male-to-female ratios were tested against the expected 1:1 ratio for all diploid and triploid clones. Next, the gender ratio for the diploid clones was used as the expected ratio to test for deviations in the gender ratio expressed in the triploid clones. A total of 418 diploid progeny and 110 triploid progeny from 50 hybrid families were used in the analysis.

RESULTS AND DISCUSSION

Of the 1219 random 10-mer primers examined, 381 revealed no amplified bands among the bulked samples. The remaining 838 primers yielded 2488 bands, with an average of 2.7 bands per primer for the Operon primers and 1.5 bands for the UBC primers. The majority of the bands were monomorphic between the male and female bulked samples. Seven primers did reveal nine polymorphisms between male and female bulks (Table 1). The segregation ratios among individual genotypes are presented in Table 1. None of the polymorphic bands occurred with 100% fidelity within a single gender. That is, the frequency of each polymorphic band was higher in one gender or the other, but no bands were found solely in all individuals of one gender and absent in the other. Linkage analysis for these segregating bands indicated that none of the bands were significantly associated with gender (LOD scores ranged between .25 and 1.05, critical LOD score=3.5 at $P \leq 0.05$). The estimates of linkage distance ranged from 62.6 cM to 27.9 cM.

Of the 418 diploid progeny, 23.9% were flowering; 46 were male and 54 were female. Fifty-four percent of the triploid progeny were flowering, resulting in 28 male and 31 female progeny. Chi-squared values for testing deviations in gender ratios in diploid and triploid progeny revealed no significant departures from the expected 1:1 ratio ($X^2 = 0.51$ and 0.17, respectively, critical $X^2 = 3.841$ at $P \leq 0.05$). In addition, there were no significant differences in the observed male-to-female ratios between the diploid and triploid progeny.

It was anticipated that DNA markers could accurately identify the gender of the tested individuals. The bulked segregant analysis and subsequent linkage analysis failed to identify such markers. It is possible that different primers may probe a portion of the genome not sampled by the tested primers used in this study, thus revealing markers that are linked to gender-determining loci. This seems unlikely given the number of primers tested and the nature of the random 10-mers. Bulked segregant analysis has been used successfully to identify gender-related markers in *Silene latifolia* (Mulcahy et al. 1992). Here four markers were found after examining only 40 primers, although it was previously known the *Silene latifolia* contained sex chromosomes. The lack of gender-related markers detected in this study would argue against the presence of sex chromosomes in *Populus*. It is still possible that gender is controlled by separate loci operating in an additive, threshold manner or in an interacting epistatic manner (C. Alstrom 1994, personnel communication, Dellaporta & Calderon-Urrea 1993). Bulked segregant analysis would not be effective in detecting such loci outside of a structured pedigree segregating for a single gender-determining locus.

Table 1. The presence (+) or absence (-) of randomly amplified polymorphic DNA markers in a segregating F_1 hybrid poplar family for nine out of 1219 selected 10-mer primers

Genotype	Gender	Random 10-mer Primer [1]								
		UBC523-575	UBC269-340	AG5-550	AG5-500	P9-850	P9-550	P14-650	R15-550	S11-280
50-180	male	+	+	-	-	+	+	-	+	-
50-181	male	-	-	+	-	+	-	+	+	-
50-183	male	+	+	+	-	+	+	+	+	-
50-186	male	-	-	-	-	+	+	-	-	+
50-190	male	+	-	+	-	+	-	+	-	+
50-194	male	+	+	+	-	-	+	-	+	+
50-185	female	-	-	-	+	-	+	-	-	+
50-188	female	-	-	+	-	-	-	-	+	+
50-191	female	-	-	-	+	-	-	-	-	+
50-192	female	-	-	-	+	+	+	-	-	+
50-195	female	-	-	-	-	-	-	-	+	+
50-196	female	-	-	+	-	+	-	-	-	-

[1] The UBC primers were purchased from the University of British Columbia, Vancouver, BC, Canada; the remaining primers were purchased from Operon Technologies Inc., Alameda, CA. Primer sequences are available upon request

The similarities between the male and female ratios for diploid and triploid progeny suggest that it is unlikely that gender is determined via ratios of autosomes to gender-determining loci. In other words, the additional haploid set of chromosomes, contributed by the female parent (Bradshaw & Stettler 1992), did not bias the gender ratio in the triploid progeny. Additionally, across the 50 families used in this analysis, there appears to be no bias towards one gender or the other, though this ratio does vary on a within family basis. It is possible that as more individuals within these 50 families begin to flower there will be a shift towards female biased ratios, given that male clones tend to flower at an earlier age (Kaul & Kaul 1984).

As Karlin & Lessard (1986) indicate gender determination in dioecious species usually occurs at syngamy. It may be possible that in *Populus* gender is controlled by the environment (e.g. temperature, nutritional status, etc.) at the moment of syngamy and once determined it remains tightly canalized during ontogeny. Similar environmental gender determination mechanisms occur in nematodes, amphibians, fish, and reptiles (Bull & Vogt 1979; Charnov & Dawson 1989; Ross et al. 1983). Furthermore, Freeman et al. (1980) and Charnov & Dawson (1989) suggest environmental gender determination offers some advantages to perennial plant species that occupy patchy environments, as do members of the Salicaceae family.

CONCLUSIONS

Reported literature would suggest that gender is genetically controlled in *Populus* and that gender is consistently expressed across developmental age and environments. Bulked segregant analysis and chi-squared analysis failed to identify molecular markers associated with gender or relationships between gender and ploidy level, respectively. The genetic basis of gender determination in *Populus* must thus involve multiple interacting loci, or alternatively involve environmentally determined effects early in ontogeny.

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Differentiation of poplar and willow clones using RAPD fingerprints

D. LIN, M. HUBBES & L. ZSUFFA
Faculty of Forestry, University of Toronto, Toronto, Ontario, Canada

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RAPD (Random Amplified Polymorphic DNA) fingerprints were used to differentiate species and hybrids of the genera *Populus* and *Salix* as well as to identify individual clones. Fifty-five poplar clones and 81 willow clones were included in this study. Of the 17 random DNA primers tested, only 4 primers (Chl-1, Deca-2, 7, 10) were needed to differentiate 55 poplar clones into species and hybrids. Individual clones were further characterized by their DNA polymorphism obtained with primer Chl-1 or Deca-7. DTAC clones from different parentage origin, i.e., *P. deltoides x trichocarpa* and *P. trichocarpa x deltoides*, could be distinguished by primer Chl-1. Although species and hybrids of tree-type willow clones could be differentiated with primers 2114, 2115 and 2116, the identity of several clones needs to be verified. DNA polymorphisms obtained with primers Chl-2 and Chl-4 distinguish species and hybrids of shrub willow clones originating from N. America. The influence of parentage on hybrid clones was also evident from their DNA polymorphisms. Species of *S. udensis*, *S. dasyclados* and *S. viminalis* could be differentiated by primers 2114 and Chl-1. Willow clones from the U.K. showing different reactions against *Melamspora* rust could not be distinguished into species and hybrids. Genetic relationships among these clones were analyzed with a dendrogram.

Key words: DNA fingerprinting, DNA polymorphisms, PCR, RAPD markers, *Salix*, *Populus*.

D. Lin, Faculty of Forestry, University of Toronto, Toronto, Ontario, M5S 3B3, Canada

Interest in the use of poplars and willows in short rotation intensive culture plantations for energy, chemicals and fibre has been developing over a decade (Zsuffa et al. 1984). Due to easy clonal propagation, species and hybrid materials were inadequately defined. Recent progress in the field of molecular biology has resulted in the development of new analytical tools that are well suited for taxonomic investigations.

RFLPs have been used successfully to differentiate clones and species of *Populus* (Keim et al. 1989; D'Ovidio et al. 1990, 1991: D'Ovidio 1992; Faivre-Rampant et al. 1992a, 1992b). The procedure of RFLPs is time consuming and expensive, requiring restriction enzymes, Southern blotting, and specific probes for DNA labelling (Castiglione et al. 1993). The recently introduced polymerase chain reaction (PCR) (Saiki et al. 1988)

combined with random amplified polymorphic DNA (RAPD) are powerful tools for detecting DNA polymorphisms linked to different species or hybrids of *Populus* and *Salix* (Lin et al. 1994). This method is quick and requires only small amounts of DNA (Castiglione et al. 1993). Therefore, DNA fingerprints by RAPD markers were chosen for clonal identification of poplars and willows in the present study.

MATERIALS AND METHODS

Plant material

Fifty-five poplar clones (Table 1) and 81 willow clones (Table 2) were analyzed. Clones were selected according to frequency of cultivation in Europe and North America (Castiglione et al. 1993), and for their potential use in plantations and biomass production (Steenackers et al. 1990; Zsuffa 1990). Several willow clones obtained from UK and Sweden are used for biomass production. Dormant cuttings were collected and two cuttings taken from each clone and rooted in the greenhouse at the Faculty of Forestry, University of Toronto.

Table 1. List of Poplar Species and Hybrids for Clonal Characterization

Species	No. of clones
P. x balsamifera	3
P. x jackii	3
P. deltoides	5
P. trichocarpa x deltoides	3
P. deltoides x trichocarpa	2
P. x euramericana	25
P. nigra	6
P. nigra x maximowiczii	5
P. maximowizii	3
TOTAL	55

DNA extraction

Young leaves were collected from the rooted cuttings. After washing with distilled water and drying on a paper towel, leaf tissue (0.5 g) was ground to powder in liquid nitrogen and then transferred to 10 ml of pre-heated 2x CTAB isolation buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide ["CTAB"], 0.2% 2-mercaptoethanol). After thorough stirring the mixture was incubated at 60°C for 30 minutes, extracted twice with chloroform-isoamyl alcohol (24:1), and precipitated with 2/3 vol of cold isopropanol (Doyle and Doyle 1987). The DNA concentration was estimated by comparing serial dilutions of DNA with those in agarose gels stained with ethidium bromide.

Table 2. List of willow species and hybrids for clonal characterization

Species	Section	No. of clones
I. Tree-Type		
S. nigra	Humboldtianae	2
S. glatfelteri x alba		1
S. alba	Salix	7
S. x rubens (S. alba x fragilis)	Salix	3
S. fragilis	Salix	4
II. Shrub-N. America		
S. exigua	Longifoliae	2
S. exigua x eriocephala		1
S. eriocephala x exigua		1
S. eriocephala	Cordatae	6
S. eriocephala x petiolaris		1
S. petiolaris x eriocephala		1
S. petiolaris	Vetrix	2
S. purpurea	Helix	3
III. Shrub-UK and Sweden		
S. bebbiana	Vetrix	1
S. burjatica		2
S. x calodendron		1
S. daphnoides	Daphnella	1
S. disperma		1
S. hirtei		2
S. mollisima		1
S. sericans		2
S. x stipularis		1
S. udensis	Vimen	4
S. miyabeana or *S. rorida*	?	1
S. miyabeana	Helix	1
S. dasyclados	Vimen	6
S. viminalis	Vimen	23
TOTAL		81

Primers

The sequences of 17 primers used for the PCR reaction are given in Table 3.

Amplification of DNA by PCR

The amplification reaction was performed in 15 ul of buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 200 uM each of dATP, dCTP, dGTP and dTTP, 0.5 units of Taq DNA polymerase (Boehringer Mannheim), 15 pmol primer and 10 ng of DNA. The amplification reaction was performed in the Perkin-Elmer DNA Thermal Cycler (Model TC-1), programmed for 40 cycles of 2 minutes of denaturing at 94°C, 1 minute of annealing at 40°C and 5 minutes of extension at 72°C before an initial denaturation of 2 minutes at 94°C. The program was followed by an extension phase at 72°C for 10 minutes.

DNA from all clones was subjected to three independent amplifications, and only fragments that were repeatable in all assays were scored for species and clonal characterization.

Table 3. Sequences and sources of primers used in the RAPD analysis

No	Name	Sequence	References
1.	Chl-1	5'-GAGGCCTACGCCCCATAGAA-3'	Castiglione et al. 1993
2.	Chl-2	5'-AATGCGTTGAGGCGCAGCAG-3'	
3.	Chl-4	5'-TTCCCGTGTCTTCCGGCTTAC-3'	
4.	Chl-10	5'-TTCTTCTCCTACCAGTATCG-3'	
5.	Deca-2	5'-GCGATCCGGC-3'	
6.	Deca-4	5'-CGTTGGCCCG-3'	
7.	Deca-5	5'-CCAAGGGGGC-3'	
8.	Deca-7	5'-CCGCCCGGAT-3'	
9.	Deca-9	5'-TGGCCCCGGT-3'	
10.	Deca-10	5'-AGCCGGCCTT-3'	
11.	Deca-11	5'-ATCGGCTGGG-3'	
12.	Deca-12	5'-CTTGCCCACG-3'	
13.	Deca-13	5'-GTGGCAAGCC-3'	
14.	2114	5'-GACTGCCTCT-3'	Operon Technologies
15.	2115	5'-GAAACGGGTC-3'	
16.	2116	5'-GTGACCGAGT-3'	
17.	2117	5'-CAGAAGCGGA-3'	

Electrophoresis in agarose gel

Amplification products (15 ul) were analyzed by electrophoresis in 1.5% agarose gel (15 x 15 cm) at 100 V until the tracking dye (tartrazine) migrated toward the end of the gel. Gels were stained in 0.5 g/ml ethidium bromide solution and photographed in UV-light.

RESULTS AND DISCUSSION

Poplar clones used in this study represented 8 species and hybrids (Table 4). Willow clones, according to their shape and origin, were separated into tree willows (Table 5), shrub willows of N. America (Table 6), and shrub willows of UK and Sweden (Table 7).

Poplars

Of 17 primers tested, only 4 primers (Deca-10, Deca-2, Chl-1 and Deca-7) (Figs. 1-4) were needed to distinguish all 55 poplar clones into species and hybrids (Table 8). Species and hybrids were also differentiated by the molecular sizes of RAPD markers (Table 8). Individual clone could be characterized by primer Chl-1 (Fig. 3) and/or Deca-7 (Fig. 4). DTAC clones from different parentage origin, i.e., *P. deltoides x trichocarpa* and *P. trichocarpa x deltoides*, could be distinguished by primer Chl-1 (Fig. 3).

Table 4. Poplar (*Populus* L.) clones and their origin for RAPD analysis (Figs. 1-4)

Clone no.	Species and hybrid	Origin
Upper Gel		
1. TAC 8	*P. balsamifera*	Ontario
2. TAC 21	*P. balsamifera*	Ontario
3. TAC 51	*P. balsamifera*	Manitoba
4. JAC 4	*P. x jackii*	Ontario
5. JAC 7	*P. x jackii*	Ontario
6. JAC 28	*P. x jackii*	Manitoba
7. D 35	*P. deltoides* (W-1-2) Larsson	Ontario
8. D 39	*P. deltoides*	Ontario
9. D 196	*P. x deltoides* cv. (northwest)	Sask
10. D 391	*P. deltoides* (Mixoploid) (Brockville #C136)	Ontario
11. DTAC 7	*P. trichocarpa x deltoides* cl. 'Unal'	Geraardsbergen, Belgium
12. DTAC 8	*P. trichocarpa x deltoides* cl. Beaupre	Geraardsbergen, Belgium
13. DTAC 26	*P. trichocarpa x deltoides* cv. 'Boelare'	Geraardsbergen, Belgium
14. DTAC 9	*P. deltoides x trichocarpa* cv. 'Donk'	Wageningen, Holland
15. DTAC 10	*P. deltoides x trichocarpa* cl. 'Barn'	Wageningen, Holland
16. DN 5	*P. x euramericana* cv. 'Gelrica'	W Germany
17. DN 14	*P. x euramericana* cv. 'Harff'	W Germany
18. DN 16	*P. x euramericana* cv. 'Batarde d'Hauterive'	France
19. DN 17	*P. x euramericana* cv. 'Robusta'	France
Lower Gel		
1. DN93	*P. x euramericana* "Triplo" cl. I-37/61	Casale Monferrato, Italy
2. I-45/51	*P. x euramericana*	Casale Monferrato, Italy
3. I-214	*P. x euramericana*	Casale Monferrato, Italy
4. I 455	*P. x euramericana*	Casale Monferrato, Italy
5. DN 21	*P. x euramericana* cv. 'I-78B' (LW42)	Italy
6. DN 30	*P. x euramericana* cv. 'Canada Blanc'	Spain
7. DN 173	*P. x euramericana* (cl. 'Dorskamp') (Koster 925)	Holland
8. DN 177	*P. x euramericana* (cl. 'Spijk') (Koster 2195)	Holland
9. N 75	*P. nigra* (#113)	Hungary
10. N 84	*P. nigra* "Italica" (#555/50)	W Germany
11. N 91	*P. nigra* "Purkersdorf" (#44/62(10))	Austria
12. N 100	*P. nigra* L. Kunovice 312/65(002/66)	Czechoslavakia
13. NM 1	*P. nigra x maximowiczii*	Japan
14. NM 2	*P. nigra x maximowiczii* (cl. "Max-1")	W Germany
15. NM 8	*P. nigra x maximowiczii* (cl. #62-7)	Korea
16. M 900	*P. maximowizii*	
17. M 901	*P. maximowizii*	
18. M 908	*P. maximowizii*	

Table 5. Tree willow (*Salix* L.) clones and their origin for RAPD analysis (Figs. 5-8)

Clone no.	Species and hybrid	Origin
1. SN1, W435	*S. nigra*	New York
2. SN3, W437	*S. nigra*	Ontario
3. SGA3, W300	*S. glatfelteri x alba* (SG2 x V95)	
4. SA2, W4	*S. alba* sanguinea (Novi Sad 29)	Hungary
5. SA8, W10	*S. alba* coerulea (Novi Sad 51)(CALVA) (via Austria & Yugoslavia)	England
6. SA13, W15	*S. alba* (I-1/59)	Italy
7. SA16, W18	*S. alba* (I-4/59)	Italy
8. SA20, W22	*S. alba* (V95)	Yugoslavia
9. SA25, W27	*S. alba* (SI-2-61)	?
10. SA27, W29	*S. alba* (VALLEE R-546)(via Quebec)	Romania
11. SA18, W20	*S. x rubens* (*S. alba x fragilis*) (V99)	Yugoslavia
12. SA41, W42	*S. x rubens* (*S. alba x fragilis*) (V160)	Bulgaria
13. SN11, W445	*S. alba x fragilis*	Ontario
14. SF3, W273	*S. fragilis*	Ellice Swamp, Ontario
15. SF8, W278	*S. fragilis*	Blackburn, Ontario
16. SF17, W287	*S. fragilis*	Port Carling, Ontario
17. SF21, W291	*S. fragilis*	Alliston Twp., Ontario

Table 6. Shrub willow (*Salix* L.) (N. America) clones for RAPD analysis (Figs. 9-10)

Clone no.	Species and hybrid
1. S353(INT42)	*S. exigua*
2. S354(INT62)	*S. exigua*
3. S301(INT62 x ER276)	*S. exigua x eriocephala*
4. S625(ER39 x INT42)	*S. eriocephala x exigua*
5. S259(ER16)	*S. eriocephala*
6. S267(ER39)	*S. eriocephala*
7. S289(ER294)	*S. eriocephala*
8. S599(ER39 x PET47)	*S. eriocephala x petiolaris*
9. S71(PET311 x ER294)	*S. petiolaris x eriocephala*
10. S282(PET311)	*S. petiolaris*
11. S400(PET47)	*S. petiolaris*
12. S408(PUR12)	*S. purpurea*
13. S409(PUR34)	*S. purpurea*
14. 097/07(UK)	*S. purpurea* 'Uralensis'

Table 7. Shrub willow (*Salix* L.) (UK and Sweden) clones for RAPD analysis (Figs. 11-12)

Clone no.	Species and hybrid
Upper Gel	
1. 110/01(UK)	*S. bebbiana*
2. 033/01(SAG)	*S. burjatica* 'Korso'
3. 033/03(UK)	*S. burjatica* 'Germany'
4. 041/04(LA3111)	*S. x calodendron*
5. 053/05(UK)	*S. daphnoides* 'Meikle'
6. 068/01(UK)	*S. disperma*
7. 049/03(SR3222)	*S. hirtei* 'Reifenweide'
8. 026/01(UK)	*S. hirtei* 'Delamere'
9. 113/13(UK)	*S. mollisima* 'Q83'
10. 097/07(UK)	*S. purpurea* 'Uralensis'
11. 044/04(SHYB2)(W342)	*S. sericans* '6/18'
12. 044/03(SR3215)	*S. sericans* 'Coles'
13. 029/01(SR3219)	*S. x stipularis*
14. SX61	*S. udensis*
15. SX64	*S. udensis*
16. SX67	*S. udensis*
17. SX68	*S. udensis*
18. SX69	*S. miyabeana* or *S. rorida*?
19. SR3049	*S. miyabeana*
Lower Gel	
1. SC1(ESO77056)(UK)	*S. dasyclados* (=*S. caprea* var. *aquatica*)
2. SD1	*S. dasyclados*
3. ESO79097(UK)	*S. dasyclados*
4. ESO78146	*S. dasyclados*
5. ESO81090(UK)	*S. dasyclados* (SAG)
6. SV1(W555)	*S. dasyclados*
7. 115/49(SV2)(W556)	*S. viminalis* 'mullatin'
8. 115/05(SV4)(W558)	*S. viminalis* 'Q683'
9. 115/04(SV5)(W559)	*S. viminalis* 'Q699'
10. 115/18(SV7)(W561)	*S. viminalis* 'Campbell'
11. 115/34(SV8)(SR3200)	*S. viminalis* 'Bowles Hybrid'
12. ESO78021	*S. viminalis*
13. ESO78101	*S. viminalis*
14. ESO78112(UK)	*S. viminalis*
15. ESO78183(115/54)	*S. viminalis*
16. ESO78195	*S. viminalis*
17. ESO79046(UK)	*S. viminalis*
18. QUE5027	*S. viminalis*

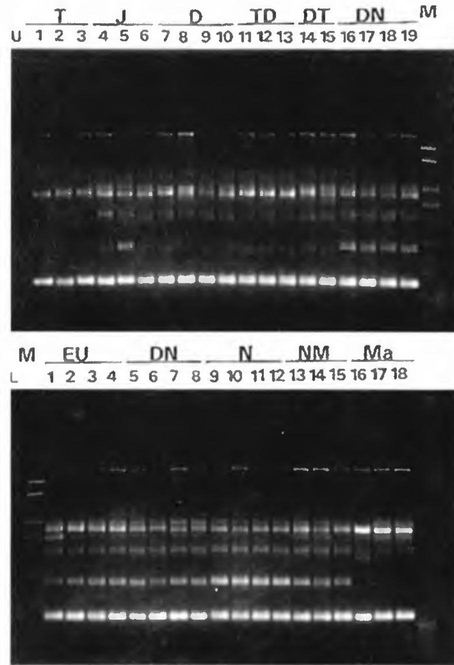

Figure 1. DNA polymorphism of poplar clones amplified with primer Deca-10. M: Molecular Markers VI from BMC. → indicates species differences. The putative species and clone identity is given in Table 4.

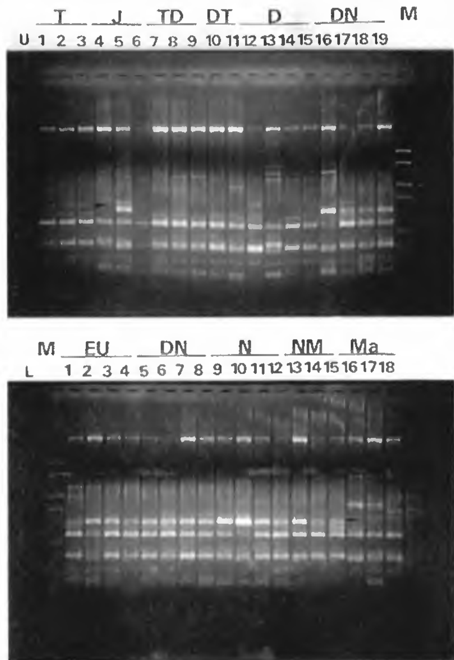

Figure 2. DNA polymorphism of poplar clones amplified with primer Deca-2. M: Molecular Markers VI from BMC. → indicates species differences. The putative species and clone identity is given in Table 4.

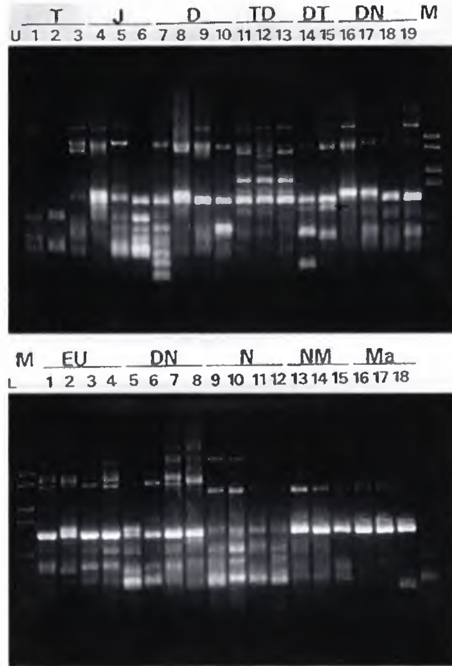

Figure 3. DNA polymorphism of poplar clones amplified with primer Chl-1. M: Molecular Markers VI from BMC. → indicates species differences. The putative species and clone identity is given in Table 4

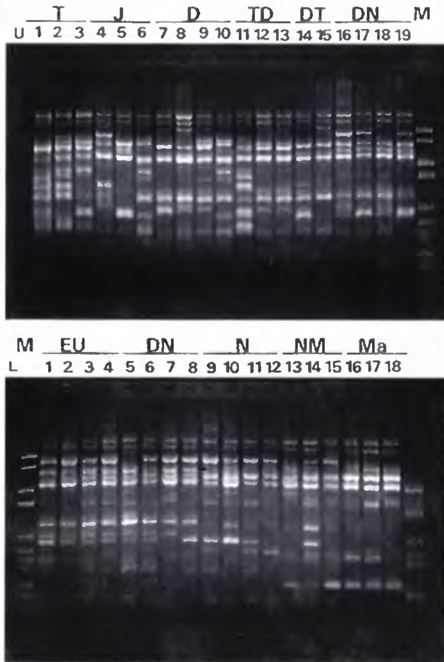

Figure 4. DNA polymorphism of poplar clones amplified with primer Deca-7. M: Molecular Markers VI from BMC. → indicates species differences. The putative species and clone identity is given in Table 4

Table 8. Identification of poplar species and hybrids by primers and molecular marker

Primers	Marker size	Species and hybrids
Deca-10	922	J, D, TD, DT, DN, EU, N, NM, M
	807	M
	600	DN, EU, N, NM
	583	J, D, TD, DT
Deca-2	1102	M
	953	T
	884	DN, EU, N, NM
Chl-1	1710	N, NM, M
	1096	TD
		DT
Deca-7	1649, 1474	DN, EU
	272	NM, M

T: *P. balsamifera*
J: *P. x jackii*
D: *P. deltoides*
DT: *P. deltoides x trichocarpa*
TD: *P. trichocarpa x deltoides*
DN: *P. deltoides x nigra*
EU: *P. euramericana*
N: *P. nigra*
NM: *P. nigra x maximowiczii*
M: *P. maximowiczii*

Willows

Tree willow - Species and hybrids of tree willows were differentiated by primers 2114, 2115 and 2116 (Figs. 5-7). DNA polymorphisms obtained with primers 2115, 2116 and Chl-2 differentiated clones of *S. fragilis* from other species and hybrids (Figs. 6-8). However, many points need clarification for the taxonomy of tree willows. As shown in the results, DNA polymorphism of clone SN11 (*S. alba x fragilis*) resembled that of *S. fragilis*. DNA polymorphisms of clones SA2 and SA13 (*S. alba*) were similar to clone SA41 (*S. alba x fragilis*) with several primers tested (Figs. 6-8). Same result was obtained for clones of *S. nigra* (SN3 and SGA3) and *S. alba* (SA20) (Fig. 8). Since clone SGA3 is a hybrid between *S. glatfelteri* (clone SG2) and *S. alba* (clone SA20), one may assume that clones with the similar DNA polymorphism might come from the same genetic origin.

Shrub willow (N. America) - Only two primers (Chl-2 and Chl-4) were needed to differentiate species of shrub willow originating from N. America (Figs. 9-10). The genetic impact of the female parent was noticed by the DNA polymorphisms obtained from their hybrid clones (Figs. 9-10). For example, clone S301 is a hybrid between *S. exigua* (INT62) and *S. eriocephala* (ER276) with its DNA polymorphisms resembling that of *S. exigua*, whereas clone S625 is a hybrid between *S. eriocephala* (ER39) and *S. exigua* (INT42) with its DNA polymorphisms resembling that of *S. eriocephala*.

Shrub willow (UK and Sweden) - Clones from species of *S. udensis*, *S. dasyclados* and *S. viminalis* show a similarity in their DNA polymorphisms obtained with primers 2114 (Fig. 11) and Chl-1 (Fig. 12). However, clones from other willow species exhibited individual DNA polymorphism. This is probably due to their parentages originating from various genetic sources (Table 8). Some of these clones obtained from the U.K. showing

different reactions against *Melampsora* rust (D. Royle, personal communication). Genetic relationships among these clones is shown in the dendrogram by scoring the polymorphisms of different primers used in band-sharing analyses (Fig. 13).

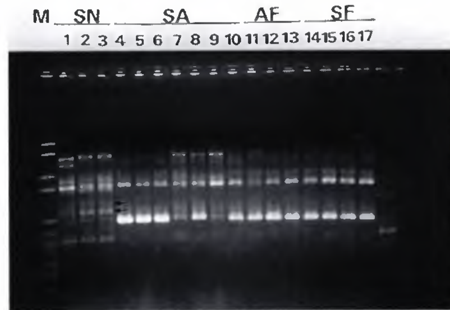

Figure 5. DNA polymorphism of tree-type willow clones amplified with primer 2114. → indicates species differences. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 5

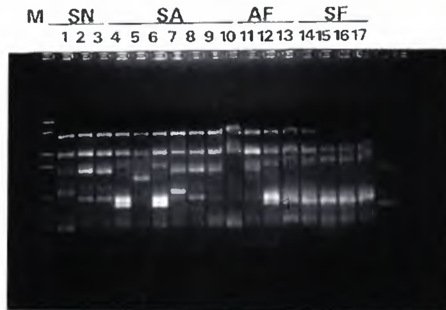

Figure 6. DNA polymorphism of tree-type willow clones amplified with primer 2115. → indicates species differences. ▷ indicates similarity among clones. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 5

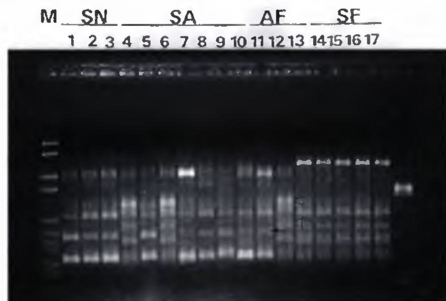

Figure 7. DNA polymorphism of tree-type willow clones amplified with primer 2116. → indicates species differences. ▷ indicates similarity among clones. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 5

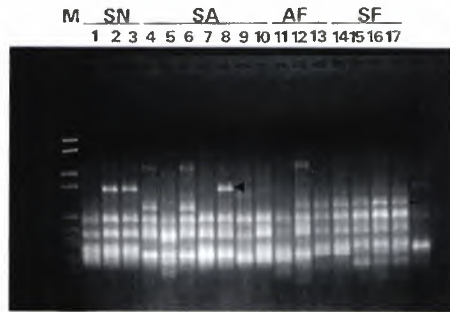

Figure 8. DNA polymorphism of tree-type willow clones amplified with primer Chl-2. → indicates species differences. ▷&▸ indicates similarity among clones. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 5

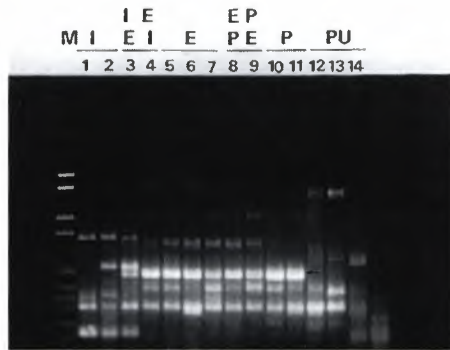

Figure 9. DNA polymorphism of shrub willow clones (N. America) amplified with primer Chl-2. → indicates species differences. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 6

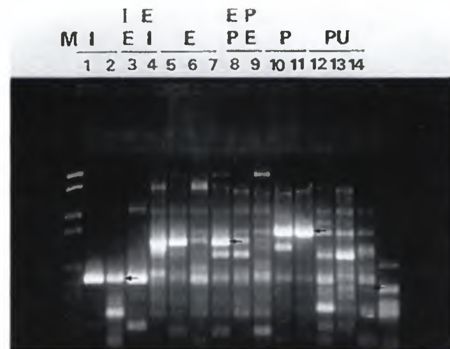

Figure 10. DNA polymorphism of shrub willow clones (N. America) amplified with primer Chl-4. → indicates species differences. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 6

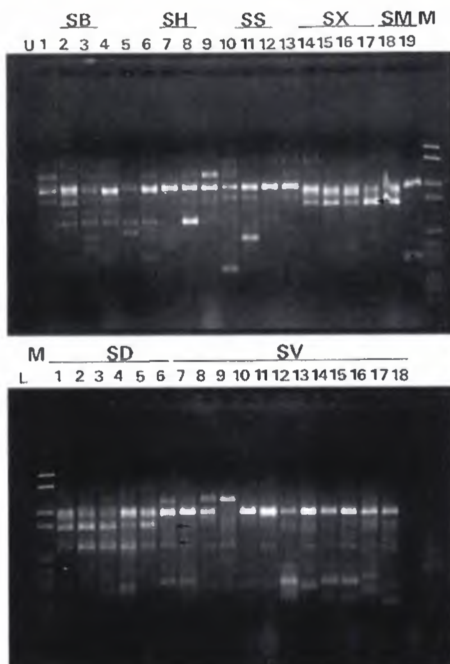

Figure 11. DNA polymorphism of shrub willow clones (UK and Sweden) amplified with primer 2114. → indicates species differences. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 7

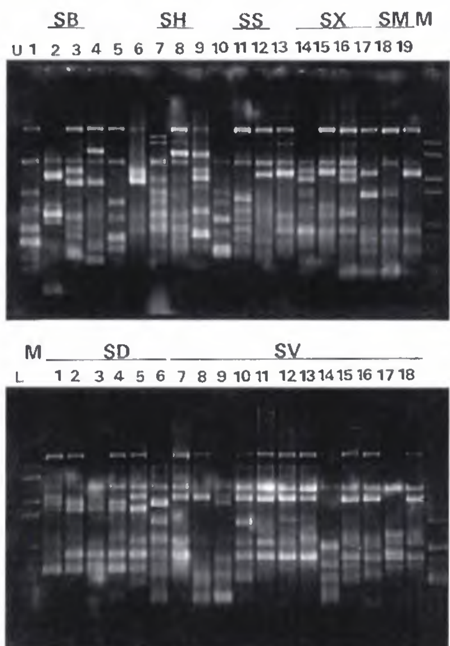

Figure 12. DNA polymorphism of shrub willow clones (UK and Sweden) amplified with primer Chl-1. → indicates species differences. M: Molecular Markers VI from BMC. The putative species and clone identity is given in Table 7

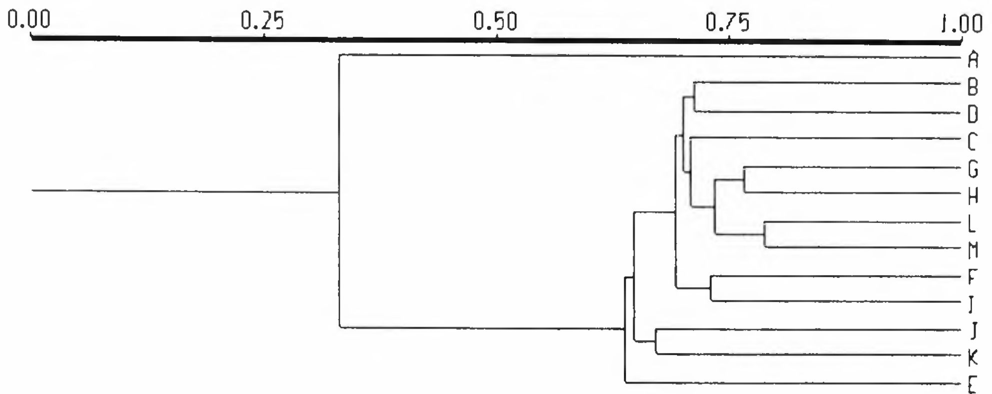

Figure 13. UPGMA dendrogram of shrub willow clones (UK). A: *S. bebbiana*, B: *S. burjatica* 'Korso', C: *S. burjatica* 'Germany', D: *S. x calodendron*, E: *S. daphnoides* 'Meikle', F: *S. disperma*, G: *S. hirtei* 'Reifenweide', H: *S. hirtei* 'Delamere', I: *S. mollisima* 'Q83', J: *S. purpurea* 'Uralensis', K: *S. x stipularis*, L: *S. sericans* '6/18', M: *S. sericans* 'Coles'

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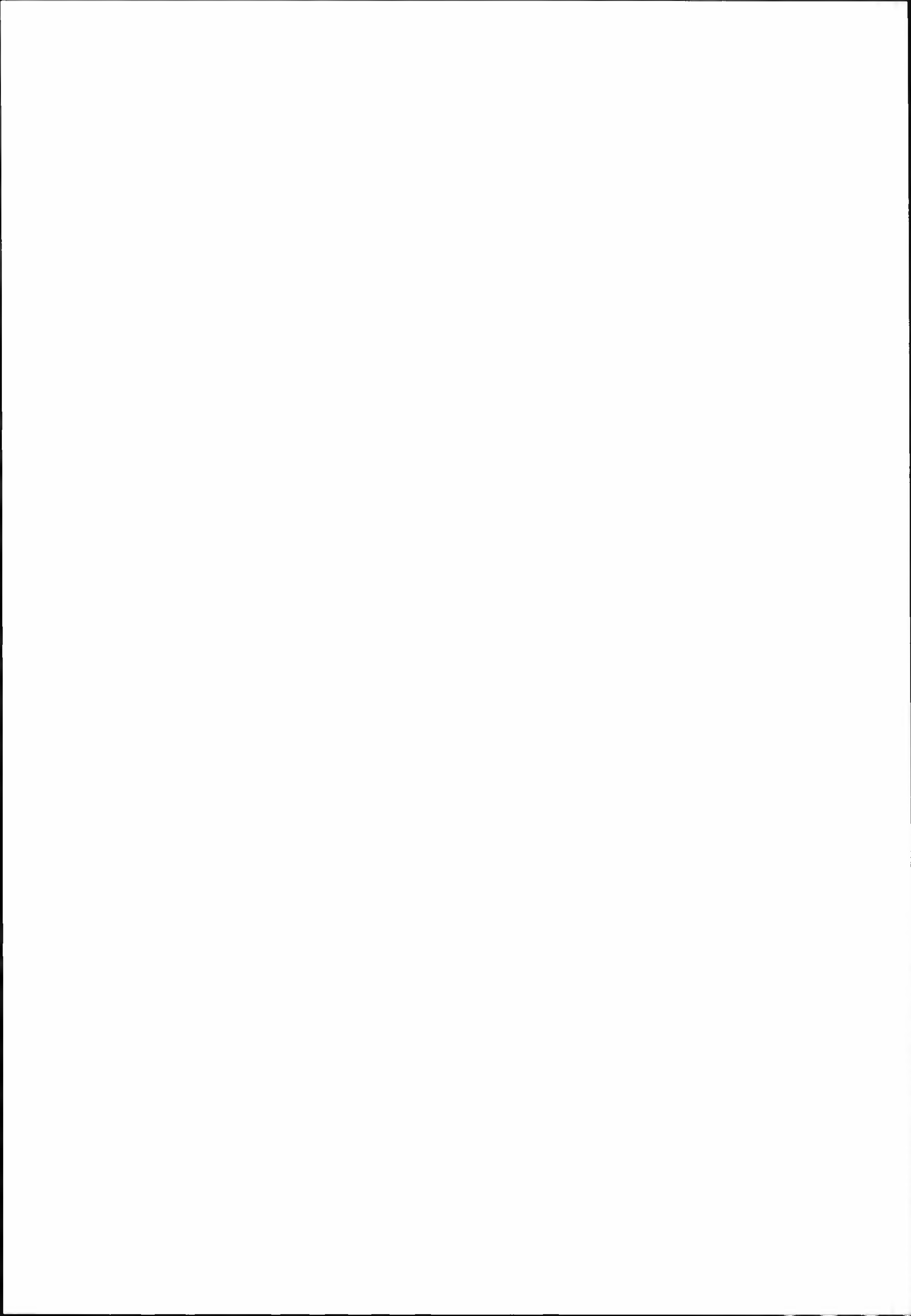

Preliminary trials of woody biomass species tolerance to irrigation with pulp and paper mill wastewater

MICHAEL R. WAGNER[1] & MOHAMADU AW[2]
[1]Northern Arizona University, School of Forestry, Flagstaff, AZ, USA
[2]La Station Terrienne de Toujounine, Nouakchott, Mauritania

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Seventy-eight selections of woody plants were experimentally tested for their tolerance to irrigation with saline pulp and paper mill wastewater in east-central Arizona. *Cupressus arizonica*, *Eleagnus angustifolia*, and *Ulmus pumila* survived well and grew at a rate of greater than 40 cm per year in height. Numerous species and genotypes in *Populus* spp., *Robinia* spp., and *Salix* spp. also performed well. There are strong indications that many of these species can be grown in an intensive biomass plantation under irrigation with wastewater.

Key words: Biomass plantation, selection, species, wastewater.

Michael R. Wagner, Northern Arizona University, School of Forestry, Flagstaff, AZ, USA

The disposal of wastewater generated in the commercial pulp and paper industry is a worldwide concern. There is an increasing trend within the waste treatment industry to utilize biological methods of waste treatment (Dall-Bauman et al. 1990). One biological treatment option under consideration by the pulp and paper industry is land application of sludge and wastewater (Ritter 1992; Nutter 1978). The application of wastewater to grow short rotation intensive biomass plantations is one promising solution to the waste disposal problem that can provide economic benefits to the industry.

Such a land application wastewater treatment system is currently under development by the Stone Container Corporation in Arizona (Aw & Wagner 1993, Aw et al. 1993). One of the many important experimental issues under examination is the selection of the most suitable plant species for the climatic conditions and waste characteristics that exist at the Stone Container facility. A major contaminant in the wastewater that is of concern for plant growth is salt (primarily sodium chloride); a detailed description of the wastewater characteristics is provided elsewhere (Aw et al. 1993).

In this manuscript we report annual growth and survival for three growing seasons of woody species irrigated with saline pulp and paper mill wastewater in east central Arizona. Substantial variation in performance was obtained among the selections tested.

METHODS

A total of 78 selections (58 unique species and 20 hybrids or selected genotypes) were planted in the spring of 1992-1994 at an experimental plantation located approximately 35 km south of Holbrook, Arizona. Plant material was obtained from numerous commercial and scientific sources and was planted as bareroot seedlings, containerized seedlings or unrooted cuttings. Detailed records of the source and condition of planting material is documented elsewhere (Wagner et al. 1993).

The experimental design consisted of a randomized block design. Each species was planted in 4 randomly located plots consisting of 36 (6x6) trees per plot (144 trees/species total). Trees were hand planted and spaced 3.7 x 3.7 m. Prior to planting the site was levelled to facilitate flood irrigation and mechanically tilled. Trees were initially planted in 1992. If a species completely failed in the first season it was replanted with an alternate species in 1993. A few species were selectively replanted in 1993 and 1994 when there was evidence of poor planting stock or damage from animals or mechanical weeding. The planting history of each species is noted in Table 1. All fields were irrigated exclusively with wastewater approximately weekly for about 8 months/year from mid-March to mid-November. We estimate about 300 mm of wastewater was applied per year and an additional 30 mm of natural rainfall occurred at the site.

Survival and annual height growth was determined at the end of the growing season (late September - early October) each year. Measurements were taken only on the interior 16 trees within each 36 tree block to avoid any edge effect. Survival data was considered categorical and analyzed using Chi-squared tests. Tree heights were analyzed using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Survival

Species survival was significantly different based on Chi-squared analysis (Pearson's Chi-squared = 1020, $P \leq 0.000$) and is summarized in Table 1. A variety of factors may be responsible for very low or no survival of some species. The lack of cold tolerance is probably responsible for the non-survival of the following species: *Acacia fornesiana, A. greggi, A. melanoxylon, Casuarina cunninghamiana, Cupressus arizonica* Smoothbark, *Eucalyptus pauciflora, Leucaena retusa, Pinus eldarica, Prosopis juliflora*. When initially included in the species trials, these species were considered to be marginally tolerant of the cold conditions. However, we believed some of these species would have the potential to be used as an annual crop or perhaps would survive below ground and resprout each season. We observed one or two plants of *Eucalyptus pauciflora* and *Prosopis juliflora* that did indeed survive the winter and resprout in two seasons in another experiment at this same site. We believe the approach of using these extremely salt-tolerant and fast-growing species as annual crops still has some validity, but additional research is needed.

A second group of species that survived very poorly was the following hardwoods: *Alnus glutinosa, Cercis canadensis, Cladrastis lutea,* and *Juglans nigra*. We speculate that these species are sufficiently cold tolerant but are not tolerant of the alkali soils (average

pH - 8.2) and the extreme diurnal temperature fluctuation and evaporative demand of the site.

Table 1. Survival and growth of woody species irrigated with saline pulp and paper mill after three growing seasons in east-central Arizona

Species	Planting History		1994	1994
	Initial	Replant 1) 2) 3) 4)	Growth ($\bar{x} \pm SD$)[5]	survival [6] ($\bar{x}$)
Acacia fornesiana	92	1)	-	-
Acacia greggi	92		-	0
Acacia melanoxylon	92	1)	-	-
Acer negundo	93	4)	12.8 ± 16.0	59.2
Ailanthus altissima	92		25.5 ± 22.3	56.3
Albizia julbrissin	92		-21.9 ± 17.1	29.7
Alnus glutinosa Ul	92		-	-
Atriplex canescens	92		19.8 ± 6.6	53.2
Caragana arborescens ASL	92		28.3 ± 6.7	59.4
Casuarina cunninghamiana	92		-	-
Catalpa speciosa	94		-	59.4
Cercis canadensis	92	1)	-	-
Celtis occidentalis	93	4)	4.4 ± 3.7	67.2
Cladrastis lutea	92	1)	-	-
Cupressus arizonica Roughbark	92		47.5 ± 6.5	15.6
Cupressus arizonica Smoothbark	92	1)	-	-
Eleagnus angustifolia	92	4)	68.8 ± 6.4	96.9
Eleagnus angustifolia 'Kings Red'	93	4)	46.8 ± 12.5	79.7
Eucalyptus pauciflora	92	1)	-	-
Fraxinus pennsylvanica	94		-	90.7
Fraxinus velutina	92		39.2 ± 4.1	82.8
Ginko biloba	93	4)	-10.4 ± 1.1	20.3
Gleditsia triacanthos	92		27.2 ± 1.3	84.4
Gymnocladus dioicus	92		11.1 ± 0.6	42.2
Juglans nigra		1)	-	-
Juniperus chinesis	94		-	0
Juniperus scopulorum	92		20.6 ± 4.6	96.6
Juniperus virginiana	93	4)	9.9 ± 3.5	28.2
Larix decidua	92	1)	-	-
Larix laricinia	92	1)	-	-
Larix leptolepsis	92	1)	-	-
Larix siberica	92	1)	-	-
Leucaena retusa	92	1)	-	-

Table 1. Cont.

Species	Planting History Initial	Replant 1) 2) 3) 4)	1994 Growth ($\bar{x} \pm SD$)[5]	1994 survival [6] ($\bar{x}$)
Morus alba 'Tatarica'	93		5.2 ± 7.9	53.2
Picea pungens	92	2)	-	-
Pinus contorta	92	1)	-	-
Pinus contorta contorta	94		-	1.6
Pinus eldarica	92	2)	-	-
Pinus nigra	92		4.8 ± 1.8	81.3
Pinus ponderosa 88 EDU	92		4.8 ± 1.8	79.7
Pinus sylvestris	92		3.5 ± 1.9	71.9
Pinus thumbergii	92	2)	-	-
Poplus alba x P. grandidentata	92		76.7 ± 6.0	57.9
Poplus angustifolia ASL	92		59.2 ± 10.0	70.3
Populus deltoides SCS- Los Lunas	92		-	-
Populus deltoides 14392	93	4)	72.0 ± 0.0	4.7
P. deltoides x P. nigra 1471	93		70.5 ± 9.5	84.4
P. deltoides 'Siouxland'	92	4)	67.6 ± 30.6	57.9
P. deltoides x P. nigra	92		97.7 ± 21.3	79.7
P. euroamericana 'Imperial'	92		107.1 ± 29.2	82.9
P. euromericana 'Norway'	92	4)	68.9 ± 27.3	65.7
P. euromericana 'Robusta'	92	4)	64.0 ± 19.0	43.8
P. fremontii NAU	92	4)	90.6 ± 38.7	68.8
P. fremontii SCS Cot	92	4)	118.7 ± 58.1	51.6
P. fremontii wisliz	92	4)	94.9 ± 13.4	43.8
P. jackii	93	3)	41.7 ± 5.3	76.6
P. nigra 'Italica'	92		54.6 ± 43.1	81.3
Prunus armeniaca var. manchshurica	93		24.4 ± 5.659.4	
Prunus virginiana	92		18.3 ± 4.1	40.7
Prosopis juliflora	92		-	0
Pyrus ussuriensis 'McDermand'	93	4)	11.1 ± 6.3	40.7
Robinia fertilis Schma	93		14.3 ± 0	23.5
Robinia hispida var. fertilis Schma	93		-4.9 ± 0.0	14.1
Robinia neomexicana 90-101	92		46.8 ± 29.1	45.4
Robinia neomexicana 91-Shf	92		13.7 ± 7.6	51.6
Robinia pseudoacacia Schma	92		65.4 ± 44.7	76.6
Robinia pseudoacacia 6663 Baktal	92		40.3 ± 10.7	46.9
Robinia pseudoacacia Bareroot	92		67.1?	95.3
Salix alba	92		35.7 ± 8.1	65.7
Salix alba 'Vitellina'	92		45.8?	34.4

Table 1. Cont.

Species	Planting History Initial	Replant 1) 2) 3) 4)	1994 Growth ($\bar{x} \pm SD$)[5]	1994 survival [6] ($\bar{x}$)
Salix exigua	92		13.1 ± 2.9	95.4
Salix gooddingii VCD	92		42.3 ± 13.2	92.2
Salix gooddingii SCS	92		50.0 ± 16.1	90.7
Salix matsudana x S. alba	92		67.9 ± 2.5	87.5
Saphora japonica	93	4)	3.6 ± 3.7	95.3
Tamarix ramosissima	92		22.2 ± 10.4	95.4
Ulmus pumilla 'Dropmore'	93	4)	17.7 ± 4.0	73.5
Ulmus pumilla	92		83.1 ± 30.7	100

1) Species was replaced with a new species in 1993 because of zero survival
2) Species was replaced with a new species in 1994 because of zero survival
3) Species was replaced with the same species in 1993 and interplanted in 1994
4) Species was interplanted in 1994
5) Mean +/- standard deviation, n = 4, negative values generally indicate shoot dieback

A third group of species that somewhat surprisingly did not survive well were most of the conifers including: *Juniperus chinesis, Larix decidua, Larix laricinia, Larix leptolepsis, Larix siberica, Picea pungens, Pinus contorta, Pinus contorta contorta*, and *Pinus thumbergii*. One possible explanation for the poor survival of this group is the lack of appropriate mycorrhizal fungi. While all of these species were inoculated with either a local mycorrhizal crude inoculum or commercial strains of *Psiolithus tinctorus*, these may not be suitable mycorrhizal strains for the alkali conditions at the study site. We also speculate that the lack of irrigation during the typical winter season of November - March may have resulted in winter desiccation. Anecdotally, we are aware that ornamental plantings in nearby communities require winter irrigation to avoid desiccation.

A small group of conifers did, however, survive reasonably well including: *Cupressus arizonica* Roughback, *Juniperus scopulorum, Juniperus virginiana, Pinus nigra, Pinus ponderosa,* and *Pinus sylvestris*. All of these species are known to be drought and/or salt tolerant.

The best surviving species were all hardwood species and included the following: *Acer negundo, Ailanthus altissima, Atriplex canescens, Caragana arborescens, Catalpa speciosa, Celtis occidentalis, Eleagnus angustifolia, Fraxinus* spp., *Ginko biloba, Gleditsia triacanthos, Gymnocladus dioicus, Morus alba, Populus* spp., *Prunus armeniaca* var. *manchurica, Prunus virginiana, Pyrus ussuriensis, Robinia* spp., *Salix* spp., *Saphora japonica, Tamarix ramosissima* and *Ulmus pumila*.

Growth

ANOVA indicated a highly significant difference in growth between species ($F = 2.647$, $P \leq 0.000$). Growth of species is summarized in Table 1. Among the 27 species that

survived reasonably well, the following grew at an average rate of greater than 40 cm/year: *Cupressus arizonica, Eleagnus angustifolia, Populus* spp., *Robinia* spp., *Salix* spp., and *Ulmus pumila*. The fastest growing species was a species of cottonwood native to this area of Arizona, *Populus fremontii*.

From these experiments, which include three field seasons of irrigation with saline wastewater, it is clear that a range of species and genotypes have potential to be used in a land application wastewater treatment system. Many of the successful species include species known to have potential for biomass including *Populus* spp., *Robinia* spp., *Salix* spp., and *Ulmus pumila*. Since none of the species have yet reached commercial harvest size we cannot yet conclude that biomass can be produced using this system. We do, however, estimate that at current growth rates some selections of *Populus* will achieve a harvestable size of 10 cm diameter at breast height within two years. Soil analyses indicates that salt is accumulating in the soil, but insufficient data is available to project when salt levels in the soil will reach levels that will negatively influence growth.

The experimental treatments above are expected to continue for at least two additional growing seasons when the feasibility of biomass harvest from pulp and papermill wastewater irrigated plantations will be determined.

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Poplar disease research: host resistance and pathogen variability

MICHAEL E. OSTRY
USDA Forest Service, North Central Forest Experiment Station, St. Paul, MN.

Ostry, M.E. 1994. Poplar disease research: host resistance and pathogen variability. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 89-94. ISSN 0802-1600.

Diseases have been the major limiting factor in growing hybrid poplars and aspen for biomass production in the north-central United States. One of the most critical needs for the development of poplar biomass production systems is the availability of high yielding clones that have greater disease resistance than the ones now being planted. Recent research has concentrated on screening clones for disease resistance, increasing disease resistance in poplar clones through the use of tissue culture and somaclonal selection technologies, and studying pathogen variation. Our understanding of pathogen populations affecting poplars is incomplete. We are uncertain how much variation in pathogenicity may exist among species, races, or pathotypes and we are unsure of their distribution. This complicates disease resistance screening and can jeopardize the success of biomass plantations. Research results on biological control of Septoria leaf spot and canker, caused by *Septoria musiva*, have been encouraging and these studies are being continued. Investigations of the early host responses of selected aspen genotypes to wounding and invasion by *Hypoxylon mammatum* have provided preliminary information that may be useful in developing resistance screening bioassays.

Key words: Aspen, *Hypoxylon mammatum, Melampsora* spp., *Populus*, *Septoria musiva*, somaclonal variation, tissue culture.

Michael E. Ostry, USDA Forest Service, North Central Forest Experiment Station, St. Paul, MN.

Poplar biomass plantations managed for fiber or energy production are costly to establish and maintain so minimizing disease risk is important. The biology of poplar pathogens makes them difficult to control using chemicals so planting clones that have been screened for disease resistance is the recommended management strategy (Ostry & McNabb 1990). In addition, environmental concerns and application costs limit the practical use of pesticides for control of poplar diseases in most cases.

Poplars (*Populus* spp.) are damaged by many disease agents that can kill or reduce the productivity and quality of diseased trees (Ostry et al. 1989). Hybrid poplars with parentage in the section Tacamahaca grown under short rotation forestry systems in the north-central United States have primarily been damaged by *Septoria musiva*, the causal agent of Septoria canker and leaf spot. In addition, many clones are affected by leaf rust,

caused by *Melampsora* spp. which causes premature defoliation and subjects trees to winter injury and invasion by secondary organisms. Susceptibility to these diseases have limited the number of clones suitable for use in biomass plantings. Clones in the section Aigeiros are more resistant to *S. musiva*, but generally are lower yielding than clones from the section Tacamahaca (Ostry and Berguson 1993).

Hypoxylon canker, caused by the fungus *Hypoxylon mammatum* is the major killing disease of aspen (*Populus tremuloides*) in native stands and plantations throughout the region. Aspen clones vary widely in their susceptibility to the disease, however, the mechanism of host resistance is poorly understood and rapid methods of screening for resistance are not available.

Disease resistance screening to select suitable hybrid poplar and aspen clones for management under various silvicultural systems has been the focus of our research for the past several years. The following is a brief overview of the poplar disease research projects that are underway in our laboratory, research progress, and the disease research areas that need to be addressed in the future to maximize biomass yields.

TISSUE CULTURE AND SOMACLONAL SELECTION

Tissue culture has been most often used in the past to clonally propagate plants. However, the number of examples of in vitro isolation and regeneration of somatic mutants, a phenomenon known as somaclonal variation (Larkin and Scowcroft 1981), has rapidly increased. Many authors have reported recovering plants with valuable traits, including plants expressing increased disease resistance. Tissue and cell culture technologies may provide tools to augment breeding strategies to rapidly develop trees with improved characteristics, and may yield new sources of genetic diversity.

We have used tissue culture to recover plants of hybrid poplar clones that have expressed increased resistance to Septoria leaf spot in laboratory bioassays (Ostry & Skilling 1988). The incidence of somaclonal variation in disease resistance differed widely among poplar genotypes. Preliminary evidence suggests that the somatic variation in these plants may have a cytological basis.

Resistant plants were vegetatively multiplied and outplanted in field trials in Wisconsin in 1986 and Minnesota in 1991 to evaluate the stability of the resistance in the field and to ensure that tree growth characteristics and resistance to other pathogens had not changed. Several of the somaclones have retained their resistance and these plants will undergo further testing at additional sites. In contrast, the non-tissue cultured plants have mostly been killed by the disease.

Obtaining a thorough understanding of the controlling factors associated with in vitro-induced variation is important to either enhance or avoid the regeneration of mutant plants. Random somatic variation may also complicate results of transformation systems that rely on cell and tissue culture systems. Explant source and culture method were shown to influence the frequency that variant poplars were recovered (Ostry et al. 1994). Variant plants are being examined in detail and compared with the non-tissue cultured plants in order to determine the underlying cause for the somatic variation in disease resistance.

FIELD DISEASE RESISTANCE SCREENING

One of the most critical needs for the development of successful poplar biomass production systems is the availability of high yielding clones that are disease-resistant and adapted to the sites where commercial plantings will be established. To meet this need, more than 140 poplar selections have been under test in 61 clonal trials across a 5 State region in the north-central U.S. since 1986 (Hansen et al. 1994). These trials are now providing us with data on yields and disease resistance of poplar selections from many breeding programs, including several newer selections that have not previously been tested in this region.

Disease susceptibility among clones was usually expressed by the second year after planting. In most instances Septoria canker and Septoria leaf spot were most severe on sites with lower water availability. As expected, the parentage of the clones greatly influenced their susceptibility to the various pathogens.

We have identified a group of clones that have acceptable disease resistance and biomass production across a range of sites. However, these results are still preliminary since clone performance can change during the next several years of the projected rotation.

PATHOGEN VARIABILITY

The need to monitor pathogen populations has been underscored by recent reports of the detection of the Eurasion species of leaf rust in the United States (Newcombe and Chastagner 1993a) and the detection of native species of leaf rust in areas where they were not known to occur (Newcombe and Chastagner 1993b, Moltson et al. 1993). Obtaining meaningful data on disease resistance from laboratory and field studies requires knowledge of pathogen variability and distribution. We have evidence that *S. musiva* is not present in the Pacific Northwest region of the United States. This may explain why Septoria canker is not a problem in hybrid poplar plantings in this area and should serve as a warning about the possible inter- and intra-country movement of pathogens.

The population genetic structure of *S. musiva* is unknown, making it difficult to confidently select poplars that will be resistant over a wide geographic area. Over 700 *S. musiva* and *S. populicola* isolates from native poplars and 45 hybrid clones across 16 locations in 5 States have been collected throughout 3 growing seasons to study the temporal and spatial variability among isolates (Ward et al. 1994).

Randomly amplified polymorphic DNA (RAPD) assays based on the polymerase chain reaction (PCR) have revealed a large amount of genetic variation among the isolates. Greenhouse-grown plants from selected clones of known levels of resistance to *S. musiva* are being inoculated with various isolates to detect any variation in pathogenicity or host specificity among the isolates.

BIOLOGICAL CONTROL OF SEPTORIA LEAF SPOT

Septoria canker and Septoria leaf spot continue to be the most serious diseases of hybrid poplars in the north central region of the United States. Chemical controls are of limited

value and only a few proven high yielding resistant clones are available. This makes biological control an attractive management alternative, especially when combined with an integrated pest management program. We reported promising results using suppressive strains of *Streptomyces* spp. as a biological control of *S. musiva* (Ostry and Anderson 1992). In addition, investigators have recently reported success using the fungus *Phaeoitheca dimorphospora* as a biological control agent against *S. musiva* (Yang et al. 1994).

Co-plating, antibiotic, and leaf disk bioassays have been used to demonstrate significant inhibition of *S. musiva* on poplars by strains of the bacterium *Streptomyces* (Shimizu 1994). On agar plates, *Streptomyces* inhibited spore germination of *S. musiva,* indicating that the antibiotic activity of *Streptomyces* may have value for the control of Septoria leaf spot and canker in nurseries and plantations by reducing inoculum levels. Greenhouse and field tests are now underway to test the feasibility of using this approach to reduce the damage by *S. musiva*.

EARLY SELECTION FOR HYPOXYLON CANKER RESISTANCE

There is a need to identify clones of aspen (*P. tremuloides*) with resistance to Hypoxylon canker. Currently there are no rapid screening techniques to identify resistant clones making it necessary to rely on expensive, long-term field trials. We are conducting research to test the hypothesis that a major difference between a resistant and susceptible clone is the early defense response against the pathogen. Studies are aimed at evaluating the histochemical and enzymatic changes in a resistant (Pike Bay™) and a susceptible clone that have been wounded or wound-inoculated with *H. mammatum*.

Greater and more rapid enzyme activity of phenylanine ammonia-lyase (PAL), *O*-methyl transferase (*O*-Met), and cinnamyl alcohol dehydrogenase (CAD) were detected after 4 days in green branches of the wounded or wound-inoculated resistant clone relative to the susceptible clone (Bucciarelli et al. 1993). Presence of the pathogen increased levels of PAL and CAD activity in the resistant, but not the susceptible clone.

Fluorescence microscopy has been used to study early cell wall responses of these clones after being wounded or wound-inoculated (Bucciarelli et al. 1994). The major difference between the clones was the greater amount and earlier deposition of phenolic or lignin-like substances at the wound site of the resistant clone. This type of information may assist in the development of bioassays that can be used for detection of resistant genotypes.

MANAGEMENT NEEDS

Interest in growing poplars as an alternative agricultural crop for fiber and energy production is rapidly growing. However, many critical areas of poplar management remain in need of further research and development before large-scale plantings can be successfully established and attain maximum biomass yields.

Of immediate need in the north-central United States are more reliable clones. This requires a major breeding program and long-term clonal performance trials across a wide

range of sites. To avoid confusion and costly losses caused by planting unsuitable clones, a certification program is needed so that clonal identity can be maintained. In addition, we need better information on how to deploy clones in plantings to minimize disease risks and to maximize yield.

We need to exercise extreme care in the movement of poplars from one region to another to avoid the introduction of new pathogens. Techniques are needed to easily detect and identify pathogen species and the presence of races or pathotypes. Native poplars may also be in jeopardy if we do not use care in preventing the introduction of non-native pathogens. It is clear that we need to monitor pathogen populations within poplar plantings as a routine management strategy.

Molecular biology is providing researchers the tools allowing them to make rapid progress in the study of host-parasite interactions and genetic control of disease resistance in trees. These biotechnologies are increasingly being applied to the study of poplars and have already dramatically increased our knowledge that may assist in developing poplar clones that are far superior for biomass production systems to what are now available.

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International energy agency rust/clone trials, 1991-93

T. HUNTER, D.J. ROYLE & M.H. PEI
Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Bristol, UK

Hunter, T., D.J. Royle & M.H. Pei 1994. International energy agency rust/clone trials, 1991-93. Norwegian Journal of Agricultural Sciences. Supplement No. 18: 95-100. ISSN 0802-1600.

Willow trials were established at sites in the UK, Sweden and Canada to determine the pathotype composition of the rust populations in the different regions. Twenty four willow clones, 12 from the UK, 6 from Sweden and 6 from Canada were selected for their ability to differentiate rust pathotypes to cover a wide range of sensitivity to rust based on observed reactions in the field and for their potential as commercial clones. The sites were planted in 1991 and monitoring of the rust populations has been done annually. Rust levels between sites were compared using a standardised disease assessment key. Samples of rust were collected annually from the three UK sites to identify rust pathotypes using a standard set of willow differentials. Three species of Melampsora rust, *M.epitea*, *M. capraearum* and *M. amygdalinae* were identified in the trial sites, with *M. epitea* being the most prevalent. In general willow clones of European origin were infected by rust in the UK and Sweden but not in Canada. In contrast, those of Canadian origin were infected only in Canada. Clones differed in the severity of the attacks. The results suggested that rust pathotype populations markedly differ between N. America and Europe.

Key words : Melampsora, pathotypes, willow differentials.

T. Hunter, Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Bristol BS18 9AF, UK.

Surveys done in UK, Sweden and Canada and co-ordinated by Long Ashton Research Station, had shown that rust caused by *Melampsora* spp. was the most important disease or pest limiting the production of biomass as a renewable energy source by short-rotation, coppiced willows (Royle & Hubbes, 1992). Rust occurrence and severity were found to vary markedly between sites, clones and also within the same clone at different sites. Some clones have become either progressively more susceptible to rust or have suddenly succumbed to heavy rust infections. This is likely to be due to the intrinsically variable nature of the rust populations and the selection pressure imposed from planting a limited number of willow clones. In such a situation it would be expected that the rust population would be composed of a number of different forms or pathotypes of *Melampsora* each of which can infect a distinct range of willow clones. Three species of *Melampsora : M. epitea, M.capraearum and M.amygdalinae* have been identified in the UK on willow clones

grown as potential biomass (Pei *et al*, 1993). Using pathogenicity testing it has been shown that there are at least 10 distinct pathotypes of *M. epitea*, but the distribution of species and pathotypes between regions or countries was not known.

To study the occurrence and composition of the rust populations globally a series of trials were established using an agreed common set of willow clones at a number of sites in the UK, Sweden and Canada.

METHODS

Plots of twenty four willow clones were planted at Long Ashton Research Station (England), Loughgall (N. Ireland), Aberdeen (Scotland), Uppsala (Sweden) and Orono (Canada) during Spring 1991. Each plot comprised 5 x 5 (= 25) stools of each clone at 1 x 1m spacing. A further site in S. Sweden was planted in 1993 using the same collection of willow clones (Table 1).

Table 1. Willow clones used in the experiments

Clone	Parentage[1]
UK clones	
1. *S. x stipularis*	*aur. x vim. x cap.*
2. *S. x calodendron*	*cap. x cin. x vim.*
3. *S. burjatica* Korso	S.A.G.
4. *S. x dasyclados* (Wimm)	*cap. x cin. x vim.*
5. *S. viminalis* Bowles Hybrid	
6. *S. viminalis* Mullatin	
7. *S. x* 445 de Biardii	*cap. x cin. x vim.*
8. *S. x sericans* Coles	*cap. x vim.*
9. *S.* x *hirtei* Reifenweide	*cin. x vim. x aur.*
10. *S. burjatica* Germany	S.A.G.
11. *S. x mollisima* Q83	*tri. x vim.*
12. *S. triandra* Black Maul	
Canadian clones	
13. *S. eriocephala*	
14. *S. eriocephala x petiolaris* 601	
15. *S. eriocephala x exigua*	
16. *S. eriocephala*	
17. *S. purpurea* 012	
18. Shrubby willow ex Orient	unknown
Swedish clones	
19. *S. viminalis* 78183	
20. *S. viminalis* 79046	
21. *S. "dasyclados"* 77056	S.A.G.
22. *S. "dasyclados"* 79097	S.A.G.
23. *S. viminalis* 78112	
24. *S. "dasyclados"* 81090	S.A.G.

[1] *aur. = S. aurita, vim. = S. viminalis, cap. = S. caprea, cin. = S. cinerea, tri. = S. triandra*, S.A.G.= *S. aquatica gigantea*

After each year's growth all shoots were cut back to allow fresh shoots to develop the following year. Natural development of rust within each plot was monitored using a standard protocol to allow results to be compared between the sites.

At least two rust assessments were carried out during the growing season (early July/August and late September/October). Detailed records were taken on the centre stool of each replicate plot. The leaf area assessment key developed at Long Ashton by Parker *et al.*(1993) was used to help standardise results between the sites.

Rust samples were taken from each infected clone at the UK sites to establish the species and pathotype of the rust present. During 1991, three species of *Melampsora* were identified. *M. amygdalinae*, the only autoecious willow rust, was found on *S. triandra* Black Maul whilst *M. capraearum* was detected on *S. x hirtei* Reifenweide at all three UK sites. The identity of these species was confirmed from the morphology of the uredinial and telial stages. The remaining rust types all belonged to *Melampsora epitea* var. *epitea* and required the use of selected differential willow hosts to establish their pathotype (Table 2).

Table 2. Pathotypes of *Melampsora epitea* detected in 1992 on clones in the UK sites, showing their virulence on a standard set of diffential hosts

Salix host	Pathotypes									
	LET1	LET2	LET3	LET4	LET5	'SIF'[b]	LR1	LR2	LD1	RP1
viminalis 'Mullatin'	+	-	-	-	-	-	-	-	-	-
vim x *tri* Q83	-	-	-	+	-	-	-	-	-	-
viminalis 'Bowles Hybrid'[a]	-[c]	-	-	-	-	+	-	-	-	-
x *stipularis*	+	+	-	+	+	-	-	-	-	-
x *calodendron*	-	+	+	-	-	+	+	+	-	-
burjatica 'Korso'	-	-	-	-	-	+	+	+	-	-
disperma (himalayas)	-	-	-	-	-	-	-	+	-	-
daphnoides 'Meikle'	-	-	-	-	-	-	-	-	+	-
purpurea 'Uralensis'	-	-	-	-	-	-	-	-	-	+

' + susceptible reaction, uredinia well developed; - resistant reaction, no symptom or negligible pustule development, often with necrosis; NT, not tested.

[a] Only folded leaves at shoot-tips can be used. [b]'SIF' needs 9 days to produce uredinia.[c] expanding and fully open leaves are susceptible to LET1.

RESULTS

Rust severity

By the end of 1991, the establishment year, rust had been recorded on all the UK-selected clones at the Long Ashton site and on all but one, *S. x mollisima* Q83, at Craibstone and Loughgall. Rust also occurred on all the Swedish selections at the UK sites. Of the Canadian clones only *S. eriocephala x petiolaris* 601 became infected, and only at Loughgall very late in the season.

In 1992 rust was found on the same clones at the 3 UK sites but in varying degrees

of severity. An exception was that recorded on *S. burjatica* Germany at Loughgall but not at the other sites. The most severe infection (mean 52% leaf area infected) was found on *S. x stipularis* at Long Ashton and on *S. "das"* 81090 at Loughgall (mean 67%). In Scotland, less rust was found and *S. x stipularis* showed greatest levels of infection (mean 5%). There was a similar pattern of clones infected in Sweden compared with the UK sites, but the level of infection was much lower. In Canada, rust was not found on the clones of European origin and was restricted to clones with *S. eriocephala* parentage.

During 1993 at Long Ashton, rust was generally found on the same clones as in 1992 but was more severe. *S. x stipularis* was again the most severely infected with defoliation associated with rust. Of the European clones, only *S. burjatica* Germany was free of rust. A substantial increase in rust severity was recorded on *S. x mollisima* Q83 with more than a ten-fold increase from 1992. At Loughgall, *S. burjatica* Korso, Germany and *S. "das"* 81090 were significantly worse than other clones with no rust encountered on the Canadian clones. At Craibstone, the most severely infected clones were *S. sericans* Coles and *S. x hirtei* Reifenweide which were both subsequently shown to be infected by *M. capraearum*. In general rust levels were very low at this site, even the usually susceptible *S. x stipularis* showing only slight infection. However, rust occurred on 4/6 Canadian clones, albeit at minimal levels which were insufficient to provide material for pathotype determination. In contrast to the other two UK sites, infection did not become established on *S. x mollisima* Q83 despite the occurrence at Craibstone of pathotype LET4 in 1992.

In Sweden, rust was generally more severe at the southern site with infection restricted to clones of European origin. At Uppsala, rust was found on these clones with most severe symptoms on *S. burjatica* Korso (mean 0.7%); low infection occurred on *S.eriocephala* ER65. No comparable late season assessment was done for the Canadian site but earlier in the season infections had been recorded on the same clones as in 1992 with *S. eriocephala* 558 the most severe. No defoliation due to rust was reported from the sites in Sweden or Canada.

Pathotype distribution at the UK sites

During 1991, pathotype LET1 was generally associated with *S. viminalis* clones and LR1 with *S. burjatica* clones; the same clones were affected by the same pathotypes at the three sites. The majority of the clones which were infected with rust appeared to support only a single pathotype.

Compared to 1991 a more complex pattern of pathotype distribution occurred in 1992. *M. capraearum* was more prevalent, being found on *S. x dasyclados* (Wimm), *S. x sericans* Coles and *S. x hirtei* Reifenweide. *M. amygdalinae* was confined to *S. triandra* Black Maul on which it caused early leaf infections and often cankering of the stems. Other rust infections were caused by pathotypes of *M. epitea*. More willow clones than in 1991 were infected by more than a single pathotype. On *S. x stipularis* at Loughgall, three pathotypes were detected. Differences between the sites mainly occurred in the extra pathotypes encountered. Rust developed for the first time on *S.* x *mollisima* Q83 at all sites in 1992, in each case due to the new pathotype LET4. Another new pathotype (LET5) was found on *S. x stipularis* for the first time, but only at Loughgall. The *S. burjatica* (Swedish *"dasyclados"*) clones were widely infected with LR1 and LR2, whereas in 1991 only LR1 had been recorded. The stem-infecting pathotype (SIF) occurred at all sites on *S. viminalis*

Bowles Hybrid, but otherwise only on *S. viminalis* 79046 at the Craibstone site, as in 1991.

A complex pattern of pathotype distribution was again observed in 1993. *M. amygdalinae* was detected on *S. triandra* Black Maul at all three sites. Pathotypes LET2 and LET3 occured on willows with *S. cinerea* parentage, e.g. *S. x calodendron* and *S. x 445 de Biardii*. Since *S. cinerea* is the most common wild willow in the UK these pathotypes represent a well-established population. Pathotype determinations confirmed the presence of LET2 and LET3 at the UK sites but causing only limited infections. *S. x mollisima* Q83 continued to be attacked and, when sampled at Long Ashton and Loughgall, the rust was found to belong to the LET4 pathotype. Pathotypes LR1 and LR2 occurred together on the *S. burjatica* (Swedish *"dasyclados"*) clones at Long Ashton and Loughgall, but only LR1 occurred at Craibstone. Indeed, at the Scottish site, pathotype LR2 has only been detected on one occasion (1992) on *S. "das"* 77056. This year the stem infecting form (SIF) was found exclusively on *S. viminalis* Bowles Hybrid. There was a general similarity in the frequency of pathotypes between sites. A previously unrecorded pathotype, LET6, was identified on *S. viminalis* 78183. A further rust form belonging to the *Ribes*-alternating group (*M. ribesii-purpurea*) was found on the Canadian *S. purpurea* 012 at Long Ashton.

DISCUSSION

(a) Rust severity

At all UK sites, and with the exception of the Canadian clones, more severe rust occurred on more clones early in 1993 than in 1992. Apart from the Canadian clones no clone was consistently resistant to rust. Rust became more severe on *S. burjatica* Germany at Loughgall, although the pathotypes responsible, LR1 and LR2, were the same throughout. At both Long Ashton and Loughgall the sensitivity to rust of *S. x mollisima* Q83 changed appreciably from highly resistant to susceptible, due apparently to the appearance of the LET4 pathotype. Also at these two sites the Swedish *S. viminalis* clones all became more severely infected in 1993 than in the previous year.

S. x stipularis was consistently one of the most severely infected clones, although at Craibstone levels of infection on this and other clones were less than on the corresponding clones at the other UK sites.

(b) Distribution of pathotypes

Although certain rust species and pathotypes of *M. epitea* are widespread, the composition of pathotype populations can and does differ between regions of the country. Long Ashton and Loughgall are situated in areas where there are extensive, long-term plantings of willows which contain many species and clones. In contrast, Craibstone is a more isolated site where rust has a relatively simple pathotype population structure. The pathotype LET4, which arose for the first time on *S. x mollisima* Q83 in 1992 at all three sites, failed to occur at Craibstone in 1993 suggesting that it has not yet become established in Scotland. By 1993 the LET4 pathotype population was well established and at Long Ashton and Loughgall caused severe rust infections. Many of the same pathotypes are distributed widely in the UK and appear to be selected by compatible clones very quickly once these clones are grown in a region. Rust severity may then be determined more by environmental

conditions than interactions between a host and its pathotypes. Comparisons of rust pathotype occurrence on clones from different continents has established that the European rust pathotype populations differ fundamentally from those in Canada. All the willow rusts in N. America are *M. epitea* var. *epitea* (Ziller, 1974) and yet there were only trace infections on the Canadian clones at the UK sites in this study. UK rust populations do not seem to have adapted to these types of willow. From the patterns of infection on the Swedish selected clones it would appear likely that the Swedish rust populations have similar pathogenicity patterns to those found in UK.

The results also showed that *M. capraearum* is becoming progressively more important in UK willows with *S. caprea* parentage. Of the pathotypes encountered at the three UK sites, LET1 was the most predominant, readily infecting *S. viminalis* and its hybrids, e.g. *S. x stipularis*, *S. x sericans* Coles and *S. x hirtei* Reifenweide.

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Experiences in the use of mixed-clonal stands of *Salix* as a method of reducing the impact of rust diseases

A.R. McCRACKEN[*] & W.M. DAWSON[!]
Applied Plant Science Division, Dept. Agriculture for N. Ireland
[*] Newforge Lane, Belfast, N. Ireland BT9 5PX, UK
[!] Northern Ireland Horticulture and Plant Breeding Station, Loughgal, Co. Armagh and Dept. Applied Plant Science, Queen's University of Belfast, Newforge Lane, Belfast, N. Ireland, UK

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When *Salix* spp. clones are grown in intimate mixtures of up to six different clones there are significant increases in the yield compared to that from equivalent mono-clonal stands. These differences are evident after one year of growth but only significant two and three years after coppicing. Part of the yield effect is due to a reduction of the impact of *Melampsora* sp. rust on the willow. In mixed-clonal stands the onset of disease is delayed, its build up is slower and the final levels of disease are less than those in mono-clonal stands. These differences are most pronounced in the first year of regrowth. The survival and growth of *Salix* clones in mixed-clonal stands is variable.

Key words: Disease reduction, *Melampsora* spp. rust, mixed-clonal stands, *Salix*, survival, yield.

A.R. McCracken, Applied Plant Science Division, Dept. Agriculture for N. Ireland, Newforge Lane, Belfast, N. Ireland BT9 5PX, UK.

Probably the major problem of growing *Salix* in high density as a short rotation coppice crop is the effect of foliar rusts caused by *Melampsora* spp. Rust was first observed as a major problem in Northern Ireland in 1985 on the cultivar *Salix burjatica* 'Korso' which had been grown virtually disease free as a short rotation coppice crop in that area for ten years (Dawson 1987). At that time the pathogen was identified as *Melampsora epitea* Thum. Since then *M. epitea* has been shown to exist in at least 10 pathotypes infecting a wide range of *Salix* clones (Royle 1994). In addition there are at least two other *Melampsora* species found regularly.

The incidence and severity of the rust diseases has also increased. In a survey in 1987 only 46% of the clonal plots examined in N. Ireland had severe rust compared to 96% in 1988 (Hunter, Royle & Stott 1989). Furthermore during the past ten years rust has been

detected, often at severe levels on clones which hitherto had not shown any symptoms or where symptoms were considered unusual.

The impact of rust on the survival, growth and yield of *Salix* clones grown in short rotation coppice can be devastating. In an experiment using the highly susceptible clone *Salix burjatica* 'Korso' Dawson & McCracken (1994) showed that disease control using fungicides reduced percentage shoot death at flushing the following spring from over 60% to less than the 10%. The corresponding increase in dry matter yield at the end of the growing season was more than double in the treated, compared to untreated plots.

However, it is clear that the use of fungicides as a disease control strategy is unacceptable for at least three reasons (Dawson & McCracken 1994):

a) **economic** - margins at present are small and the cost of repeated fungicide application throughout the growing season could not be borne.

b) **environmental** - often willow is grown close to water ways or in environmentally sensitive areas eg. Co. Fermanagh, N. Ireland. The use of large quantities of agrochemicals would therefore be unacceptable.

c) **practical** - while fungicides can be applied relatively easily and effectively in the first few weeks following regrowth it becomes increasing difficult to obtain effective penetration of the canopy as the plants grow. By the end of the first year of growth and in subsequent years fungicide application becomes an impractical option.

Consequently an alternative disease control strategy was required. The possibility of introducing diversity and hence stability into the system was investigated using polyclonal or mixed stands. This paper presents data on the effect of growing *Salix* spp. clones in polyclonal stands on yield, survival and disease.

MATERIALS AND METHODS

Two large areas were planted with *Salix* at a density of 20000/ha:

Site 1: Five clones were planted in 1987: *Salix* x *dasyclados*, *S. burjatica* 'Korso', *S. viminalis* Bowles Hybrid, *S. viminalis* 683 and *S. burjatica* 'Germany'. Plots were established using hardwood cuttings in either monoclonal plots or in intimate mixtures as polyclonal plots. Harvesting was carried out in 1991 and 1994 on three year old material.

Site 2: Six clones were planted in 1989: *Salix* x *dasyclados*, *S. viminalis* Bowles Hybrid, *S. viminalis* 683 and *S. burjatica* 'Germany', *S. mollissima undulata* SQ83 and *S.* X *calodendron*. Plots were established in 1989 as both monoclonal and polyclonal stands.

In both sites small sub-plots (400 stools) were treated with the fungicide myclobutanil (Systhane) at fortnightly intervals from early May until late August to control rust.

Leaf samples were taken every two weeks from 10 shoots chosen at random and

assessed for disease as the percentage number of leaves with rust pustules. At the same time 10 random shoots from each of the clones was removed and the dry matter determined. At the end of the growing season 10 randomly selected stools were used to measure dry matter yield.

RESULTS

1 Yield

After one years growth the dry matter yield from polyclonal plots was not significantly different from the mean of the four constituent clones grown as monoclonal stands (Fig. 1)

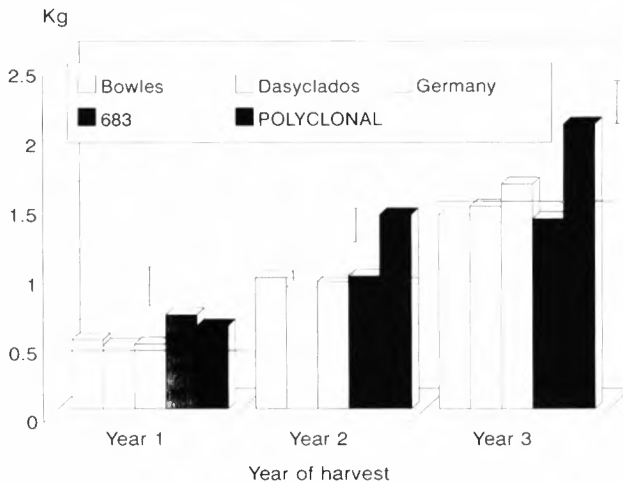

Fig. 1. Yield (kg) after one, two and three years growth of individual stools of four *Salix* clones grown in mono- and polyclonal plantations. Horizontal lines are the mean of the four clones grown in mono-clonal plots. Vertical lines are the Least Significant Differences of means ($p > 0.05$)

At the end of the second year of growth the yield from the polyclonal plots was significantly ($P > 0.05$) greater than the mean yield from the constituent clones grown as monoclonal stands and also significantly greater than the individual yields of each of the component clones. There were no significant differences between yields from any of the monoclonal stands.

The greatest differences were observed at the end of the third year of growth. As in year two there were significantly ($P > 0.05$) higher yield from the polyclonal plots compared with the mean of the monoclonal plots or the individual yields of the component clones grown in monoclonal stands.

The contribution of individual clones to the increased dry matter yield from mixed clonal plots varied considerably when assessed on the basis of individual stool dry matter yields. *S. viminalis* Bowles Hybrid gave approximately a 20% improvement in yield from

the mixed clonal plots while the improvement with *S. burjatica* 'Germany' was almost 50%. Small improvements were observed with *S. viminalis* '683' but with *S. x dasyclados* almost no yield increases occurred at any stage during the growing cycle.

2 Survival

In Site 1 where five clones,(*Salix* x *dasyclados*, *S. burjatica* 'Korso', *S. viminalis* Bowles Hybrid, *S. viminalis* 683 and *S. burjatica* 'Germany') were originally planted at a density of 20,000/ha only four survived. *S. burjatica* 'Korso', which is particularly susceptible to rust infections very quickly died out until less than 5% were found in May 1994 following the second three year harvest. There was no significant difference (P>0.05) between the percentage survival of the remaining clones which was between 77 and 82%. Similarly there was no difference between survival in the polyclonal plots compared to those in the monoclonal plots (Fig 2).

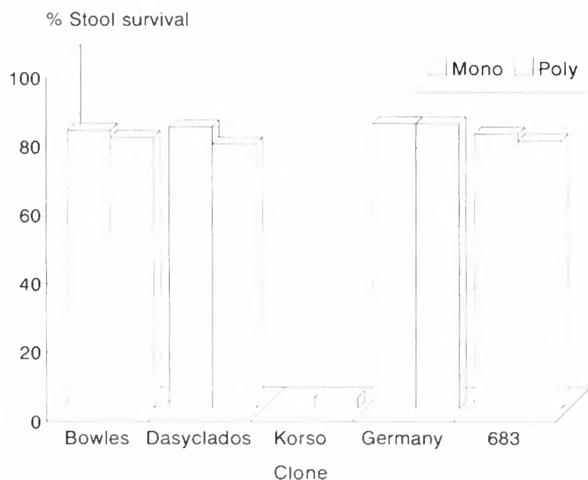

Fig. 2. Percentage stool survival at Site 1 of five clones growing in mono- and polyclonal plantations, recorded at flushing after the second three year harvest

In Site 2 six clones had been planted, (*Salix* x *dasyclados*, *S. viminalis* Bowles Hybrid, *S. viminalis* 683, *S. burjatica* 'Germany', *S. mollissima undulata* SQ83 and *S.* x *calodendron*). There were no significant differences in the number of stools of *Salix* X *dasyclados*, *S. viminalis* Bowles Hybrid, *S. burjatica* 'Germany', and *S. mollissima undulata* SQ83 surviving following the first harvest after three years growth. The survival rate ranged from 59 - 68%. This was lower than survival in monoclonal plots which was for each of the clones between 90 and 96%. Survival of *S. viminalis* 683 was significantly (P. 0.05) better than any of the other clones in the mixture at 84%. In contrast only one third of the *S.* X *calodendron* planted in the mixed stands survived compared to (98% in the pure stands) (Fig 3).

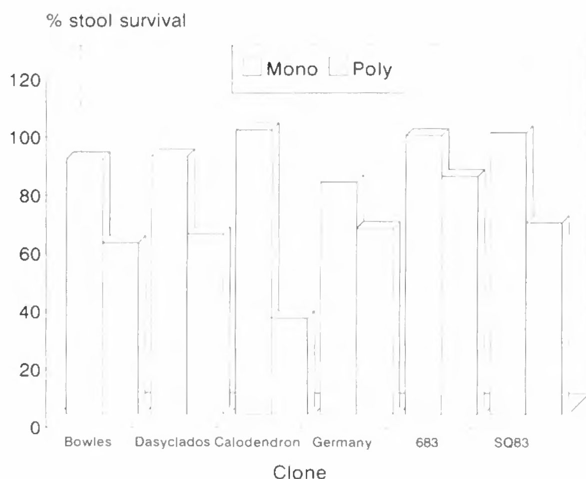

Fig. 3. Percentage stool survival at Site 2 of six clones growing in mono- and polyclonal plantations, recorded at flushing after the first three year harvest

3 Disease

The general observation on the effect of mixed clonal stands on disease incidence was that rust was observed latter in polyclonal stands compared to mono-clonal stands, its build up was slower and on most clones it failed to reach the same level even by the end of the season. The effects were most pronounced during the first year of regrowth after cutting back.

Regrowth from freshly coppiced stools

Salix viminalis Bowles Hybrid: Spraying with fungicide had little impact on disease levels in either the mono- or polyclonal plots. Rust was widespread in monoclonal plots after six weeks growth and built up steadily until at least the 14th week of growth. In contrast appreciable levels of rust were not observed in either of the polyclonal plots until ten weeks growth, built up slowly and never reached the levels found in monoclonal plots, even after 18 weeks (Fig 4).

Salix x *dasyclados*: In the unsprayed monoclonal plots rust started after 6 weeks and increased rapidly till 70% of leaves were infected with rust. Fungicide treatment gave quite good control of the disease. However disease levels, even in the sprayed monoclonal plots, throughout the growing season were at approximately the same level as those found in the unsprayed polyclonal plots.

Salix viminalis 683: Rust developmed less in all plots of this clone, with significant levels of rust only being observed 18 weeks after coppicing. Once again the levels of rust in the polyclonal plots, including the unsprayed plots was very much less than in the monoclonal plots even those which had been treated with fungicide.

Salix burjatica Germany: The trends in disease incidence and development were similar to those found in *S viminalis* Bowles Hybrid (Fig 5).

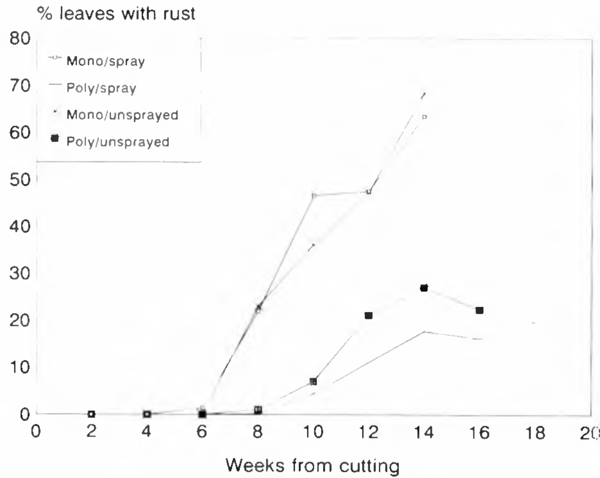

Fig. 4. Percentage leaves of *Salix viminalis* Bowles Hybrid with rust in mono- and polyclonal plantations unsprayed and sprayed with myclobutanil at weekly intervals. Disease was recorded over a twenty week period from early spring to late summer

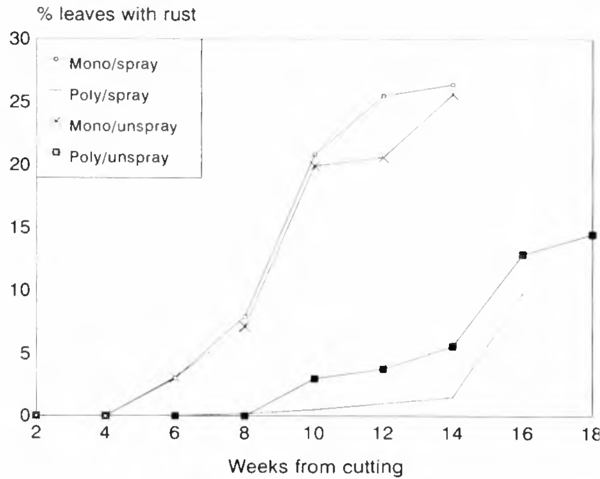

Fig. 5. Percentage leaves of *Salix burjatica* Germany with rust in mono- and polyclonal plantations unsprayed and sprayed with myclobutanil at two weekly intervals. Disease was recorded over an 18 week period from early spring to late summer

Mixed clonal stands had a limited effect on the build up of rust in older plantations. No significant differences were observed on any of the clones between mono- and polyclonal stands in terms of appearance of rust, build up or final levels.

DISCUSSION

Increased yields in plants grown as mixtures of cultivars has been well documented for several species including cereals, potatoes, grasses and various tree crops (Wolfe 1985). The problems associated with critical specifications of the product, for example in cereals related to its end use are not applicable to willow where the only objective is to maximise wood production. At least part of the yield increase in variety mixtures is due to restriction of disease. Chin & Wolfe (1984) suggest that this restriction functions at three levels:

- i) the influence of lower densities of susceptible plants
- ii) the barrier effect of disease resistant plants
- iii) the induced resistance due to the non-virulent pathogen biotypes.

Mundt, Hayes & Schon (1994) reported increased yields in some barley mixtures in the presence of scald and net blotch. Where mixtures included a susceptible cultivar with a moderately resistant cultivar disease severity was reduced by 20 - 32%. However some of the barley mixtures used did give a slight yield reduction emphasising the need for careful evaluation to identify mixtures with positive effects on disease control and yield.

It is clear that mixtures of *Salix* spp. have a marked effect in reducing disease impact, especially in the first year of regrowth, which result in increased yield. If the yield increases in polyclonal stands after two and three years are attributable to this reduced disease then the benefits must have accrued for the effects observed in the first year of regrowth. Only in this first year were significant differences in levels of disease recorded between the mono- and polyclonal plots.

There are still a number of unanswered questions relating to the practical application of mixtures in short rotation coppice willow. Careful clonal selection for a mixture is vital. Different clones planted in mixtures will only give a positive result if those clones planted represent a range of susceptibilities/resistances to the *Melampsora epitea* pathotypes present in the rust population. The number of clones included in a mixture, the plant density and mixture design potentially could all have an effect on both disease patterns and final yields. The first half of a new trial has been planted at Castlearchdale N. Ireland in the spring of 1994. Twenty clones (Table 1) selected for their yield and disease characteristics have been planted in mono clonal plots and also in 5, 10, 15 and 20 way random intimate mixtures. Planted in double rows, three densities have been used, 10,000, 15,000 and 20,000 cuttings per hectare. Establishment and survival of stools in block 1 have been very good. Block 2 of the trial will be planted in spring 1995. In addition to assessing the effects on yield a record will be made of the occurrence and build up of disease in each of the plots over three years.

The use of intimate mixtures of *Salix* clones in N. Ireland has been successful in reducing disease and increasing yield. In an intimate mixture when a susceptible clone does become infected it is separated from other stools of the same clone. Hence the rapid spread of the pathogen is slowed. Furthermore where a stool is severely affected to the point where its growth is significantly reduced growth of neighbouring stools can easily compensate for its loss utilising the space left. This may be particularly significant in the later years of the growth of a plantation.

Table 1. New planting involving a range of *Salix* clones planted in Spring 1994 as mono-clonal plots and in 5, 10 15 and 20 way mixtures

Clone
Salix burjatica Germany
Salix mollisima-undulata SQ83
Salix dasyclados x *Salix aquatica* V7511
Salix viminalis 77082
Salix dasyclados x *Salix caprea* V794
Salix viminalis x *Salix aquatica* V7503
Salix viminalis 78118
Salix viminalis 78183
Salix schwerini x *Salix viminalis* x *Salix dasyclados* V7531
Salix viminalis 870148 (ULV)
Salix viminalis x *Salix caprea* V789
Salix viminalis 77683
Salix viminalis 78101
Salix viminalis 78195
Salix schwerini x *Salix aquatica* V7534
Salix viminalis 77699
Salix viminalis Gustav
Salix schwerini x *Salix aquatica* V7533
Salix viminalis 870082 (ORM)
Salix viminalis Gigantea

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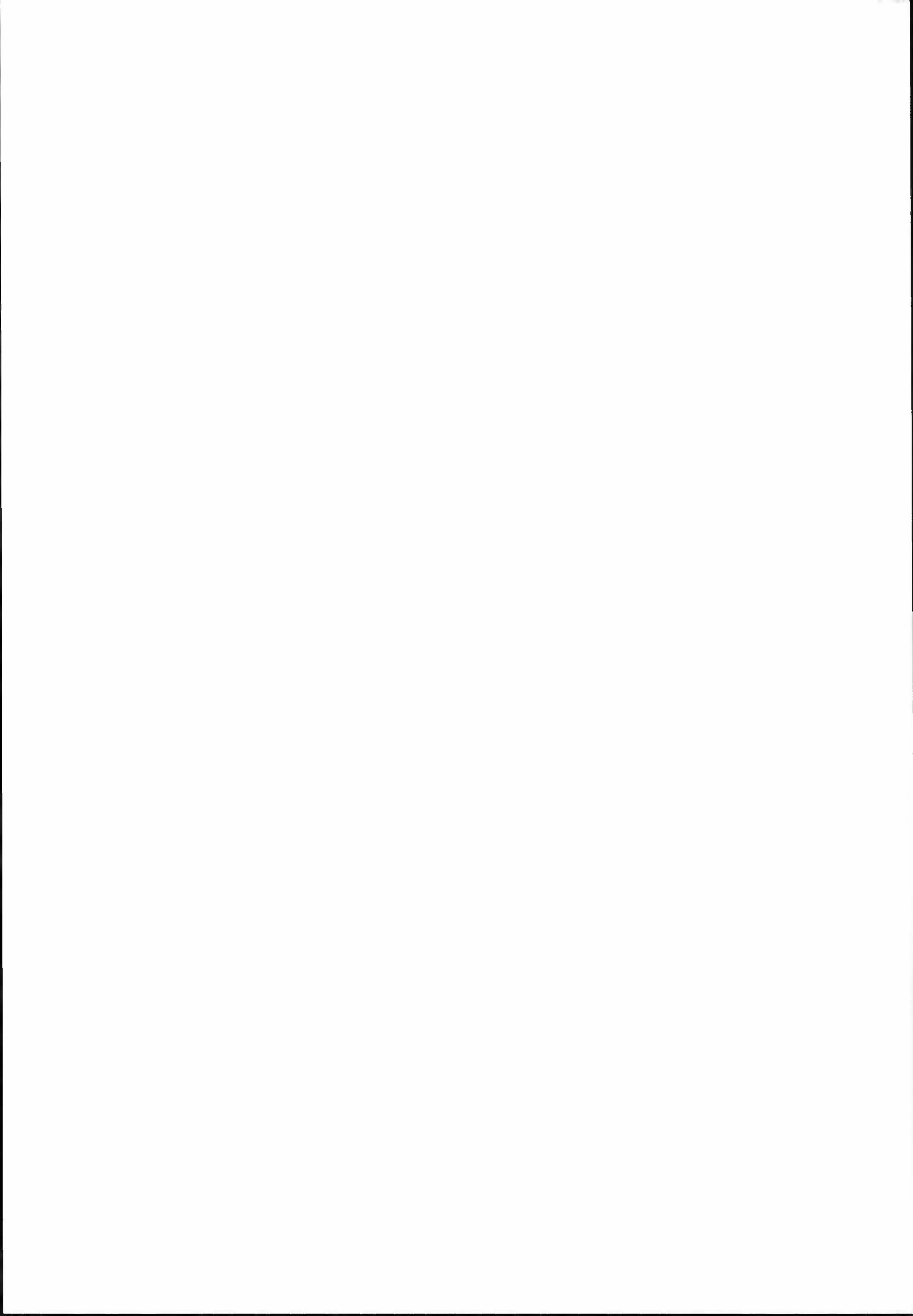

Chrysomela scripta performance on five poplar clones

SYLVIE AUGUSTIN[1], MICHAEL R. WAGNER[2] & KAREN M. CLANCY[3]
[1]INRA Station de Zoologie Forestiere, Ardon, France
[2]School of Forestry, Northern Arizona University, Flagstaff, Arizona, USA
[3]U.S.D.A. Forest Service, Rocky Mountain Forest and Range, Experiment Station, Flagstaff, Arizona, USA

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The cottonwood leaf beetle, *Chrysomela scripta* Fabricius (Coleoptera: Chrysomelidae) is an economically important defoliator of poplars (*Populus* spp.) in the United States, especially in the first years after plantation establishment. Our objective was to obtain information about potential resistance to *C. scripta* among five poplar clones (*Populus fremontii* var. *wislenzensii*, *P. deltoides* 14392, Robusta poplar, Imperial poplar, Northwest poplar) used in a biomass plantation in east-central Arizona. Parameters of *C. scripta* performance were measured including survival, pupal fresh weight, and duration of development from egg to adult. Resistance related to antibiosis was evident in some of the clones. Performance of *C. scripta* varied among the clones and was best on Robusta poplar and worst on *P. fremontii* var. *wislenzensii*.

Key words: *Chrysomela scripta*, genetic resistance, herbivore performance, host plant resistance. *Populus*.

Sylvie Augustin, INRA Station de Zoologie Forestiere, Ardon, France.

Poplars are one of the most commonly used species in short rotation intensive culture for biomass production because of their vigor, productivity, and adaptability to various edaphic and climatic conditions. However, biomass production can be limited when the trees are attacked by some species of insects (Bassman et al. 1982; Larsson 1983; Solomon 1985). Consequently, new poplar clones are currently being evaluated for their susceptibility to insects and diseases. Among defoliating insects, chrysomelid beetles are the most important pests of poplars.

Clonal resistance of poplars has been studied for several species of beetles. In Belgium, the chrysomelid *Phratora vitellinae* preferred Aigeros Section x Tacamahaca Section hybrids, (*Populus trichocarpa* x *P. deltoides*) and *P. trichocarpa* clones from Section Tacamahaca for feeding and oviposition over *P. deltoides* x *P. nigra* clones from Section Aigeros. *P. nigra* was the least susceptible to beetles (Finet and Gregoire 1982). This insect also discriminates between *Salix* spp. and *Populus* spp. (Rowell-Rahier 1984).

In France, two other chrysomelid beetles, *Chrysomela populi* and *Chrysomela tremulae* are serious defoliators on poplar. Within Leuce Section these beetles prefer aspen (*Populus tremuloides*) to white poplar clones (*Populus alba*) for feeding and oviposition; performance of the insects was also better on aspen (Augustin et al., 1993 a, b). *Chrysomela tremulae* prefers Tacamahaca clones when compared to Aigeros clones (Augustin, personal observation). In North America, clonal preference of the cottonwood leaf beetle *Chrysomela scripta* has been reported (Olivera and Cooper 1977; Caldbeck et al. 1978; Harrell et al. 1981; Moore and Wilson 1983; Bingaman and Hart 1992). Leuce poplar clones were shown to be resistant to *C. scripta*. (Caldbeck et al. 1978; Harrell et al. 1981). Harrell et al. (1981) also demonstrated that adults prefer to feed on the foliage from Tacamahaca clones versus Aigeros clones.

Chrysomela scripta is a leaf beetle feeding on *Salix* spp. and *Populus* spp. in North America; it is a particularly serious defoliating pest of poplars (Lowe 1898). This beetle damages poplars in nurseries and plantations during the first to third years of establishment. *Chrysomela scripta* is multivoltine and has from four to seven generations per year in Mississippi and may lay an average of 823 eggs per female (Head et al. 1977). Larvae and adults feed on the most succulent leaves and may attack the stem. This damage affects growth rates of trees and deforms and frequently kills the terminal shoots.

The objective of this study was to determine if there is variation in resistance to the cottonwood leaf beetle among different poplar clones used in a biomass plantation. We examined differences among the clones in *C. scripta* growth, survival and reproduction.

MATERIALS AND METHODS

Plant material

Five *Populus* spp. clones were selected for genetic resistance screening. Section Aigeros was represented by four clones: 1) *P. fremontii* var. *wislenzensii*; 2) *P. deltoides* 14392, a *P. deltoides* x *P. deltoides* cross; 3) 'Robusta', a *P. deltoides* var. *angulata* x *P. nigra* var. *plantierensis* hybrid; and 4) 'Imperial', a *P. deltoides* x *P. nigra* hybrid. One intersectional hybrid (Aigeros x Tacamahaca) was also used: *P. jackii* 'Northwest', a *P. deltoides* x *P. balsamifera* cross.

Ten trees of each clone were potted as cuttings and grown in a greenhouse with a photoperiod of 13:11 (L:D) hours and temperature of 26.6:14.4 (L:D) degrees C. Cuttings were planted in 20 liter pots with a 50-50 peat moss/vermiculite substrate. Trees were grown for 3 months prior to initiation of feeding experiments. Trees were fertilized with a 14-14-14 (nitrogen-phosphorus-potassium) slow release fertilizer after initiation of bud-break.

Experimental insects

Insects used in the experiment were progeny from a laboratory colony of *C. scripta* established in spring of 1993 from a wild population near Snowflake, Arizona, USA. The colony was reared on fresh leaves of *P. deltoides* and maintained in an incubator with a photoperiod of 13:11 (L:D) hours, a corresponding temperature of 24:18 (L:D) degrees C, and 50:60% (L:D) relative humidity.

Bioassays

Eight trees of each clone were chosen for the experiment, excluding the two smallest trees for each clone. Trees were about 100 cm tall at the beginning of the experiment. When egg masses from the *C. scripta* colony hatched, 10 unfed newly molted first instars were randomly assigned on one shoot of each tree. Larvae were caged with nylon mesh screening on the 12 apical leaves of the shoot. Larvae were examined twice a week until pupae were observed. There after, we checked for pupae every day. Pupae were removed, weighed fresh and held in an incubator until adult emergence. Adults were sexed.

Statistical analysis

Developmental times, weights of male and female pupae, and number of eggs were averaged for each tree and analyzed with ANOVA. Means were compared with Protected Least Significant Statistical Difference test of Fisher. Percentage survival from the first instar to adult stage was a categorical variable and was analyzed with a Chi square test.

RESULTS

Bioassays

Survival from the first instar to adult stage was significantly affected by clones (Chi-square = 9.709, df = 4, P = 0.0456, n = 400). Survival was the highest on 'Imperial' (96.3%) and *P. deltoides* 14392 (90%), the worst on *P. fremontii* (83%) and intermediate on *P. jackii* (92.5%) and 'Robusta' (87.5%) (Fig. 1.)

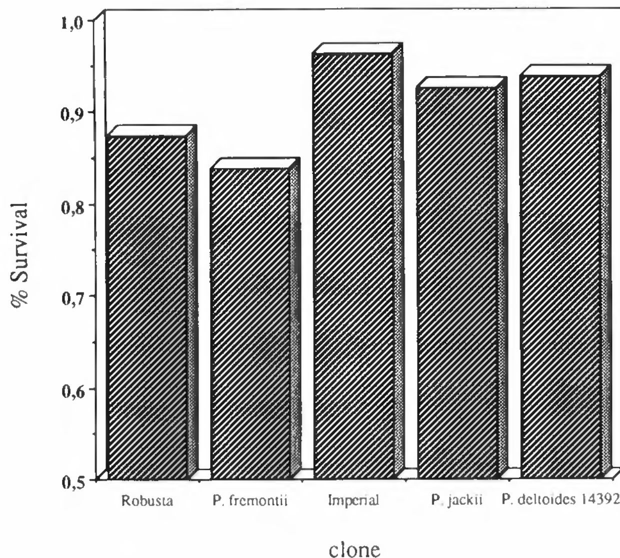

Fig. 1. *C. scripta* survival on five poplar clones (means are plotted with ± 1 SE)

Development time (days from egg hatch to adult) was affected by clone (Table 1; Fig. 2). Sex had no detectable effect on *C. scripta* development time (Table 1). Insects on 'Robusta' had a significantly shorter development time from neonate larvae to adult compared to the other four clones (Fig. 2). Differences between the other clones were not statistically significant. No correlations were observed between survival and development time.

Table 1. Analysis of variance results for larval development time and pupal weight for *C. scripta* reared on five *Populus* clones

Source of variation	F	df	P
Development time, days			
Clone	9.887	4	<0.0001
Sex	0.754	1	0.3881
Clone x sex	0.224	4	0.9242
Pupal weight, mg			
Clone	5.390	4	0.0008
Sex	436.257	1	<0.0001
Clone x sex	1.399	4	0.2432

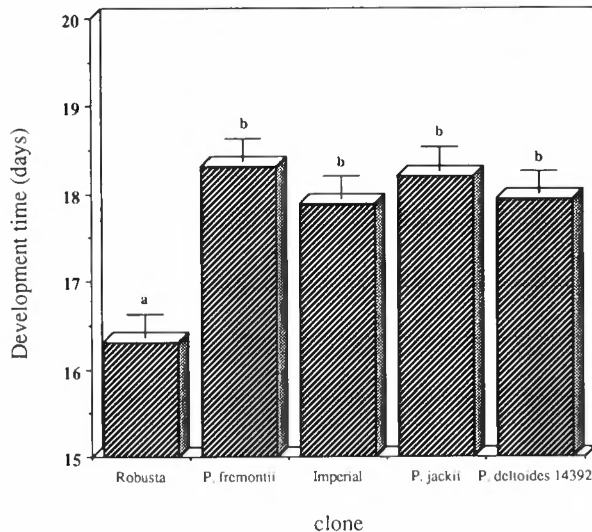

Fig. 2. *C. scripta* development time on five poplar clones. Means are plotted with ± 1 SE. Means for columns with the same letters are not significantly different at p >0.05 according to Fisher PLSD test

Pupal weight was used as an estimate of reproductive potential. Pupal weight was affected by clone and sex but there was no significant clone x sex interaction (Table 1). As expected, female pupae were heavier than males (Fig. 3). Male and female pupae were significantly lighter on *P. fremontii* and heavier on 'Robusta' (Fig. 3). A negative correlation was observed between development time and pupal weight of males (r = -0.605, P = <0001 n = 40) and between development time and pupal weight of females (r = -0.675, P = <0001 n = 40).

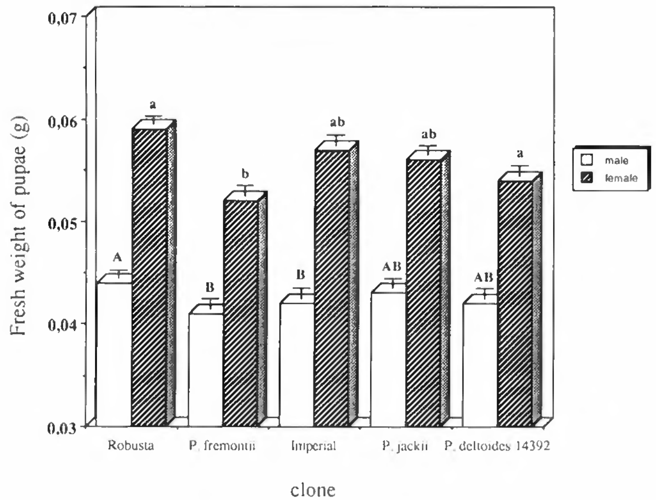

Fig. 3. *C. scripta* pupal weight on five poplar clones. Means are plotted with ± 1 SE. Means for columns with the same letters are not significantly different at p >0.05 according to Fisher PLSD test

DISCUSSION

These results suggest a mechanism of resistance related to antibiosis in the poplar clones. Performance of *C. scripta* was affected by clones. Development time was fastest and pupal weights were heaviest on the Robusta (*P. deltoides* x *P. nigra*) clone whereas the measures of performance were worst on the *P. fremontii* var. *wislenzensii* clone (Figs. 2, 3). *P. fremontii* var. *wislenzensii* is native to Arizona while Robusta is exotic. The slow larval development time on *P. fremontii* could theoretically be detrimental to *C. scripta* in the field because of prolonged exposure to natural enemies.

According to Harrell et al. (1981), we predicted that *P. jackii*, a Tacahamaca x Aigeros hybrid, would be more resistant when compared with the other clones which are all from the Aigeros Section. In this study, performance on *P. jackii* was not particularly negatively affected compared with the performance on the other clones. Two explanations are possible: first, the clones preferred by adults for feeding may not be the best for the performance of the offspring. Alternatively *P. jackii* had very poor growth under our

experimental conditions compared to the other clones (unpublished data) and this may have affected insect performance.

Various foliar characteristics of the leaves could explain the differences in performance among clones. Physical factors like leaf thickness, cuticule characteristics, surface waxes and leaf toughness may modify the palatability and digestibility of the leaves. Differences in foliar nitrogen levels, which may affect survival and reproduction of herbivores, could be linked to observed differences in larval development time and pupal weights. Variation in foliar mineral composition and concentration may influence the observed clonal differences in beetle development. Finally, allelochemicals may be responsible for the differential performance. Phenolic glycosides, the main allelochemical present in poplar and willow leaves, have been implicated in poplar resistance to *C. scripta* (Bingaman and Hart, 1993). Relationships between foliar characteristics of these clones and *C. scripta* performance remain to be studied.

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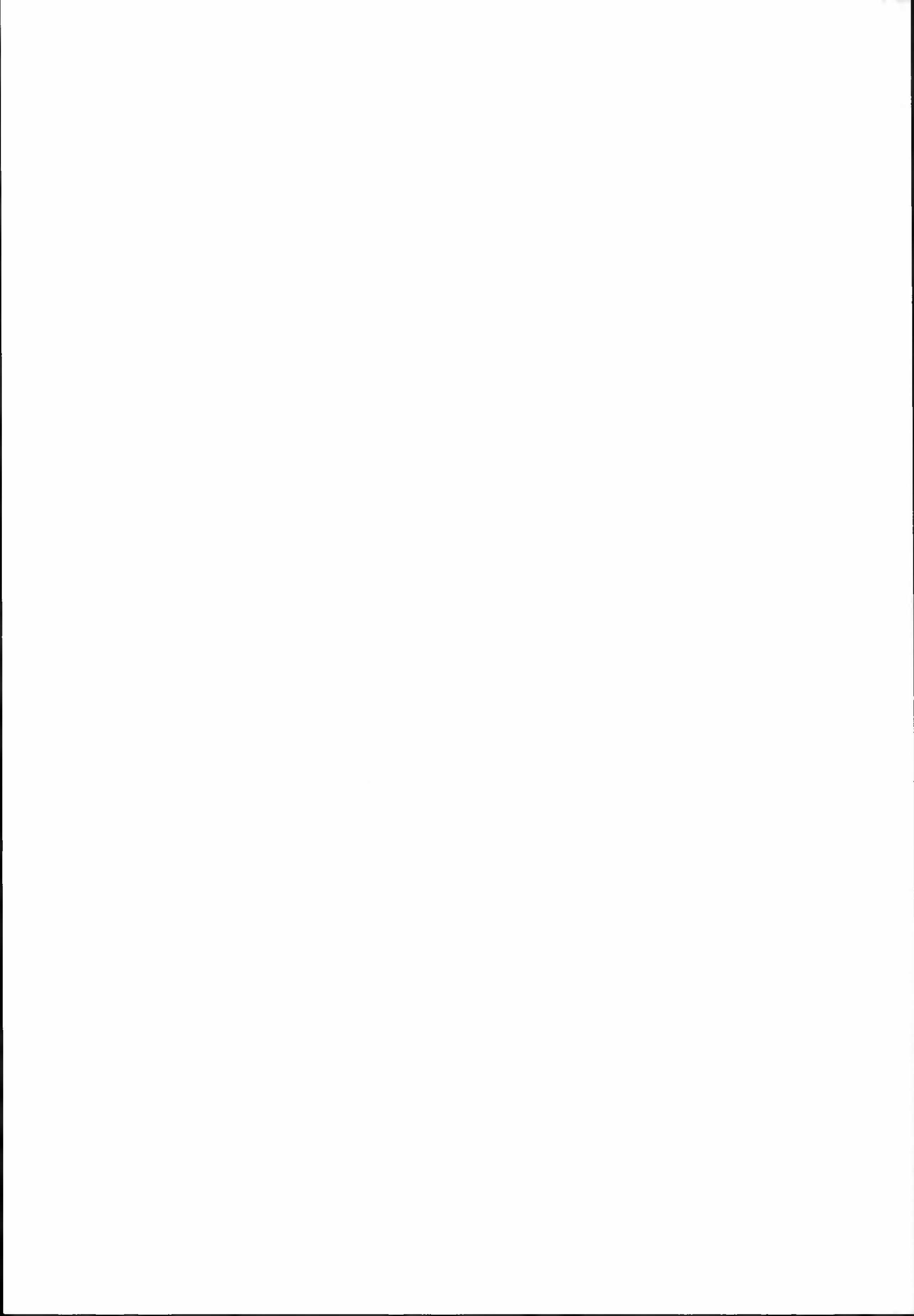

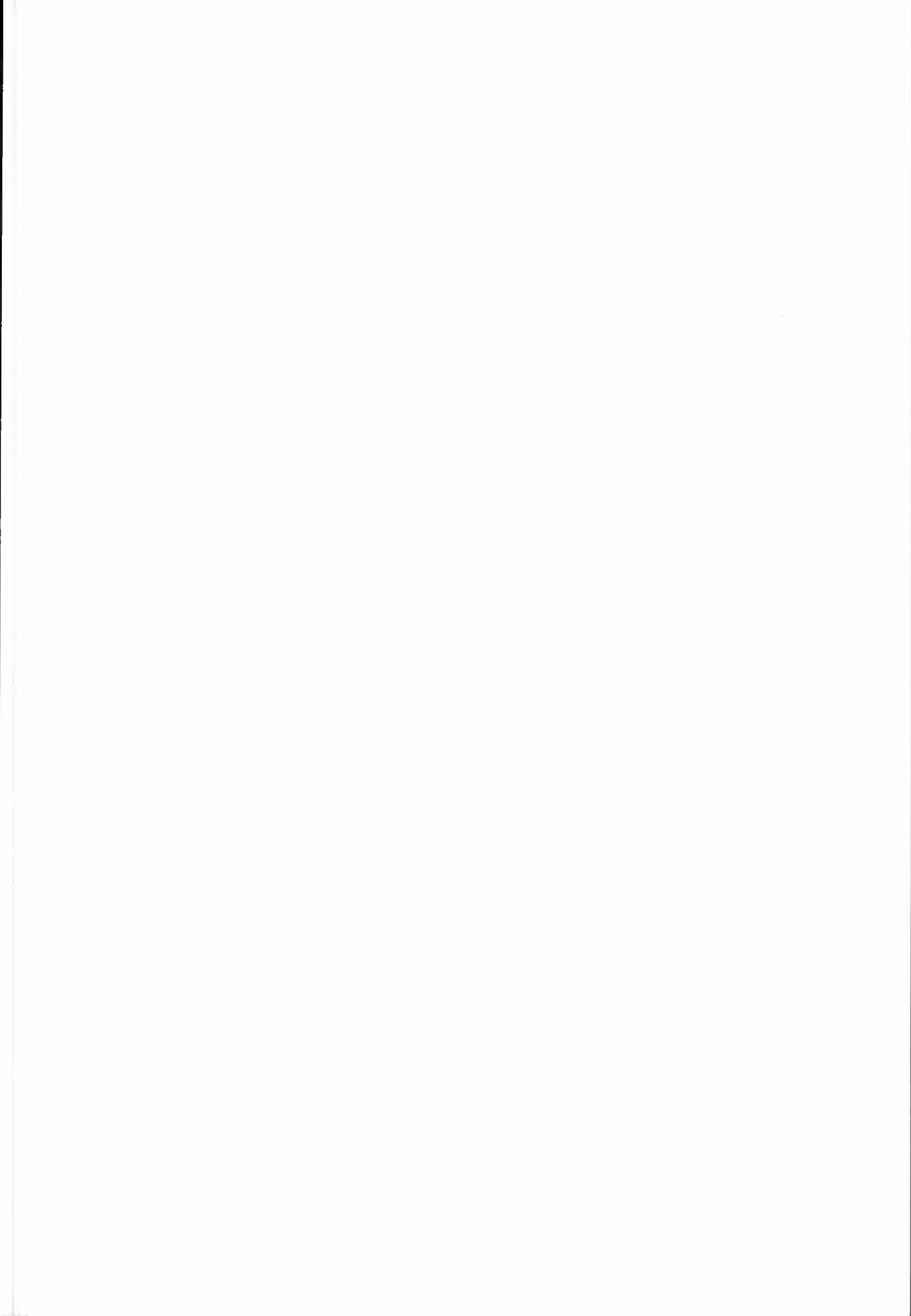

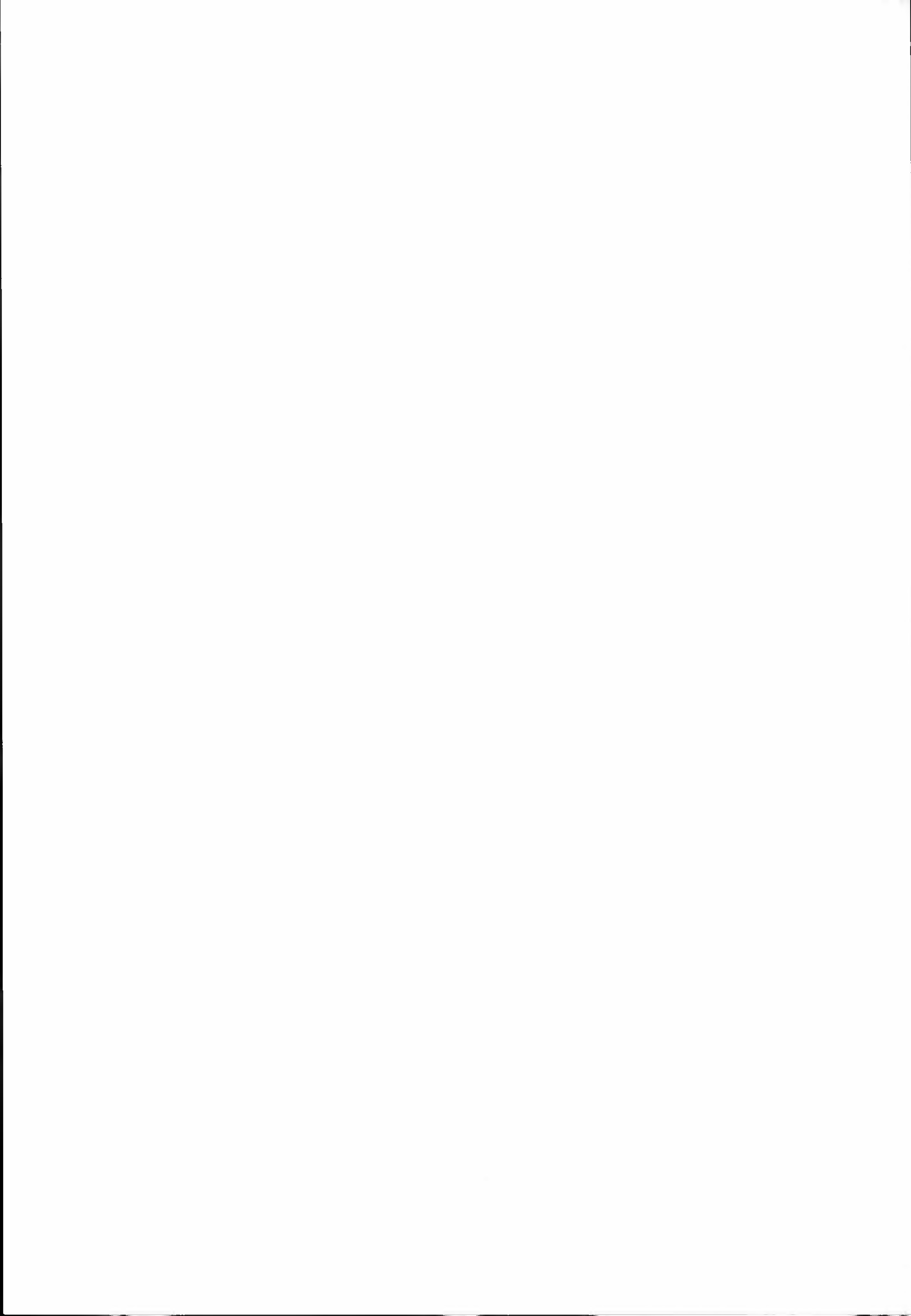

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