Possibilities and Limitations of Vegetative Propagation in Breeding and Mass Propagation of Norway Spruce

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Abstract

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The use of vegetative mass propagation in practical forestry with Norway spruce (*Picea abies* (L.) Karst.) is limited at present, although its potential to deliver high genetic gains is obvious. The objective of this thesis was to study possibilities and limitations of vegetative propagation when applied in different parts of a breeding/mass propagation system for Norway spruce.

Two vegetative propagation methods were studied: somatic embryogenesis and cutting propagation. Somatic embryogenesis was accompanied by losses of genotypes during the propagation process. The embryogenic response at proliferation and maturation was under family control, while germination was obtained for all families. Parental effects on proliferation and maturation were found for male parents but not for female. However, no correlations between embryogenic characters and breeding goal traits could be detected on parental level.

Shortening of treatment with abscisic acid (ABA) during somatic embryo development gave pronounced positive effects on height growth of regenerated plants. An improved protocol, including five weeks ABA treatment and root development in liquid medium significantly improved performance of the resulting plants. The number of plants with lateral roots at the time of *ex vitro* transfer increased substantially with this protocol. Lateral roots at *ex vitro* transfer were shown to be a marker for good height growth and clonal uniformity during the next two years.

Selection for height of cutting propagated clones in the nursery resulted in low responses in height after six years in field. The likely reason for this was low correlations between nursery traits and field traits. Genotype x environment interactions in the studied clonal test series varied from close to zero to more than 50% of the clone component. A tendency towards increased interaction components with age was obtained in one of the series. In situations with large genotype x environment interactions, clonal stability over sites should be included in the selection criteria.

Keywords: breeding, cutting propagation, early selection, mass propagation, *Picea abies*, somatic embryogenesis

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I've been waiting here for so long
And all this time has passed me by
It doesn't seem to matter now

(Banks/Gabriel/Rutherford/Hackett/Collins)

Contents

Appendix

This thesis is based on the following papers, which are referred to by the corresponding Roman numerals.

I. Högberg, K.-A., Ekberg, I., Norell, L. & von Arnold, S. 1998. Integration of somatic embryogenesis in a tree breeding programme – a case study with *Picea abies*. *Canadian journal of forest research 28*: 1536-1545.

II. Högberg, K.-A., Bozhkov, P. V., Grönroos, R. & von Arnold, S. 2001. Critical factors affecting *ex vitro* performance of somatic embryo plants of *Picea abies*. *Scandinavian journal of forest fesearch 16*: 295-304.

III. Högberg, K.-A., Bozhkov, P. V. & von Arnold, S. 2003. Early selection improves clonal performance and reduces intraclonal variation of Norway spruce plants propagated by somatic embryogenesis. *Tree physiology 23*: 211-216.

IV. Högberg, K.-A. & Karlsson, B. 1998. Nursery selection of *Picea abies* clones and effects in field trials. *Scandinavian journal of forest research 13*: 12-20.

V. Karlsson. B. & Högberg, K.-A. 1998. Genotypic parameters and clone x site interaction in clone tests of Norway spruce (*Picea abies* (L.) Karst.). *Forest genetics 5*: 21-30.

Introduction

Background

Norway spruce (*Picea abies* (L.) Karst.) is a boreal conifer of great economic importance in Swedish forestry. The current Swedish breeding programme for Norway spruce has adopted and developed the Multiple Population Breeding System presented by Namkoong *et al*. (1980). In this strategy, the breeding population is grouped into sub-populations and the breeding activities are undertaken within each sub-population. In the Swedish programme (Fig. 1), the sub-populations each include 50 parent genotypes. The genotypes are crossed in a double-pair mating design, where each parent is combined with two other, thus giving rise to 50 families. After an early screening in the nursery, 40 genotypes in each family remain as candidates for the next breeding generation. The candidates are vegetatively propagated and tested as clones in field trials. After the field tests have been completed, one genotype from each family is selected for inclusion in the next breeding generation and the breeding cycle starts again with double-pair mating. For further details regarding the Swedish spruce breeding programme, see Danell (1993) and Karlsson & Rosvall (1993).

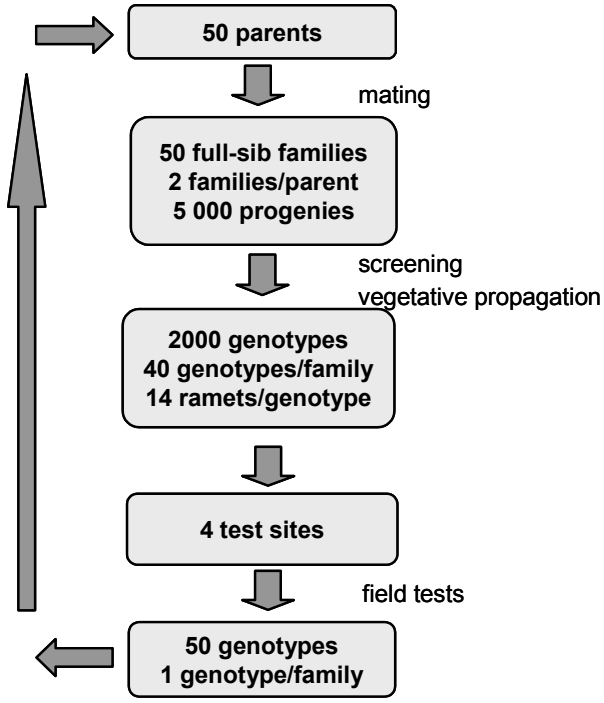

Fig. 1. The breeding cycle for a breeding sub-population in the Swedish breeding programme for Norway spruce.

In breeding, a common approach for testing candidates for the next generation involves progeny tests. Progenies are created by controlled crossings, and normally the same pollen-mix is used for all candidates. Thus, the candidates must reach the flowering stage, which can take several years in Norway spruce. Vegetative propagation allows the candidates to be directly tested as clones, instead of indirectly, as in progeny tests. Besides saving time, since it does not require trees to reach flowering competence, this strategy also increases the selection intensity, by allowing more candidates to be tested at the same cost. Studies on this subject, with varying breeding parameters, have consistently concluded that clonal candidate testing is efficient (Shaw & Hood 1985, Russell & Loo-Dinkins 1993, Rosvall *et al*. 1998).

Vegetative mass propagation can be integrated with the breeding programme at any one of three stages (Fig. 2). After crossing selected parents in the breeding programme, families can be mass propagated without knowing the value of each individual clone and without identifying the individual clones. This mode of using vegetative propagation is called bulk propagation and provides the same genetic gain as if the material was propagated via seeds. Another mass propagation option is to propagate identified and tested clones with valuable properties. Such extended use of superior clones, i.e., clonal forestry, should increase the genetic gain realised in the production forest compared with bulk propagation. However, in order to select superior clones, a period of field test is needed during which the clones must be kept in such a way that they maintain their propagation ability (”storage” in Fig. 2).

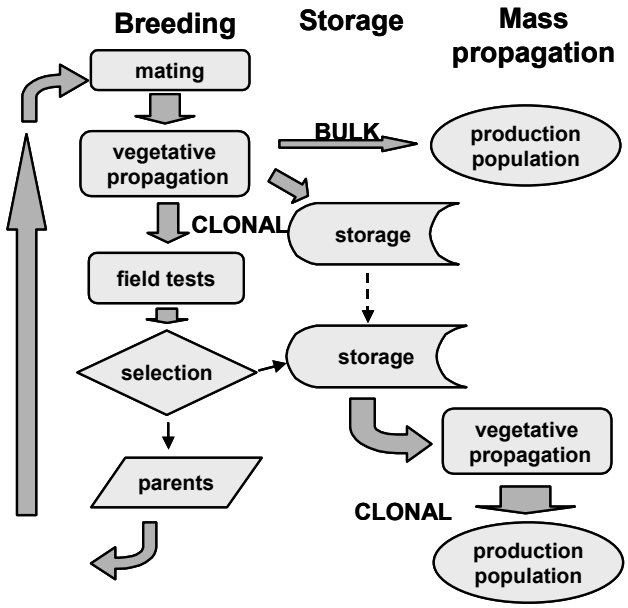

Fig. 2. Illustration of an integrated breeding – vegetative mass propagation system for Norway spruce. BULK indicates propagation without clone identification; CLONAL indicates propagation of identified clones.

Similar models have been presented by Cheliak & Rogers (1990), Sutton *et al*. (1993) and Park *et al*. (1998). Clonal testing for mass propagation can be done on part rather than all of the breeding population. If the base material is restricted to crosses with the very best parents, the genetic gain obtained after field-testing and selection will be higher than if the whole breeding population is represented in the clonal tests.

All propagation methods can introduce effects that are non-genetic, but are confounded with genetic effects. The propagation can also influence the variation within the genetic unit. Non-genetic effects that can be confounded with genetic effects are called C-effects (Lerner 1958). In connection with vegetative propagation, Libby (1976) distinguished two types of C-effects, one caused by differences in donor plant physiology and the other by differences in the environment during propagation. In the case of vegetative propagation it is normally assumed that the clone effect is equal to the genotypic effect. In a model presented by Burdon & Shelbourne (1984) the clone effect was split into a genotype effect and a C-effect. The problem is that C-effects are difficult to determine in practical propagation. In the rather few studies available concerning C-effects on conifers, the effects disappeared a few years after propagation (Foster *et al*. 1984, Cannell *et al*. 1988, Stoehr & Farmer 1988).

The genotype effect consists of two components: additive and non-additive. Non-additive effects are caused by specific gene combinations in a particular clone. The contributions of non-additive effects to the genotype effect can be either positive or negative, depending on the clone. When the clone is used in sexual propagation, recombination takes place and the non-additive effects cannot be exploited. The potential to utilise non-additive effects is one of the attractive features of clonal forestry, since it offers possibilities of capturing additional genetic gain.

Uniformity within a clone is often one of the purposes with vegetative propagation. However, vegetative propagation often results in intraclonal variation comparable to the variation within a sexually propagated population. This indicates that obtaining homogeneous propagation material from a clone is not as straightforward, for several reasons, as it might first appear. For Norway spruce, coefficients of variation in growth characters for cutting-propagated clones were initially larger than for seedlings in a study by Roulund *et al*. (1985). The coefficients of variation then gradually decreased and reached a lower level than for seedlings after eight years.

Vegetative propagation methods

A number of methods are available for vegetative propagation of trees. Advantages and disadvantages of different propagation methods are summarised in Table 1.

Table 1. *Summary of vegetative propagation techniques and their advantages and disadvantages*

Method	Advantages	Disadvantages
Layering	Simple technique.	Expensive, not applicable for mass propagation.
Grafting	Simple technique, avoids rooting problems.	Expensive, not applicable for mass propagation.
Cuttings	Cost-effective compared to other vegetative propagation methods, good plant type.	Needs rooting, low multiplication rate, large areas needed for donor plant hedges, ageing problem complicates clone testing.
Axillary shoots	Low risks of post-effects.	Needs rooting, expensive.
Adventitious shoots	High multiplication rate.	Needs rooting, risks of post-effects, expensive.
Somatic embryogenesis	Very high multiplication rate, cryopreservation can circumvent ageing, good prospects for automatic handling.	Expensive, risks of post-effects, loss of genotypes and certain selection required during the process.

Layering

Branches covered with litter or soil may form adventitious roots and each branch can develop into a new individual. Norway spruce sometimes displays this phenomenon in certain environments, e.g. at high elevations, which means that clones can be formed naturally. Formation of adventitious roots is also frequently observed in Norway spruce plants after planting in the field (Langerud *et al*. 1988).

Grafting

To multiply trees selected for seed orchards and breeding archives, scions can be grafted onto young rootstock plants. This method avoids the rooting problem connected to material from old trees, as the root is provided from a young plant. However, grafting is labour-intensive and an unrealistic approach for mass propagation and for testing large numbers of clones. Spontaneous grafting can also occur in nature, when frictional contact causes wounds on two compatible individuals followed by merger of the tissues.

Cutting propagation

Cutting propagation is a well-known method for vegetative propagation of Norway spruce. The first written description of this technique was given as early as the beginning of the 19th century (Pfifferling 1830, cited in Kleinschmit *et al*. 1973).

Cuttings are usually rooted in a greenhouse with facilities for controlling temperature and air humidity. The rooting substrate is often porous to allow good aeration of the medium during the period when frequent watering is needed at the beginning of the process. The soil is commonly heated to maintain the temperature of the substrate at an optimal level. Hormone treatment is not required if the donor plants are healthy and have not entered a stage where propagation ability has decreased.

The method has an important limitation, in that only young Norway spruce plants can be propagated at high rates in practice. When the plants get older, their rooting ability decreases and rooted cutting plants display plagiotropic growth and asymmetric branching (Kleinschmit *et al*. 1973, Roulund 1975, Dietrichson & Kierulf 1982). The requirement of field tests for identifying superior clones suggests a need for strategies that can maintain their propagation ability until field tests have been completed. One way to maintain the rooting ability is to use serial propagation (Kleinschmit *et al*. 1973), i.e., to take new cuttings from cutting plants in cycles of 3-4 years. However, Dekker-Robertson & Kleinschmit (1991) reported serious symptoms of ageing after 6-7 cycles of serial propagation. Bentzer (1981) described another strategy, so-called hedging, where low hedges are formed by recurrent pruning of the donor plants. How long propagation ability and satisfactory growth habit can be maintained with hedging is unknown, but clones will lose the capacity of cutting propagation over time also with this technique. Ageing symptoms can vary according to the part of the donor plant from which the cutting is taken. A general rule is that cuttings taken from upper positions on the donor plant display more ageing symptoms than cuttings taken further down (Fortanier & Jonkers 1975, Roulund 1975).

Norway spruce plants produced by cutting propagation differ from seedlings in various respects. For instance, their stem base diameter is larger at a given age and needles appear along the base of their stems. Higher survival and better growth have been reported for cutting-propagated plants compared with seedlings of the same size and genetic origin, up to about 10 years of age (Gemmel *et al*. 1991). It has been suggested that greater resistance to pine weevil (*Hylobius abietis*) contributes to this superiority, and cutting-propagated plants have been shown to be attacked less often, and damaged less extensively, by pine weevil (Hannerz *et al*. 2002).

Clonal forestry with Norway spruce based on cutting propagation has been tried in projects in Sweden, but has not been successful, regardless of the method used to maintain rooting ability (Högberg *et al*. 1995). Sonesson (2003) presented an overview of the present situation in the latest Swedish clonal forestry project, and concluded that the main reasons for the lack of success with clonal forestry were low rooting percentages and high degrees of plagiotropic growth. Both factors reduce the number of plants produced, as a percentage of cuttings initially taken and thus raise production costs. Hedging costs further contribute to high prices. Producing a cutting plant of a tested clone was estimated to be 100% more expensive than producing a seedling, and the cost for a bulk propagated cutting plant, with cuttings taken from a juvenile donor plant, was estimated to be 60%

higher than for a seedling. At present, bulk propagated cuttings are only produced commercially in Sweden in small numbers.

Significant bulk propagation programmes based on cuttings have been initiated for *Picea mariana* and *Picea glauca* production in eastern Canada (Tousignant 1995, Adams, pers. comm.), and for *Picea sitchensis* production both in Great Britain (Lee 2003) and Ireland (Harrington 2003). A feature shared by all of these programmes is that seedlings from selected families in the breeding population are used as donor plants.

Axillary shoots

Propagation via axillary shoots is an *in vitro* micropropagation method in which existing meristems are used as explants. The meristems are formed and develop shoots spontaneously. In some cases they are stimulated to develop by treatment with appropriate growth regulators. Further shoot multiplication can often be obtained by using shoot tips and nodal segments in subcultures. Shoots thus produced are subsequently rooted. This method is mostly used for broadleaved species belonging to genera such as *Betula* and *Populus* (Bajaj 1986, Jokinen & Törmälä 1991, Welander 1993, Chun 1993). Axillary shoot micropropagation has also been reported for many conifers (Bajaj 1986, Abdullah *et al*. 1989, Baxter *et al*. 1989). However, practical applications with conifers are hampered by low multiplication rates, difficulties in rooting, and high production costs due to the multiple manual operations required during propagation.

Adventitious shoots

This method use meristems formed *de novo* from various explant sources, e.g., embryos, stems, leaves and needles. It is closely related to the previous method and in some circumstances both axillary and adventitious shoots can be formed from the same explant. The meristems are stimulated to develop by cytokinin treatment and the elongated shoots are rooted, normally after treatment with auxin. Shoot tips and nodal segments from the elongated shoots can be used for subsequent multiplication with axillary shoot propagation. Adventitious shoot propagation has been reported for many conifers (Bajaj 1986, Lu *et al*. 1991, Kolevska-Pletikapić & Buturović-Derić 1995, Drake *et al*. 1997). In New Zealand, a practical application has been developed with *Pinus radiata* (Aitken-Christie *et al*. 1988, Gleed *et al*. 1995), in which micropropagated plants either go directly to the forest or are used as donor plants for cutting propagation (Menzies *et al*. 2001). For Norway spruce, no application of the method has been developed, even though the possibility of propagating the species by adventitious shoots has been shown (von Arnold 1982, Bornman 1983). The reasons for the reluctance to exploit the method in practice are essentially the same as for axillary shoot propagation, even though it generally gives higher multiplication rates.

Somatic embryogenesis

Vegetative propagation by somatic embryogenesis has been reported for virtually all economically important tree species. The first reports of successful plant regeneration via somatic embryogenesis for a conifer were published almost two decades ago. The species involved was Norway spruce, and this milestone was achieved simultaneously and independently in two laboratories (Hakman & von Arnold 1985, Chalupa 1985). In somatic embryogenesis with Norway spruce, embryogenic tissue is usually initiated from a zygotic embryo by transferring it to a culture medium containing cytokinin, auxin and nutrients. By renewing the medium at regular intervals, the embryogenic tissue can be stimulated to grow stably. This phase is called proliferation and can be performed using either callus cultures on gelled medium or cell suspension cultures in liquid medium. When enough tissue has been produced, somatic embryos are stimulated to develop by withdrawing the cytokinin and auxin, then adding abscisic acid (ABA). The mature somatic embryos pass through a partial desiccation treatment, in which their water content is decreased in a controlled manner, and they are then germinated on medium free of growth regulators. After germination, embryos are transferred to vessels that allow the root to develop in liquid, while the epicotyl is in the air. After further development in these vessels, somatic embryo plants are acclimatised to *ex vitro* conditions and subsequently cultivated in accordance with standard nursery practices. Embryogenic cultures in the multiplication phase can be cryopreserved in liquid nitrogen, thus providing an efficient way of maintaining the propagation ability of the clones included in field tests.

Some noteworthy details of the protocol are as follows. It has been shown that the initiation rate varies with the age of the explant tissue. Initiation of an embryogenic culture using explants from a 14-month old Norway spruce seedling has been reported (Ruaud *et al*. 1992). There is also a report of successful re-initiation of an embryogenic cell line from a 3-year-old somatic embryo plant (Harvengt *et al*. 2001). Initiation of explants taken from plants seems, however, to be rare. The best results have been attained when zygotic embryos from immature seeds were used. Using embryos from stored seeds or seedlings would be advantageous from a practical point of view, but the response is then lower (von Arnold *et al*. 1995).

Cryopreservation of embryogenic tissue has proven to be surprisingly robust. High regrowth rates after thawing were obtained in experiments with Norway spruce by Nörgaard *et al*. (1993), in accordance with results for interior spruce (*Picea glauca x engelmanni* complex; Cyr *et al*. 1994) and *Picea glauca* (Park *et al*. 1994).

The maturation of somatic embryos was early identified as a key step in somatic embryogenesis, and one in which losses can be very high. For instance, Attree *et al*. (1990) reported maturation frequencies as low as 6-8% in embryogenic tissue of two *Picea mariana* cell lines and one *Picea glauca* cell line. Clearly, as shown in a recent review by Dunstan *et al*. (1998), ABA plays an important role in the process (and also in subsequent germination). Another factor that may be

important is the osmotic potential, since Bozhkov & von Arnold (1998) showed that inclusion of the osmotically active compound polyethylene glycol (PEG) increased yields of mature somatic embryos, but adversely affected their further development. The complexity of the processes involved, including ABA metabolism, together with interactions between endogenous ABA and exogenously applied ABA, indicates the problems associated with developing a generally efficient standard protocol.

Since somatic embryogenesis includes rather strong treatments with plant growth regulators and osmotic agents, as well as cryopreservation, there is a recognised risk that genetic changes may occur during the process. However, the probability appears to be low. For instance, in a study of embryogenic cultures of Norway spruce, Mo *et al*. (1989) concluded that they were stable over time with regard to nuclear DNA content and morphology of regenerated plants. Heinze & Schmidt (1995) found no somaclonal variation in a RAPD analysis of Norway spruce clones derived from somatic embryos. Isabel *et al*. (1996) found somaclonal variation in very low frequencies in *Picea glauca* (in four plants out of 2270) and suggested that it was due to some kind of genetic instability. More recent studies on *Picea glauca* and *Picea mariana* confirm that somatic embryogenesis can induce somaclonal variation in low frequencies (1-1.6%; Tremblay *et al*. 1999). Two important sources of genetic instability were detected, clone and time in culture. Park *et al*. (1998) compared clonal performance of plants regenerated after two different lengths of storage in liquid nitrogen and found strong clonal correlations between the two plant groups. Thus, somatic embryogenesis appears to be a stable method from genetic point of view, generating very low frequencies of somaclonal variation.

The performance of interior spruce (*Picea glauca x engelmanni* complex) plants, regenerated from somatic embryos, during development in the nursery and on a reforestation site was reported by Grossnickle & Major (1994a, b) and Grossnickle *et al*. (1994). No differences between somatic embryo plants and seedlings were found in most measured characters, morphological or physiological. However, height growth rate was on average slower during the first growth period in the nursery for somatic embryo plants. Height increment after the first two years on a reforestation site was similar, but significant differences between somatic embryo plants and seedlings still remained. Nsangou & Greenwood (1998) did not find any differences between somatic embryo plants and seedlings of *Picea rubens*. Lamhamedi *et al*. (2000) found, within *Picea glauca* families, more variation in some morphological characters among clones, produced via somatic embryogenesis, than among seedlings. At the same time, family means calculated using data from clones did not differ from family means based on seedling data.

The obvious advantages associated with somatic embryogenesis, i.e. rapid multiplication and the possibility of long-term maintenance of propagation ability in storage, have aroused great interest and stimulated worldwide research efforts. Clonal test programmes with *Picea glauca* and Norway spruce are in progress in eastern Canada (Adams, pers. comm.), while programmes for large-scale propagation by somatic embryogenesis are being developed in both Canada

(Sutton 2002) and the USA (Timmis 1998). However, automatic handling of somatic embryos has not yet been reported in commercial application. Two strategies can be distinguished at the crucial germination stage (comparable to sowing of seeds): to encapsulate the embryo in an artificial seed-shell or to develop systems for handling naked embryos. For high value applications, semi-automated solutions may have a place, even if the protocol could be further optimised. Whatever techniques are eventually developed, it will be essential to reduce the cost of somatic embryo plants to a level close to that of seedlings (Cervelli & Senaratna 1995).

Breeding and mass propagation applicability

Today, cutting propagation and somatic embryogenesis appear to be the most likely options to be used for vegetative propagation in breeding and mass propagation of many conifers, including Norway spruce. Three different ways of utilising vegetative propagation can be distinguished (Fig. 2): candidate testing in the breeding programme, bulk propagation of selected families and propagation of tested, high-value clones.

With its robustness and the widespread acceptance as a propagation technique, cutting propagation is a useful tool, and it has been practiced for a decade in the Swedish breeding programme. Since it involves young plants, the cutting propagation is not subject to adverse ageing effects in this case. The rapid multiplication rates and corresponding savings of time offered by somatic embryogenesis make it a tempting option, but to date the costs of producing somatic embryo plants is too high. To replace cutting propagation in the spruce-breeding programme, the costs of producing somatic embryo plants need to be lower than the costs for cuttings, which will require a high degree of automation of the production techniques. The present standard protocol includes many laborious steps, especially in the handling of single somatic embryos, and plants during the germination and early growth stages. Examples of labour-intensive operations include picking embryos for partial desiccation and for germination, the transfer of germinated plants from germination medium to vessels with liquid medium and the transfer of plants to *ex vitro* conditions. Automation of these steps would bring the production cost down substantially.

Like propagation of next-generation candidates in the breeding programme, bulk propagation of high-value families by cuttings avoids the ageing problem. The choice of method will therefore essentially be made on the same basis as discussed in the previous paragraph.

The difficulties raised by the reduction of rooting ability with time make cutting propagation less attractive for mass propagation of tested clones. In this respect, somatic embryogenesis has obvious advantages, since it offers the cryopreservation option and high multiplication rates. The main obstacle for application of somatic embryogenesis is the production cost. With the information

currently available on this subject, it is difficult to predict when a cost-effective system will be developed.

In vegetative propagation, unintentional losses or intentional selections of genotypes occur at several points during the breeding process and associated mass propagation. One could argue that losses of genotypes also occur in sexual propagation, e.g., when some seeds fail to reach a mature stage or are damaged. Such losses are assumed to be random, but there may be differences among genotypes. If, in an extreme case, no flowering occurs for a particular genotype, the genotype is lost and the breeding population decreases in number.

In the Norway spruce breeding programme, losses of genotypes occur when candidates are vegetatively propagated, and if no candidates can be obtained from some parents, the propagation is also a selection process, which can have significant genetic influence as it decreases the number of members in the breeding population. This decrease can be neutralised by including additional genotypes in the population, but such measures will decrease the genetic gain, as lower-ranked genotypes will be added. If low propagation rates occur within families, more progenies of these families must be included. This will increase costs, but will not affect genetic gain as long as propagation ability and breeding goal traits are uncorrelated. If there is a negative genetic relationship between propagation ability and goal traits, the propagation will reduce the genetic gain.

The propagation method can introduce effects of varying nature that influence the accuracy and precision of clone performance estimates, making clonal selections less reliable, as explained earlier. If there are enough individuals representing a clone, an early within-clone selection may improve the precision. Effects that reduce the accuracy of clonal estimates are more difficult to address, but important to detect if possible.

Early selection among candidates is applied in the Swedish spruce-breeding programme. Normally, this is applied to seedlings, but it can also be applied on clonal level when vegetatively propagated plants are still in the nursery. For practical reasons, the selection intensity at this stage cannot be very strong and the selection efficiency depends mainly on the correlations between early and later traits.

Theoretically, effects in a mature trait after early clonal selection in a juvenile trait can be described by the formula for correlated response (modified from Falconer & Mackay 1996):

$$CR_{m|j} = i\, r_{TIj}\, r_{Gm|j}\, \sigma_{Gm} \quad (1)$$

where:

$CR_{m|j}$ =correlated response in trait m (mature) when selecting for trait j (juvenile)
i = standardised selection intensity
r_{TIj} = correlation between the true and estimated genotypic value for trait j
$r_{Gm|j}$ = genotypic correlation between traits m and j
σ_{Gm} = genotypic standard deviation of trait m

The selection intensity is independent of genetic relations and is decided by the breeder/selector in each situation. From the formula it can be concluded that the genetic relations determining the response to selection are expressed by the factors r_{TIj}, $r_{Gm|j}$ and σ_{Gm}. The factor r_{TIj} expresses the precision of the genotypic value estimates for the juvenile trait. The genotypic correlation, $r_{Gm|j}$, describes the degree and direction of the relationship between the juvenile and mature trait, while σ_{Gm} is a measure of the genotypic variation in the mature trait.

Clonal field tests require vegetative propagation, and the selection of superior clones is an important step in the process. The genetic gain that can be captured following clonal tests depends on genetic parameters, the selection intensity applied and the clonal variation. Field trial quality has a major impact on the accuracy of genetic parameter estimates. Low trial quality often results in low heritability estimates, thus reducing the effect of clonal selection. Results from several clonal tests of Norway spruce have been presented over the years. Published estimates of broad-sense heritabilities for height after five to 10 years in the field range from 0.16-0.25, accompanied by genotypic coefficients of variation ranging between 0.08 and 0.22 (Bentzer *et al*. 1988, Bentzer *et al*. 1989, Shaw *et al*. 1988, Högberg & Danell 1989, Karlsson *et al*. 1998). Rosvall *et al*. (2001) compiled information of tree height from a number of clonal tests, and found a mean broad-sense heritability of 0.18 and a mean genotypic coefficient of variation of 0.12.

Another important component to consider for capturing genetic gain is the genotype x environment (GxE) interaction. If this component is large, it is advisable to study the tested population carefully and, if sufficient information is available, to direct clones to appropriate, specific target zones. Shelbourne (1972) suggested that testing and selection become problematic when the GxE interaction effect is 50% or more of the clone variance component. For Norway spruce, most reports of GxE interaction for height growth in clonal trials suggest that they are lower than 50% of the clone variance component (St. Clair & Kleinschmit 1986, Bentzer *et al*. 1988, Kleinschmit & Svolba 1991, Isik *et al*. 1995). However, if frost-prone sites are included in a field test series, differences between clones in

the timing of bud flush, with corresponding differences in frost damage, can cause substantial GxE interactions. For example, in Karlsson *et al*. (2001), ratios between GxE interaction components and clone components for height growth approached values close to 100%. Considering GxE interactions when selecting clones is not straightforward, and it is only effective if the factors underlying the interaction can be accurately identified. If such factors cannot be identified, clones that show the most stable performance over sites tend to be selected. One reason why GxE interactions are more often seen as a selection problem, rather than offering possibilities to match clones to site, is that site information is usually insufficient and cannot provide a sound basis for such decisions.

Objectives

The overall objectives of the work on which this thesis is based were to study possibilities and limitations of vegetative propagation when applied in different parts of a breeding/mass propagation system for Norway spruce.

The specific objectives of this research were:

To identify ways to integrate somatic embryogenesis in the Swedish breeding programme for Norway spruce, and the expected effects (**I**).

To identify critical factors during *in vitro* stages and acclimatization in somatic embryogenesis that influence early plant development (**II**).

To estimate the effects of selection at *ex vitro* transfer on clone performance and intraclonal variation of somatic embryo plants (**III**).

To estimate the gains in field growth achieved by clonal selection in the nursery after the first cycle of cutting propagation (**IV**).

To estimate genotypic parameters and genotype x environment interactions when selecting clones after field tests established with cuttings (**V**).

Materials and Methods

In the studies of different aspects of somatic embryogenesis (**I, II** and **III**), material from a controlled crossing carried out in 1995 was used. All parents involved were members of a Norway spruce sub-population in the Swedish breeding programme. Altogether 25 full-sib families deriving from 23 parents were collected for initiation of embryogenic cultures.

Unfavourable weather conditions during crossing and embryo development reduced the material entering somatic embryogenesis to 18 families (**I**). After this reduction in the number of families, the mating design included four disconnected groups. The zygotic embryos were subjected to initiation followed by proliferation, cryopreservation, thawing, renewed proliferation, maturation, and, finally, plant regeneration. The numbers of responding cell lines at different stages of somatic embryogenesis were counted. For plant regeneration, numbers of individuals within cell lines were also counted. The data were analysed with respect to differences between parents and families using χ^2-tests. Parental effects on proliferation frequency and maturation frequency were estimated within each disconnected group and gender. Frequencies were transformed to approximate normal distribution prior to analysis. Obtained parental values for the events in the propagation were used for ranking within each group and gender, then rank correlations between embryogenic values and breeding values from field trials were calculated. Observed significance levels were calculated groupwise, and then combined for all groups by calculating the product of the derived levels. The significance level of the combined test was found by enumerating all possible combinations of rank correlations.

Data analysed in Paper **II** were collected during the first growth period, either in the growth chamber or nursery, and related to treatments during maturation and acclimatization. The material was derived from three full-sib families and included, in all, 21 genotypes. Four sets of experiments were performed. The first experiment was designed to study effects of abscisic acid (ABA) treatment for five or seven weeks during maturation. The effects of early treatment with short photoperiods to induce inwintering of recently acclimatised somatic embryo plants were studied in the second experiment. The third experiment focused on the effects of polyethylene glycol (PEG) treatment during maturation and the length of first growth period under continuous light treatment (CLT). The fourth experiment monitored the effects of an improved protocol, including a one-week maturation pre-treatment in hormone-free medium, and plant development with roots in liquid medium in later stages of germination. Seedling controls, sown at the time of *ex vitro* transfer of somatic embryo plants, were included in the first and second experiment. The results were assessed by analysis of variance, using models with fixed main effects, but no interactions, as independent variables.

In the experiments reported in Paper **III**, height was measured after the first two growth periods in the nursery on somatic embryo plants belonging to 13 clones from three full-sib families. Plant regeneration was based on the improved

protocol described in Paper **II** (fourth experiment). Plant materials were generated in two consecutive years. Plants regenerated in the first year were transplanted into larger containers in the beginning of year 2, while plants regenerated in the second year remained in the same container throughout the experimental period. The plants were kept in the nursery during the whole of this time. At *ex vitro* transfer, data were collected on epicotyl length, main root length and the presence of lateral roots. Clonal means, standard errors and coefficients of variation for these variables were calculated. Significant differences for plant height were determined by calculating 95% confidence intervals. Effects of selection that were already apparent at the time of *ex vitro* transfer were studied in four clones represented by a large number of plants. Plants within each clone were grouped in successively stronger selections for epicotyl length (2-mm classes) and main root length (5-mm classes). Finally, the plants were divided into two groups according to the presence or absence of lateral roots. Differences between groups, reflecting different selection strengths, within clones were determined by calculating 95% confidence intervals.

In the study described in Paper **IV**, 794 four-year-old seedlings from 96 full-sib families were selected and cutting-propagated. This primary selection was based on plant height. Timing of bud flush was classified in spring two years later using the obtained cutting-propagated clones. In autumn of the same year, five additional characters were assessed on the clones. Three years after rooting, the clones were divided into two groups, one with clones intended for a harsh climate and one with clones intended for a mild climate. Based on this division, the clones were planted in four field trials, two in each seed zone. In one of the field trials in the mild seed zone, the clones selected for the harsh zone were also planted. Each trial was designed as a randomised block experiment with single-tree plots and seven replications. Plant heights were measured immediately after planting and after six years in the field. On the latter occasion, three quality characters were classified. Genetic parameters were estimated by calculating variances for clones and residuals. Best linear unbiased predictors (BLUPs) of clone values were calculated using software developed by Danell (1988). BLUPs over trials were calculated by rescaling the genetic variance to a standardised variance. Pearson's product-moment correlations between nursery characters and BLUPs for field characters were estimated. Clone x site interactions were estimated by analysis of variance, using an expanded model including the site variable as a main effect. Selection was based on clonal rankings at the time of planting. Selection responses were estimated by zonewise regression of BLUPs after six years in the field against selection intensities.

In the investigation reported in Paper **V**, 311 cutting-propagated clones were planted at five field sites, with nine replications/clone and site. The clones were in the second cycle of vegetative propagation. Bud flush was scored in the spring of the sixth year in the field, while height was measured and frost damage scored in the autumn of the same year. Several characters were measured or scored after 11 years in the field. Clonal BLUPs for each site were calculated. Correlations between trials were estimated as Pearson product-moment correlations between BLUPs of the same traits in different trials. The sitewise model was expanded by

including site and clone x site interaction effects, and used in analysis of variance and estimation of variance components for the complete experimental series.

Main results

Results are summarised in Table 2, along a time axis to indicate when different effects appear or selections are made.

Table 2. *Summary of results from Papers **I-V**. The time column indicates approximately when different activities/measures occur, counting from fertilization and formation of zygotic embryos. SE = somatic embryogenesis, CP = cutting propagation*

Time	Activity/event	Results	Paper
4 months	SE-Establishment of cell lines	~50% reduction in number of cell lines. Mainly method, but also family and genotype effects.	**I**
6 months	SE -Cryopreservation	~50% reduction in number of cell lines due to exclusion of slow-growing cell lines. No reduction of selected fast-growing lines.	**I**
8 months	SE-Maturation	~50% reduction in number of cell lines. Method, family and genotype effects.	**I**
10 months	SE-Plant regeneration	No reduction of cell lines, no family effects on survival, but genotypic differences in numbers of regenerated plants. No correlations between embryogenic capacity and breeding values.	**I**
2-3 years	SE-Propagation effects and plant growth	Negative effects on plant growth of long ABA treatment during maturation and long first growth period with continuous light. Clones had lower height means than seedlings and larger intraclonal variation. Long epicotyl and presence of lateral roots at *ex vitro* transfer indicated good clone performance and uniformity. Somatic embryo plants with the same height as seedlings in year 1 grew equally well during year 2.	**II**, **III**
6 years	CP-Nursery selection	Approximately 3% gain in height after 6 years in field after a 20% selection in the nursery. Negligible effects of nursery selection on other characters, except branch angle. Stratified selection did not improve the gain.	**IV**
12 years	CP-Clonal performance in field tests	Significant clone x site interaction for many traits, and increasing trends with age for growth traits. Medium heritabilities for growth traits.	**V**

All tested families in Paper **I**, representing all tested parents, gave rise to embryogenic cell lines, except one that was lost due to infection. Significant differences in embryogenic responses were present among families, among groups and among male parents within groups, but not among female parents within groups. All cell lines survived cryopreservation, except one, with fast tissue growth as the selection criterion. Mature somatic embryos were obtained from 12 out of 15 families, representing 15 out of 20 parents. Significant differences in maturation response were detected among families and male parents within groups, but not among groups or female parents within groups. Plants with elongating epicotyls were regenerated for all cell lines entering germination. Variations among genotypes within families were large. No significant rank correlations between embryogenic characters and field performance were found, except in one case. The proportion of genotypes that could successfully generate plants was approximately 12% (Fig. 3). Data from experiments with an improved protocol showed that this number could be increased to about one third (Fig. 3).

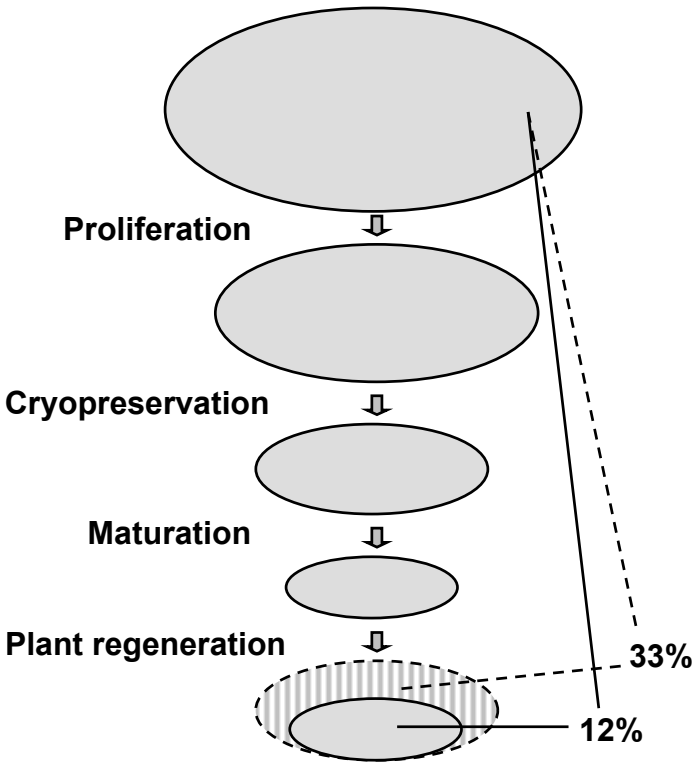

Fig. 3. Loss of genotypes during somatic embryogenesis (**I**). The shaded areas correspond to the proportions of genotypes remaining after each respective step. The barred area indicates the expected remaining proportion of genotypes for an improved protocol. N.B. Only the final outcome of the improved protocol is marked in the figure, summarising improvements in each step.

The data presented in Paper **II** show that two factors involved in somatic embryogenesis had a clear impact on early plant height development: the duration of abscisic acid (ABA) treatment during embryo development and the length of continuous light treatment (CLT) during the first growth period. Shortening the ABA treatment from seven to five weeks increased plant height after the second growth period by approximately 60%. No differences between the two durations of ABA treatment were found in terms of survival. Eleven months of CLT resulted in slight, but significant, reductions in plant height after two growth periods compared with nine months of CLT. The negative influence of long CLT was strongly significant for height increment during the second growth period. Immediate inwintering after acclimatization improved survival of somatic embryo plants but was followed by poor growth during the next growth period. A third factor, polyethylene glycol (PEG) treatment during embryo development, affected neither plant height nor survival in the following two growth periods. A protocol including a pre-treatment of cultures in growth regulator-free medium for a week before ABA treatment for five weeks, and root development in liquid medium during later stages of germination (Fig. 4), had a pronounced positive effect on survival and plant height at the end of the first growth period. The improved protocol gave a much higher frequency of plants with lateral roots at the time of *ex vitro* transfer. The presence of lateral roots was strongly, positively correlated with survival and height after the first growth period, while no effects of main root length were detected.

Fig. 4. Early plant development in a vessel that allows root growth in liquid medium improved the standard protocol. Presence of lateral roots at this stage is beneficial for further growth. (Photo: Christine Devillard, Skogforsk).

Survival within somatic embryo clones ranged from 83% to 100% after two growth periods in the nursery (**III**). In comparison, the survival of seedling

controls ranged from 99 to 100%. Height after one and two growth periods was, with a few exceptions, lower for somatic clones. The relationship between heights attained after the first and second growth periods was similar for seedlings and clones within their common range of height values. The coefficients of variation within seedling families were consistently smaller than within clones, due to a large proportion of small plants in the latter category. Early selection for presence of lateral roots at *ex vitro* transfer improved the clonal height means considerably (Fig. 5). If epicotyl length was also included as a selection criterion, the effect was further improved. Intra-clonal variation was reduced with increased selection strength and reached levels comparable to intra-family variation of seedling controls when the presence of lateral roots and epicotyls exceeding 8 mm were applied as selection criteria. This procedure imposed selection strengths within clones of 13-37%. Selection for main root length had minor effects on clonal mean heights and intra-clonal variation.

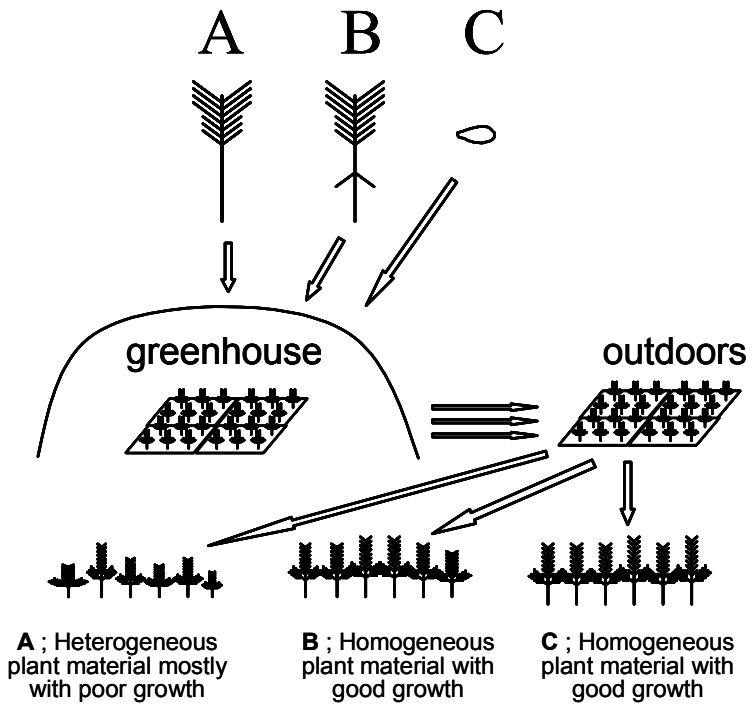

Fig. 5. Effects of absence and presence of lateral roots at the time of *ex vitro* transfer on later growth in the nursery. A = somatic embryo plants without lateral roots at *ex vitro* transfer, B = somatic embryo plants with lateral roots at *ex vitro* transfer, C = seedlings.

Clonal means, broad-sense heritabilities and genotypic coefficients of variation for field characters were homogeneous over trials in the study described in Paper **IV**. Heritabilities for height, crookedness and branch index typically reached values of around 0.20, while heritabilities exceeding 0.40 were found for branch angle. Genotype x site interaction accounted for small proportions of the random variance in height after six years in the field. Correlations between nursery characters and field characters were generally weak. Regressions of field height on nursery height, in successively smaller groups following the clonal ranking in the nursery, had consistently high correlation coefficients. Direct selection of the top 20% clones with respect to height at planting resulted in gains in field height of about 3%. Selection gains were not improved by stratified selection based either on differences in plant height between nursery beds or differences in growth rhythm.

In Paper **V**, high broad-sense heritabilities were obtained for bud break and branch angle. Other branch traits displayed heritabilities of 0.20 or less. For growth traits, heritabilities of around 0.20 were derived, except in one trial where it exceeded 0.30. Correlations between trials were slightly less than 0.50 for growth traits, but more variable for quality traits (Table 3). The lowest growth trait correlations between trials were found between the two most divergent sites in terms of recorded frost damage. Comparatively large clone x site interaction components were found for growth traits, while they were smaller for most quality traits. For height after 11 years in the field, the clone x site component was equivalent to 54% of the clone component. The corresponding value for height increment in the last five years was 77%. Branch symmetry and vertical branches had clone x site components as high as 150% of the clone component. In contrast, time for bud break was very stable over sites with a component ratio of 10%.

Table 3. *Mean correlations between trials for height after six years in the field (H6), height after 11 years in the field (H11), height increment in years 7-11 (INC), stem crookedness (CRO), branch angle (BRA) and number of whorls with vertical branches (VER)*

Trait	Mean correlation coefficient
H6	0.47
H11	0.46
INC	0.40
CRO	0.49
BRA	0.67
VER	0.12

Discussion

Application of somatic embryogenesis in the breeding programme

Propagation via somatic embryogenesis is under genetic control. A clear influence of parents and family was found in the study described in Paper **I**. This finding corresponds well with the results of Park *et al*. (1993, 1994) in experiments with *Picea glauca*. In spite of the clear family influence on the propagation capacity, it is likely to cause very limited loss of families, if any, provided that a large enough quantity of zygotic embryos from each family can enter the initiation step. Norway spruce is notorious for its 0irregular flowering, and this feature could complicate any attempts to propagate the species by somatic embryogenesis for breeding purposes. It can be difficult to fulfil requirements for large numbers of zygotic embryos with mothers that seldom flower. Thus, it is not straightforward to integrate somatic embryogenesis in a breeding programme with respect to family representation, even though there will probably be few losses. The rapid multiplication of tissue and thus rapid production of cloned candidates can reduce the breeding cycle time by 2-3 years compared to cutting propagation. The significant family effects on embryogenic capacity would, in practice, give different numbers of candidates within each family. Alternatively, to ensure that enough candidates successfully pass through the propagation stage, it would be necessary to include more zygotic embryos at initiation. Assuming no *a priori* knowledge of embryogenic capacity of the families, this implies that a large number of zygotic embryos would be needed from all families.

For a number of reasons, some genotypes are inevitably lost during propagation. The degree of reduction depends on the methods used. Losses of genotypes at cutting propagation of young Norway spruce donor plants are not generally reported in the literature, presumably because such losses have been very small and considered negligible.

For somatic embryogenesis, a substantial loss of genotypes occurs at different stages during propagation. As indicated in Paper **I**, the current standard protocol leads to losses of ca. 90% of the genotypes (Fig. 3). A standard protocol is a dynamic procedure that will be modified as knowledge about the processes involved increases, so the reduction reported in Paper **I** should be seen as a maximum level. Preliminary data from other experiments suggested improvements to the standard protocol that would enable successful propagation of at least one third of the genotypes (Fig. 3). It should also be possible to include more than one protocol, a measure that would allow even more genotypes to be propagated. However, this would require a screening of the genotypes to identify the optimal protocols for each of them. This strategy requires more work and may cause loss of time. Propagation systems that include more than one protocol were recently presented by Becwar *et al*. (2003).

The recovery rate after cryopreservation was almost 100%, which is encouraging. However, only fast-growing cultures were subjected to cryopreservation, and selection for this criterion would further reduce the number of genotypes. Adjustment of protocol to suit each genotype would increase the numbers of fast-growing cultures, but would also necessitate an early screening of genotypes for different protocols.

Easily propagated genotypes with high multiplication rates will be economically attractive and probably tend to be selected. This will increase the percentage reduction in genotypes, and the loss in plant production for clonal field trials is likely to be in the region of 75%, even if the improved protocol we development is applied (Fig. 3).

An important possibility to consider is that such strong genetic selection on embryogenic capacity could be correlated with unfavourable responses in other traits. However, the data presented in Paper **I** gave no indication that propagation capacity in different stages of somatic embryogenesis is correlated with important traits in breeding. The material was small, so the finding cannot be taken as a definitive demonstration that there is no risk of adverse correlations, although it is supported by results of Passerieux *et al*. (1999). Using RAPD markers, they found that an 80% reduction in the number of genotypes, when applying somatic embryogenesis to a Norway spruce seed lot, did not present a risk of reduced genetic diversity within the resulting clonal population. Furthermore, Ekberg *et al*. (1993) found no correlations between embryogenic capacity and phenological traits in two populations of Norway spruce. Haines & Woolaston (1991) found that the genetic gain will be substantially reduced only in the following situations: (i) when the proportion of genotypes that can be propagated is very low, (ii) when the proportion that is retained is high and the proportion that can be propagated is low and (iii) when strong adverse correlations between propagation capacity and breeding traits are combined with a high retained proportion and/or low propagation rate. Mass propagation of selected clones after field tests implies a low retained proportion. Furthermore, it should be possible to propagate tested clones without losses as they already have passed the sieve in the clonal test propagation. All stages including cryopreservation have then been successfully tried and mass propagation should be safe for tested clones. Genetic gains in the production population would thus be expected to be unaffected by loss of genotypes. However, very low propagation rates (<0.10) introduce a risk for reduced genetic gains.

Bulk propagation can tolerate a more skewed distribution of clones among families, and of plants among clones. However, this can affect the genetic gain, depending on correlations between propagation traits and breeding goal traits. There is a high probability that a skewed distribution will also reduce the genetic diversity of the produced plant population.

In vitro effects on early plant development

Development of somatic embryos is a very important process in propagation via somatic embryogenesis. The negative effect of prolonging abscisic acid (ABA) treatment on subsequent plant growth (**II**) illustrates the importance of the embryo development step. If this effect differs among genotypes, a C-effect will be generated. How long this effect is likely to persist is unknown.

Extension of the first growth period under continuous light was advantageous in that it increased plant size during this time, but it had negative effects on growth in the next two growth periods. This negative effect was not very pronounced (**II**), but significant. It should be pointed out that factors other than the continuous light might have caused these effects. For example, confinement of a growing amount of roots in the container might have imposed stress on the plants. However, it is important to reach a satisfactory plant size as rapidly as possible. A technique for accelerating early plant development was developed, including a two-step germination procedure, in which the germinating plantlets were initially grown on solidified medium and then transferred to liquid medium (see Fig. 4). This modification enhances the root development that seems to be of great importance to subsequent plant growth. This procedure allows earlier acclimatization in a less protected environment, e.g., a greenhouse.

Data in Paper **II** and Paper **III** (even more so) indicate that early formation of lateral roots provides a marker for fast plant growth. Selection for this character significantly improved plant growth during the first two years in the nursery. Another factor that influenced growth positively (albeit less strongly then the lateral root factor) was epicotyl length at *ex vitro* transfer, while main root length at the same point was not significant in this respect.

As well as improving clonal means for first and second year height, selection for the characters mentioned above reduced intraclonal variation in height. With a strong selection at *ex vitro* transfer, somatic embryo plants and seedlings performed equally well. Lateral root formation and rapid shoot development *in vitro* proved to be signs of high embryo quality and can be suggested as early quality criteria when evaluating different treatments that control embryo development. Even with improved procedures, some variation between embryos within cell lines will remain, and low-quality embryos with poor potential will be formed. Such low-quality embryos or plantlets should be culled as soon as possible, preferably even prior to desiccation. Sorting of embryos by computerised one-by-one vision analysis has been tried for agricultural crops (Harrell *et al*. 1993, Hämäläinen *et al*. 1993), but both speed and accuracy were too low for practical application. Another way to remove inferior individuals would be to cull plantlets or plants at a later stage. This would require one-by-one-handling, either mechanically or manually, of low-quality embryos and plantlets, and add to the plant production cost.

Clonal selection and responses in field growth

Clonal selection of cutting-propagated plants in the nursery gave rather poorly correlated responses in height after six years in the field (**IV**). Lack of replications in the nursery made it impossible to calculate the correlation between true and estimated values for traits (r_{TI}). For the same reason, juvenile-mature correlations ($r_{Gm|j}$) could not be calculated. However, the regression analysis performed should provide good estimates of the response in practical situations, even though it cannot give any information about the components involved.

Results from other studies of similar materials (Roulund *et al*. 1986, Bentzer *et al*. 1989) suggest broad-sense heritabilities for height of 0.30 to be realistic, corresponding to an r_{TI}–value of 0.55. Genetic correlations between juvenile and mature traits ($r_{Gm|j}$) often show great variation and, for a species like Norway spruce, are strongly affected if frost-prone sites are included in the field trials. The field trials in Paper **IV** were not subjected to severe frost damage, and the $r_{Gm|j}$–value between nursery height and field trial height can be expected to be about 0.5 (see, for instance, Skröppa & Dietrichson 1986, Roulund *et al*. 1986). Selection of the best 20% of the clones gives a selection intensity of 1.4. With a genotypic coefficient of variation of 0.10, formula 1 leads to a selection response of 3.8%. The estimated response in Paper **IV** was 3.4%, i.e., close to what could be expected under these conditions.

Early growth of Norway spruce plants has several features that contribute to the difficulty of establishing strong correlations with field growth, the most striking of which is the capacity of young plants for free growth, i.e., growth that is not predetermined in buds formed in the previous year. This ability gradually declines, and after five to six years it has disappeared (Ununger *et al*. 1988). If the speed of this age-dependent decline of free growth differs from clone to clone, predictions of future growth based on early height measurements will lose precision.

For Norway spruce, growth rhythm traits often show high heritabilities when measured early (Worrall 1975), and large genetic variance components (Ekberg *et al*. 1991). Reported correlations with growth in the field have varied from absent or weak (Ekberg *et al*. 1994) to strong (Hannerz *et al*. 1999). In the latter case, frost damage caused unfavourable correlations between time for bud burst and field growth. In the present study (**IV**), no effects of frost were detected in the field trials and, accordingly, the stratified selection according to growth rhythm traits did not improve the response in field growth.

Another reason for low correlations between nursery and field traits could be C-effects, caused either by donor plant differences or environmental differences among clones during propagation. Indeed, the set-up of the clones during cultivation in the nursery generated C-effects related to the nursery beds where the clone was grown (**IV**). A significant difference was obtained between the two beds, but stratified selection based on this variable was not an efficient means of raising the effect on height growth in field. Apparently, the C-effects did not persist in the field.

Early selection efficiency could be improved by adjusting many factors, but improving the juvenile-mature correlation is likely to be the most important. Different genes determining growth in different situations could cause low correlations. These differences may be age-dependent (von Wühlish & Muhs 1986, Ununger *et al*. 1988, Ekberg *et al*. 1991), environment-dependent (von Wühlish & Muhs 1991, Kaya 1992), or both. Environmental conditions in the plant's early life are generally more favourable than in the field, so it is not surprising to find low correlations. In retrospective studies on Norway spruce families, attempts have been made to manipulate conditions during early cultivation to be more similar to field conditions. Sonesson *et al*. (2002) found that drought treatment at a very young age improved the juvenile-mature correlations at the family level. Larsen & Wellendorf (1990) reported significant correlations between water use efficiency, measured in the nursery, and growth in older field trials. However, in another study on a material similar in structure to that used by Sonesson *et al*. (2002), drought tolerance correlated poorly with field growth (Sonesson & Eriksson 2003). The lack of juvenile-mature correlations in this case could be explained by the young age of the field trials and their location, suggesting that competition for water had not occurred (Sonesson & Eriksson 2003).

To summarise, growth is a complex trait with many components, varying according to both age and environment, and there is a long way to go before we have an efficient system for selecting families or clones in the nursery that are likely to display superior growth characteristics as mature trees in the field.

The more efficient selection achieved for branch angle (**IV**) is consistent with theoretical expectations, as this trait generally displays high heritabilities and is less sensitive to different environments.

The progress in molecular genetics may provide powerful tools for early selection in future tree breeding. To accomplish this, molecular markers showing strong correlations with target traits must be identified. Molecular markers for traits regulated by a few genes will probably be easiest to develop. However, developing markers for growth traits will be more difficult, because of their complexity. Clapham *et al*. (2000) reported successful gene transfer by particle bombardment of embryogenic cultures of Norway spruce, and subsequent regeneration of transgenic plants. Somatic embryogenesis appears to be a suitable propagation method for gene transfer. Nevertheless, development of gene transfer applications requires knowledge of functional genes and their expression. Again, it will be difficult to efficiently improve complex traits by gene transfer.

Estimated broad-sense heritabilities and genotypic variation in the clonal field trial series described in Paper **V**, agree well with previous results from comparable materials (Roulund *et al*. 1986, Bentzer *et al*. 1989, Högberg & Danell 1989). In this respect, selection of clones is straightforward and should result in expected gains of traditional magnitude. However, the GxE interaction components for height derived in Paper **V** were higher than corresponding values reported in

studies by other authors (Bentzer *et al*. 1989, St Clair & Kleinschmit 1986, Isik *et al*. 1995) and in Paper **IV**. In Paper **V**, the interaction components were calculated without transformation of data to get homogeneous variances among genotypes. Thus, the components may have been overestimated due to scale effects (Lynch & Walsh 1996). Referring to the rule of thumb suggested by Shelbourne (1972), GxE components that are 50% or more as high as the clone component may have serious effects on selection gain. Even considering the possible overestimation, the GxE components probably lie near the limit according to the rule of thumb.

One way to handle selection where there are large GxE interaction components is to select stable clones. However, as different clones can contribute to GxE interactions in different traits, selecting clones that are stable with respect to two or more traits becomes difficult. Such relations have been shown by Sonesson & Eriksson (2000) for *Pinus sylvestris* families. In a study of nitrogen use efficiency and mycorrhiza only a few of the tested Norway spruce families were unstable over nitrogen and mycorrhiza treatments in a 2 x 2 factorial design (Mari *et al*. 2003). However, one of them was among the best performers in an environment with low nitrogen and mycorrhiza. Thus, culling of unstable clones should be made with caution. By increasing the number of test sites with fewer ramets per clone on each site, stable clones can be selected with better precision. This strategy is supported by findings of Russell & Loo-Dinkins (1993).

Another possibility for increasing efficiency in cases where GxE interactions are large is to group the sites and select different clones for different site conditions. This strategy works well when site conditions are determined by easily identified factors. However, the environment is usually as complex as growth in terms of the number of factors that can influence it. Easily determined factors, such as latitude, longitude and altitude are not sufficient to explain the interactions, and more detailed systematic information is generally not available. This means that selections made to suit specific site conditions will be made on weak information and include a high risk of error.

Since Paper **V** was published, the Swedish regulations regarding clonal forestry have changed. Instead of having to comply with a stipulated maximum number of ramets of a clone, and a minimum number of clones per hectare, clonal selection can now be made freely. However, the area in which vegetatively propagated plants can be deployed is limited to 5% of a forest property larger than 20 hectares. These new rules mean that the risk/gain analysis is now an issue for the plant user to gauge, and he or she must decide how many clones to select and how to deploy them. In this context, it should be remembered that selection errors are more likely to be made when GxE interactions are high and few clones are selected.

Conclusions

Somatic embryogenesis can be integrated into breeding programmes provided that: (a) a high frequency of genotypes can be successfully regenerated, (b) there are no strong negative correlations between embryogenic capacity and breeding goal traits, and (c) the production costs are reduced. There is scope for improvement at all stages of current propagation methods, but the somatic embryo development phase is the most important. Use of more than one protocol may also be advantageous. To reduce costs, development of automatic handling techniques will probably be essential, but biological improvements of the propagation process could contribute significantly to reductions in cost.

In the context of clonal testing and mass propagation of tested clones via somatic embryogenesis, losses of genotypes are not as critical as they are in breeding. Provided the number of genotypes that can be propagated is high enough to allow strong selection intensities after testing, the selection response can be kept at a high level. The losses will mainly affect production economics during propagation for clonal testing, as costs will be incurred for handling genotypes in the laboratory that will not enter clonal tests. These costs can be tolerated if they can be absorbed by the mass propagation of plants from the selected clones that can be propagated and tested. Cryopreservation seems to be robust and reliable from a biological perspective, and improvements for this stage could be concentrated on increased capacity.

Somatic embryo development is a very important stage in propagation via somatic embryogenesis, both biologically and economically. It affects both the number of genotypes that can be propagated and the development of the resulting embryos and plants. Treatments during this stage could be further refined, and balanced to ensure that high-quality somatic embryos are obtained.

Clonal performance and the uniformity of clones propagated by somatic embryogenesis can be improved substantially by selecting plants with lateral roots at *ex vitro* transfer. Inclusion of epicotyl length as a selection criterion further improves these characteristics, while main root length does not provide a criterion for good further growth. With the present standard protocol, strong selection is required within a clone to reach the levels of performance and variation shown by control seedlings. Future protocols should be designed to generate a high frequency of plants with desired characteristics at *ex vitro* transfer.

The efficiency of selecting clones for growth in the nursery in order to increase growth in the field is low. The main reason for this seems to be that correlations between traits in the nursery and field are low. A possible way to improve the correlations would be to adjust conditions in the nursery to make them more similar to field conditions. Attempts to do this have not been very successful so far, but development of a reliable method for early selection would be valuable, and further research should be encouraged.

Since genetic parameters in clonal tests of Norway spruce are generally quite consistent, it should be possible to make general estimates of genetic gains. The genotype x environment interactions seem to be more variable. Substantial interactions can often be difficult to consider in selection decisions in practice. Until better understanding of the GxE interaction is attained, the strategy of selecting well-performing clones that are stable over sites should be preferred.

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