Conifer clonal propagation in tree improvement programs

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Abstract

Clonal propagation of conifers is achieved mostly by rooting of cuttings, organogenesis and somatic embryogenesis (SE). Of these, SE is the most powerful in obtaining genetic gain because SE cultures can be maintained in a juvenile state indefinitely by cryopreservation. This allows for long-term field testing of clonal lines while part of these lines are maintained in a juvenile physiological state until the field test has shown which are the best clonal lines for mass-production of propagules. This makes within family selection possible which is not the case with rooting of cuttings or organogenesis. However, as is explained in this review, one can expect that with advances in culture proceedings and in particular with increasing use of modern DNA analysis, within family selection may become possible for rooting of cuttings and organogenesis as well. Furthermore, issues such as deployment and field performance of clones and the cost of mass cloning are discussed.

Keywords: cost, cryopreservation, deployment, genetic gain, field tests, rooting of cuttings, organogenesis, somatic embryogenesis

1. Introduction

Forest plantations are increasingly needed to satisfy the growing demand for wood. Kirilenko and Sedjo reported in 2007 that only 3% of the world's forested land was plantation forest. However, despite this small percentage already more than one third of the industrial round wood production is provided by plantations and it is expected that by 2050 this production will rise to about 75%. Clearly, plantations are highly productive and with further improvement in genetic composition of planting stock and the application of biotechnology additional productivity increases can be envisioned (Fenning and Gershenzon 2002).

To operate plantations is expensive and requires a high productivity per hectare to make them economically viable. To achieve such productivity requires that good quality, i.e., genetically improved planting stock is used. Traditionally

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this was done by using high quality seed provided by seed orchards, either obtained by open pollination or more effectively by controlled pollination between superior parents. Although this has resulted in a marked improvement of planting stock much further improvement is potentially possible by combining traditional tree breeding with new biotechnology technologies. As summarized by Lelu-Walter et al. (2013) and El-Kassaby and Klápště (2014) seed orchards have limitations; not all parents contribute seed, some seed results from self-pollination thus producing poor progeny, poor quality pollen blows in from outside the orchard and there can be a negative correlation between fertility and vegetative growth. Therefore, many forest companies are currently considering clonal propagation in addition or in conjunction with their breeding programs.

2. Clonal propagation

2.1 Introduction

A comprehensive review of benefits, risks, risk management and technical problems of clonal propagation has been presented by Burdon and Aimers-Halliday (2003). The main benefits include capture of non-additive genetic gain, uniformity of the product and tailoring of the product to the growing site. The risks include genetic uniformity, biotic risks, inability to adapt to for example climate change and changing markets.

Clonal propagation can be achieved by various means, grafting, rooting of cuttings, coppicing or *in vitro* propagation. For many species clonal propagation occurs naturally often creating clonal populations that are very large. For example, root suckering has produced clonal populations of *Populus tremuloides* as large as 81 ha (Cook 1983) and one such clone is perhaps the largest living organism known (Mitton and Grant 1996). Clones can also be very old, e.g., it has been estimated that clones of the seagrass *Posidonia oceanica* have existed hundreds of thousands of years (Arnaud-Haond et al. 2012). Natural clones of conifers are rare; they occur occasionally in high stress environments, i.e., in arctic areas or at high altitudes near the tree line. Black spruce (*Picea mariana*), for example, will reproduce as a low bush in arctic northeastern Canada by rooting of low branches (air-layering) when environmental stress limits vertical growth (Pereg and Payette 1998). Similarly, clonal *Picea abies* is found at high altitudes in Sweden, these clones being thousands of years old and having living stems that show up to 600 growth rings (Öberg and Kullman 2011). One Gymnosperm is known that propagates vegetatively but lives in a temperate rather than severe climate environment, namely *Lagarostrobos franklinnii* (Huon pine) a native species in Tasmania (Shapcott 1997).

Many horticultural crops such as fruit trees have been cloned by grafting for centuries. For example, the *Vitis vinifera* cultivar Cabernet sauvignon has been propagated clonally since Roman times and since then has existed only in adult form. Interestingly, propagation *in vitro* of this cultivar has resulted in the reappearance of its juvenile form (Mullins et al. 1979). Some agricultural crops have been cloned for almost as long as the start of agricultural practices about

13.000 years ago (Allard 1999). Clonal forestry is not new either. In China *Cunninghamia lanceolata* (Chinese fir) has been mass propagated by rooting of stump sprout cuttings for at least 800 years (Minghe and Ritchie 1999).

2.2 Various methods of clonal propagation

Several methods of clonal propagation are being practiced with conifers. Of these the discussion in the following will be mainly focused on rooting of cuttings, organogenesis and somatic embryogenesis (SE). Grafting is not used for mass-propagation of conifers but is mainly restricted to the establishment of seed orchards. Coppicing, which is effective for many hardwood species (Wendling et al. 2014) is not an option for conifers because with the exception of a few species, i.e., *Sequoia sempervirens* (Bon et al. 1994), *Pinus serotina* (Bramlett 1990), *Taxodium distichum* (Wilhite and Toliver 1990) and *Cunninghamia lanceolata* (Minghe and Faxin 2001), conifers do not produce the juvenile stump or root sprouts needed for coppicing.

A comparative evaluation of rooting of cuttings, organogenesis and SE has recently been published (Bonga 2015). In this past review it is argued that in spite of the fact that with SE at present greater genetic gain is possible than with rooting of cuttings and organogenesis, the latter two still have a major function in conifer clonal propagation. It also points out that with expected future developments, especially by increasing use of genomic selection the advantage in genetic gain currently possible with SE may diminish. The objective of the current review is to elaborate on these subjects.

2.3 Rooting of cuttings

Rooting of cuttings has been highly effective for several hardwoods, in particular for several *Eucalyptus* species (Ondro et al. 1995). This success is based on the fact that rooted cuttings can easily be obtained from juvenile stump sprouts that are obtained from ortets (trees from which the cuttings were taken) that have shown superior characteristics. In Brazil clonal propagation of *Eucalyptus* has increased volume production from 33 m^3/ha/yr with unimproved seedlings to 70 m^3/ha/yr with rooted cuttings from selected trees (Zobel 1993). Unfortunately, for most conifers suitable cuttings are available only from plants that are too young to have demonstrated their growth potential. Nevertheless, rooting of cuttings can lead to genetic improvement. In breeding experiments improved seed is obtained but often in numbers far too low for planting. By producing seedlings from improved seed and by mass-propagating these by rooting of cuttings a large population is obtained with the same genetic makeup as the seed family. With this process selection within the family is not possible while, as explained later, with SE it is, making selection with the latter more powerful than with rooting of cuttings. Even though the period during which stock plants (ortets) can provide rootable cuttings can be extended by hedging, this extension is generally not long enough for proper long-term testing (Bonga 2015). Nevertheless, by rooting cuttings of plants obtained from seed that had been improved by breeding the gain

obtained can still be substantial. For example, a gain greater than 30% in height growth has been reported for *Picea glauca* when rooted cuttings of improved seed origin are compared with those obtained from non-improved seed (Weng et al.2010). *Pinus* species are sometimes mass-propagated by rooting of elongated fascicle buds instead of stem cuttings (e.g., *Pinus radiata,* South et al. 2005). How well rooted cuttings behave in comparison to seedlings depends on many factors, as described in a review by Ambebe et al.(2013).

2.4 Organogenesis

Because of age related maturation problems with rooted cuttings there is a tradition of trying to obtain clonal propagation by *in vitro* means. The first success in that respect was achieved with *Pinus palustris* by Sommer et al. (1975) who used excised zygotic embryos as explants. Adventitious shoots are formed on the zygotic embryo explant or on parts thereof or from meristematic nodules. These shoots are subsequently given a rooting treatment to form plantlets. However, for most conifer species this technology never reached a practical application stage primarily because of low plantlet formation rates and excessive handling and costs. For only a few species, most notably *Pinus radiata* (Aitken-Christie et al. 1988), have reasonable production levels been obtained. Instead of zygotic embryos another suitable type of explants are needle fascicles. For some *Pinus* species the use of needle fascicles has resulted in regeneration by organogenesis (e.g., *Pinus brutia ,* Jericó et al. 2012).

Organogenesis never became as popular as somatic embryogenesis because organogenic cultures can generally not be kept in a juvenile state by cryopreservation as is possible with SE cultures. Thus with organogenesis selection between but not within families is possible while with SE within family selection is possible (see below). Attempts have been made to prolong the juvenile state of organogenic cultures by low, non-freezing temperatures. Adventitious shoots of *Pinus radiata* have been stored for up to $5^1/_2$ years at 4^oC but rooting was restricted to shoots that had been stored for only 17 months (Aitken-Christie and Singh 1987) which is not long enough for a proper field test. However, since then it was discovered that partially desiccated cotyledons of *Pinus radiata* can be cryopreserved without killing them. After thawing the cryopreserved cotyledons produced the same number of adventitious shoots and plants as the non-cryopreserved control (Hargreaves et al. 2004). This procedure looks promising because it may allow long term testing in a fashion similar to that achieved with SE and cryopreservation.

2.5 Somatic embryogenesis (SE)

2.5.1 *Why SE?*

SE has become popular because SE cultures can be cryopreserved and retrieved in a viable state after cryopreservation. Cryopreservation of SE cultures makes it possible to select within the family rather than just between families.

Because of the large within family genetic variability such selection is more powerful than when selection only achieves the family average as is the case with other forms of vegetative propagation (see Park et al. in this volume). This makes SE attractive and generally the preferred method of clonal propagation. Unfortunately there are still many problems in its universal application. In the following part of the focus will be on such problems and on their resolution. Only a few technical aspects of SE will be discussed; for more detail several extensive reviews are available (Klimaszewska et al. 2007; Lelu-Walter et al. 2013).

2.5.2 *Initiation and maturation*

Initiation of SE in conifers is generally easier with immature than with mature zygotic embryos (Park et al. 1993; Miguel et al. 2004; Kvaalen et al. 2005). This creates problems because it limits collection of suitable material for SE initiation to a short period each year. Furthermore, zygotic embryos at an immature stage of development do not survive lengthy storage of cones and this limitation again restricts experimentation to short periods annually. It would be preferred to use cones with mature embryos that can be stored often for years in a viable state but for most conifer species such embryos either do not initiate SE or do so at reduced rates (Park and Bonga 2011).

For some gymnosperms initiation is restricted to a very early stage in the development of the zygotic embryo. For example, initiation of *Pinus banksiana* SE is possible only when the zygotic embryo is in the poly-cleavage stage and even then only leads to a low initiation rate. It is assumed that in that case initiation of SE is simply a continuation of the cleavage process for as long as the explant is maintained on a medium containing 2,4-D (Bonga 2012). Similarly, zygotic embryos of *Juniperus communis* can form embryogenic lines only when they are at the poly-cleavage stage (Helmersson and von Arnold 2009). *Pinus sylvestris* zygotic embryos produced somatic ones primarily when at the four-cell to the cleavage polyembryo stage whereas *Pinus pinaster* had the highest initiation rate at the stage just prior to elongation of the cotyledons (Lelu et al.1999). For some *Pinus* species initiation does not proceed directly from pro-embryogenic masses, as is usually the case, but from nodules (von Aderkas et al. 2005). SE via nodules has also been observed in cultures of *Picea abies* treated with histone deacetylase inhibitor (Uddenberg et al. 2011), in cultures of tissues from 10-year-old *Picea glauca* trees obtained by SE (Klimaszewska et al. 2011) and in cultures of adult *Larix decidua* and *L. x eurolepis* trees that formed embryo-like structures (Bonga 1996).

Once initiated, the culture consisting of masses of cloned embryos and suspensors grows rapidly. When large enough the masses are subdivided and are used to produce mature embryos that can be germinated and used for clonal field testing. Maturation generally requires transfer of the tissue to a culture medium free of auxin and containing abscisic acid (ABA) and an increased level of osmoticum to slow down growth. Mature embryos are germinated and the resulting plantlets are acclimatized and transferred to a greenhouse and eventually to the field (Klimaszewska et al. 2007; Celestino et al. 2013). Initiation and each

subsequent developmental stage of SE require a finely balanced application of various plant growth regulators that is specific for each developmental stage (for a review see Vondráková et al. in this volume).

2.5.3 *Cryopreservation*

After initiation has produced masses of embryos and suspensors large enough to be subdivided part of each mass is cryopreserved while the other part is used to produce clonal plants for field testing. Presumably, cryopreserved material can be maintained in a viable state for a very long time if not indefinitely. This allows for clones to be field tested for a time much longer than is possible with rooted cuttings, the ortets of which can, as already stated, be kept in a state capable of providing rootable cuttings for only a limited time. After the field test has determined which clones are superior, the best are then removed from cryostorage for mass production of germinating SEs ready for greenhouse and eventually field planting.

The suitability of cryopreservation depends on what effect, if any, it has on the genetic stability of the stored material. This is an important issue because if genetic stability cannot be assured the value of cropreservation is greatly diminished. For conifers this has been investigated extensively (Sutton and Polonenko 1999). Cryopreservation has to be capable of storing without ill effects a wide variety of genomes and families to be of value. These qualifications were met by Cyr et al. (1994) who obtained a 97% recovery rate of cryopreserved embryogenic cultures of 12 full-sib families of *Picea glauca engelmanni* using a large number (357) of genotypes. DNA fingerprinting showed no evidence of somaclonal variation resulting from the cryopreservation. Similar results have been obtained for other conifer species (Isabel et al. 1993; Cyr1999; Hazubska-Przybyl et al. 2013).

However, there are reports of abnormalities in tissues retrieved from cryopreservation, possibly in part due to the effect of the use of dimethylsulfoxide (DMSO) as cryoprotectant. Studies with SE cultures of some conifer species, for example *Abies cephalonica* (Aronen et al. 1999; Krajnakova 2011) and *Picea glauca* (De Verno et al. 1999), have indicated that this cryoprotectant can induce genetic and epigenetic changes. Consequently it has been attempted to achieve cryopreservation without cryoprotectant. This has been successful with cultures of *Picea glauca* and *Pseudotsuga menziessii* which could be cryopreserved without DMSO after a 4-8 week pretreatment at 5^{o}C (Kong and von Aderkas 2011). Similarly SE cultures of *Picea abies* exposed to desiccation in the absence of DMSO prior to cryo storage remained viable subsequently (Hazubska-Przybyl et al. 2013).

Regrowth after cryopreservation occurs for most clones. However, in some species, for example in an experiment with *Abies nordmanniana* regrowth depended on genotype since only one of five genotypes recovered from cryopreservation (Nørgaard et al. 1993). Cryopreserved embryo-suspensor masses are sometimes more productive embryo producers than their non-cryopreserved counterparts (Galerne et al. 1992). It appears that cryopreservation kills most

suspensor and non-embryogenic cells but not all embryogenic cells (Kristensen et al. 1994) thus presumably freeing the latter from competition.

2.5.4 *Abnormalities induced in vitro*

Conifers are generally considered to be genetically stable. Only a few cases of naturally occurring polyploids and aneuploids are known (Miksche and Hotta 1973; Saylor 1983). However, one has to consider the possibility that this natural genetic stability may not be strong enough to safeguard against changes induced by the *in vitro* culture environment. For example, Marum et al. (2009) found genetic variation at SSR loci in embryogenic cell lines of *Pinus pinaster* after prolonged proliferation and in some emblings recovered from these cell lines. Aronen et al. (2014) found that *in vitro* culture reduced telomere length of *Betula pendula*, a symptom of loss of regeneration capacity. Whether such reduction in telomere length occurs in conifer cultures is not known. Telomere length is of interest because it has been found that shortening of telomeres is associated in changes in gene expression during aging (Robin et al. 2014).

Genetic changes induced by *in vitro* culture do not necessarily show up in the trees regenerated from aberrant cultures. Many *Picea glauca* embryos regenerated after cryopreservation exhibited abnormal genetic patterns but these abnormal embryos did not form plantlets that survived transfer to soil while the embryos that were genetically normal did (DeVerno et al. 1999). Similarly, Harvengt et al. (2001) detected a high mutation rate in SE cultures of *Picea abies.* However, no allelic abnormalities were found in plants that originated from these SE cultures and the plants showed no abnormal growth behavior. These observations again suggest that there is an effective selection for normal genotypes when plants are formed by SE cultures.

2.5.5 *Problems with SE:*

Because SE is a very attractive technology a lot of research effort has gone into making it industrially applicable for most commercial conifer species. However, in spite of these efforts large scale industrial application has so far been restricted to a limited number of species. SE works well for several larches (Bonga et al. 1995), spruces and pines (Park et al. 2006; Park and Bonga 2011) but is difficult for the *Cupressaceae* (Helmersson and von Arnold 2009). For some pine species the initiation rates are still too low to be of practical value (Park and Bonga 2011). Low initiation rates are also common for some commercially important firs (Nørgaard and Krogstrup 1995; Vooková and Kormuták 2004). Furthermore, there often are considerable within species differences. Within 20 open pollinated families of *Pinus pinaster* initiation rates ranged from 35.8 to 2.0% (Miguel et al. 2004). Another experiment with that species also showed a wide variety in response depending on what parents were crossed (Lelu-Walter et al. 2006).

To be effective initiation rates should be greater than about 30%. Fortunately, initiation rate is a highly heritable trait and initiation rates can be improved if one parent capable of high initiation is included in each controlled

cross (Park et al. 1998). Furthermore, initiation rates are generally higher if immature rather than mature zygotic embryos are used as explants (Park et al. 1993; Klimaszewska et al. 2007). One consequence of low initiation rates is that only a few genotypes within the family are recovered resulting in a lack of genetic variation within the regenerated population (Högberg and Varis in this volume).

Recalcitrance in regeneration is a poorly understood problem. Several reviews have recently dealt with the subject (Zeng et al. 2007; Zavattieri et al. 2010; Bonga et al. 2010, 2012; Diaz-Sala in this volume) but the problem is persistent and difficult to solve. A variety of different potential explanations of what makes cells competent to initiate SE have been published, which suggests that several mechanisms may operate independently. For example, stress appears to reprogram deteriorating cells into a survival mode that stimulates SE (Dudits et al. 1995; Fehér et al. 2003) while Durzan (in this volume) noted that nutritional stress in embryonal initials initiates a meiotic process. On the other hand stress, even mild stress, often also results in abnormal phenotypes (Joyce et al. 2003). Zhang et al. (2010) identified four families of abiotic stress-induced miRNAs that are differentially expressed in embryogenic and non-embryogenic cultures of *Larix leptolepis*. An interesting novel approach has been proposed by Rutledge et al. (2013). They suggest that suppressing biotic defense mechanisms could perhaps initiate a physiological state that more readily initiates SE. Epigenetic factors also play a major regulatory role in SE initiation (Mahdavi-Darvari et al. 2015). Epigenetic factors are involved in developmental events such as phase change and rejuvenation and have to be manipulated to overcome recalcitrance (Us-Camas et al. 2014, Diaz-Sala this volume). Another factor that could perhaps influence initiation rates is the nutritional state of the explant. The amounts of various sugars, amino acids and soluble proteins in the megagametophyte and zygotic embryo can vary considerably depending on climate and seed source (Durzan and Chalupa 1968). Even when initiation rates are high the quality of the embryos can be low resulting in poor maturation, germination and formation of low quality plantlets (Thompson 2014; Monteuuis in this volume). Poor embryo quality was especially a problem shortly after SE was first developed. For example, Klimaszewska et al. (2007) list 15 *Pinus* species all of which produced SEs during the early years of SE experimentation. However, of these only three species produced plants that survived transfer to soil. In later years with improved protocols much better results were obtained, at least for some species. In an early experiment with *Picea glauca* (Park et al. 1998) nearly half the SEs were abnormal and showed low germination rates. Since then their culture protocol has improved and now most embryos are normal and show high germination and survival rates. Harrington (2003) reported an initiation rate of about 30% for *Picea sitchensis* but a loss of up to 50% of the cultures during proliferation. With an improved protocol for this species (Fenning and Park 2012) an initiation rate of about 70% was attained. This demonstrates that for some species unsatisfactory initial results can turn into much better ones later with improved culture procedures. However, there are still many species that in spite of extensive efforts at improvement are still intractable. An example of that is *Pinus banksiana* which has never initiated SE above rates of 3-4% in spite of years of research efforts (Park and Bonga 2011).

2.6 Combining the various propagation techniques

Combining SE with either rooting of cuttings or organogenesis can be useful. In that case SE together with cryopreservation is used to select superior genotypes that are subsequently mass-propagated by rooting of cuttings or organogenesis. This process is useful when SE maturation and germination rates are low and only a low number of field grown plants is produced for each clone. This is the case, for example, for *Pinus radiata* (Montalbán et al. 2010, 2011; Moncaleán et al. this volume). Harrington (2003) indicated that Coillte Teoranta in Ireland plans to produce up to 6 million rooted cuttings of *Picea sitchensis* using cuttings taken from 40.000 improved stock plants that had been produced by SE.

For many angiosperm species it is possible to combine rooting of cuttings with cryopreservation and organogenesis. Cryopreservation of shoot tips or dormant buds, and regeneration of plants thereof by organogenesis, is used mainly to preserve germplasm (Harding et al. 2009). Regeneration of plants from cryopreserved buds has been reported for a number of forest tree species for example, *Populus tremuloides* (Aronen and Ryyänen 2014) and *Melia azedarach* (Yang et al. 2011). With regard to gymnosperms, *Tetraclinis articulate* (Cupressaceae) (Serrano-Martinez and Casas 2011) and *Picea sitchensis* (Gale et al. 2003) shoot tips have been successfully cryopreserved.

In cases where regeneration of plants from cryopreserved shoot meristems or buds of conifers proves to be possible the following scenario can be envisioned. A few buds could be removed from seedlings and be cryopreserved while the seedling is subsequently assessed in a long-term field test. After the field test has determined which ortets are genetically superior, their cryopreserved buds could then be used for regeneration of offspring through organogenesis. This process could result in selection of superior clones similar to such selection after SE and cryopreservation and could be useful for species for which SE on a large scale is difficult.

Regeneration from shoot tips is difficult for most conifers and presumably would require that in most cases the buds are obtained from very young seedlings. Regeneration using shoots excised from buds of seedlings has been reported for some conifer species, e.g., *Pinus radiata* (Prehn et al. 2003), *Sequoia sempervirens* (Sul and Korban 2005) and *Pinus roxburghii* (Kalia et al. 2007). Whether regeneration from cryopreserved buds of these species is possible remains to be determined.

2.7 Attempts to clone mature conifers either by organogenesis or SE

Clonal propagation of adult conifers either by rooting of cuttings, organogenesis or SE is problematical. So far SE, the preferred method of propagation, has not been feasible on a commercial scale if at all (reviews see: Bonga et al. 2010, 2012; Diaz-Sala, this volume). However, propagating adult conifers by SE is still a cherished goal. It would be helpful if such propagation could be achieved at a practical level and with true-to-type offspring. In that case the period of field testing of clones, as is done when SE is obtained from zygotic

embryos, would not be necessary and much time would be gained. Furthermore, individuals could be selected for propagation that were not only superior for one trait but for many. When SEs are obtained from zygotic embryos, clones exhibiting all these good qualities combined may not appear and, furthermore, selection for traits that do not show until late in the life cycle would be difficult. Therefore, cloning of adult trees is still attractive and is being attempted in several laboratories.

There are means to return the ortet from a mature to an at least partially rejuvenated, more responsive state. These include forcing of pre-formed proventitious buds, serial grafting or micrografting and spraying with benzylaminopurine (Chang et al. 2010; Monteuuis et al. 2011and in this volume). In one case this has led to propagation of *Larix decidua* as old as 140 years (Kretzschmar and Ewald 1994). However, this technology is experimental and complex and has not yet lead to large scale application.

In an attempt to propagate adult *Larix decidua,* adventitious shoots were obtained *in vitro* but these rooted only rarely (Bonga and von Aderkas 1988). Attempts to obtain SE from tissues of adult conifers has been unsuccessful to date except in an experiment in which 10-year-old *Picea glauca* trees obtained by SE were used (Klimaszewska et al. 2011). A few instances of the appearance of embryo-like structures has been reported, i.e., *Pinus radiata* (Montalbán et al. 2010) and *Larix decidua* and *L. x eurolepis*. The *Larix* embryo-like structures arose from nodules, they germinated and formed elongating shoots but these lacked roots (Bonga 1996). Regeneration of rooted shoots from bud explants of adult trees by organogenesis has been reported for *Pinus pinaster* (De Diego et al. 2008) and *P. sylvestris* (De Diego et al. 2010).

Clonal propagation of adult conifers may lose some of its appeal as genomic selection technology progresses. It is conceivable that with expected future advances in that field it may eventually become possible to select clones with complex desired traits, quickly from among embryogenic cultures without the need to cryopreserve and long-term field test them.

3. Gains by breeding and SE

The ability to preserve SE clones over a long period of time in an unaltered state provides an effective way of improving the genetic makeup of planting stock. When SE is used for the clonal propagation of zygotic embryos in seed that was genetically improved by breeding, cryopreservation makes it possible to select and multiply the best clones within the breeding population, i.e., within family selection is possible. Thus a clonal population is obtained that on average will outperform the population obtained by breeding alone (Park 2002; Nehra et al. 2005; Lelu-Walter et al. 2013). This approach is highly effective because of the large degree of heterozygosity in most tree species, in particular in conifers (Ledig and Conkle 1983). Due to the long breeding cycle and self-incompatibility of most conifer species, little domestication has taken place. In fact, most conifers that have gone through a few breeding cycles are genetically still close to their wild populations and still are highly heterozygous and thus carry a lot of variation that one can potentially choose from (Libby 1987).

Breeding of *Pinus taeda* in the southeast US has resulted in the following gains in yield: 8% in the first generation by open pollination in a seed orchard; 11% by open pollination of the best mothers; 21% by full sib controlled pollination (Sedjo 2004). These percentages represent improvement in the average of performance of all individuals within the family. SE and cryopreservation can, as already explained, further improve these percentages. Sutton (2002) reports a 13% volume gain by using seed from open pollinated seed orchards of *Pinus taeda* while with the use of SE a gain in excess of 40% can be expected. Sorensson (2006) indicated that growth gains of 50% can be expected for *Pinus taeda* via SE. Superior genetic traits are often due to unique "non-additive" gene combinations that are difficult to capture by conventional tree breeding but that are captured by clonal propagation (Mullin and Park 1992; Bentzer 1993).

It is fortunate that for several conifers, including *Picea glauca* (Park et al 1993, 1998) and *Pinus taeda* (MacKay et al. 2006), SE initiation is under strong additive genetic control. Therefore, by having at least one parent with a high capacity for SE included in each breeding pair, families with a reasonably high SE initiation rate can be obtained. Thus some of the high qualities of the breeding partner with the low capacity for SE can still be captured by SE. Niskanen et al. (2004) found that the maternal effect was greater than the paternal effect on SE initiation. During prolonged maintenance of the cultures the effect of the mother's genotype diminished but had a significant effect during SE maturation. It has been suggested that the presence of the megagametophytes during initiation may prolong the maternal effect (MacKay et al. 2006).

A large degree of heterozygosity is not present in all conifer species. A few conifers lack diversity because their current population originated from a small remnant that survived after a catastrophe. Examples of this are *Pinus resinosa,* which appears to have originated from a small pocket of trees that survived the last ice age (Fowler and Morris 1977) and *Pinus torreyana* which became isolated otherwise (Ledig and Conkle 1983). Due to the lack of genetic variation in such species improvement by breeding and/or vegetative propagation cannot be expected and propagation by seed is the most economical and effective.

Epigenetics has attracted attention lately as a possible way of introducing beneficial traits through plant breeding (Mirouze and Paskowski 2011). In epigenesis environmental cues activate genes that initiate the formation of proteins that enable adaptation to environmental challenges. Sometimes the resulting phenotype is preserved through one or more sexual cycles which, for example, could quickly improve adaptability, especially in plants that possess limited genetic diversity. However, even though traits of interest may thus be acquired more or less permanently, the possibilities to acquire new traits presumably are far more limited via the epigenetic than via the Mendelian genetics route. A conifer example of traits that are epigenetically determined is presented by Kvaalen and Johnsen (2008). They observed that height growth and bud set in *Picea abies* are influenced by the temperature to which zygotic and somatic embryos were exposed and that the effect was still noticeable after two growing seasons. Exposure to high temperature (28^{o}C) during SE initiation of *Pinus radiata* resulted in increased drought stress tolerance one year later in the plants thus produced (Montalbán et al.

2014). In developing zygotic embryos of conifers the ability to initiate SE is reduced as the embryo matures and is rare after germination. However, it has been observed that once SE is induced in *Picea glauca* the ability to induce new SEs is maintained in some of the somatic trees that have developed from the SEs, even sometimes up to the point where these trees start to form sexual cones. It is assumed that this is due to an epigenetic fixation of the capacity to form SEs (Klimaszewska et al. 2011). Similarly, SE initiation continued in the shoots that developed from SEs obtained from shoot bud explants of adult *Larix decidua* (Bonga 1996). This repetitive SE initiation, although without the formation of proper root meristems, presumably also is epigenetic in nature. Durzan (in this Volume, Figure 7) noted that epigenetics silences embryonal initials that are not capable of SE.

4. Deployment

Clonal propagation is often seen in a negative light because of the perception that it promotes genetic uniformity in populations. However, when properly practiced uniformity problems can be limited to an acceptable level. Furthermore, plantations resulting from the application of modern biotechnology will take harvesting pressure off the natural forest (Sedjo 2005) and thus help to preserve these and their biodiversity. Because tree species have a long life cycle that requires a long-term investment, it is highly desired that clonal plantations remain risk free to the maximum possible extend.

In any plantation, clonally or sexually produced, there is a risk of insect or pathogen attack, sometimes of an unforeseen nature. For example, due to lack of diversity, problems have occurred in clonal poplar populations in several countries (reviewed by Stelzer and Goldfarb 1997; Bishir and Roberds 1999; Burdon and Aimers-Halliday 2003). Obviously, using only a few, highly productive clones may impose unacceptable risks (Burdon and Aimers-Halliday 2003). However, even though risks can decrease with a larger number of clones being deployed, too large a number can increase potential problems. It has been suggested that the risk level is unlikely to be reduced if the number of clones used exceeds 30 – 40 (Bishir and Roberds 1999). Another model suggests that approximately 18 genotypes are optimal under many conditions and that with regard to merchantable volume no more than 30 clones are needed for good risk protection and near-optimal timber yield (Yanchuck et al. 2006). This model also indicates that planting blocks with a mixture of clones has advantages over planting a mosaic of blocks with each block containing a different single superior clone. Another option is to mix clonal propagules with sexually produced seedlings and rogue these populations at regular intervals (Park et al. 1998). In general, the clones should be planted at highly productive sites and be well adapted to those sites, and a balance must be reached between genetic diversity and expected gain (Cyr and Klimaszewska 2002).

In several countries legislation has been enacted that regulates the deployment of clones. These regulations state what number and mixture of clones is to be deployed and what size of area can be planted with clones (Burdon and Aimers-Halliday 2003, Lelu-Walter et al. 2013, Högberg and Varis in this volume).

5. Field performance of clones

In a 12 year field test of *Pinus radiata,* obtained by organogenesis, clones were planted in both monoclonal blocks and blocks with mixtures of clones. Average performance was the same in both but variation was larger when clones were mixed. Uniformity is an advantage but risk is greater in monoclonal blocks (Sharma et al. 2008). Comparing SE emblings with seedlings from the same families showed that *Picea glauca x engelmannii* emblings had slower height, diameter and root growth rates than their sexually produced counterparts during the initial $2^1/_2$ months in the nursery but that growth of the former catches up after that (Grossnickle et al. 1994). Embling and seedling performance was similar over two years on a reforestation site (Grossnickle and Major 1994) and the emblings performed reasonably well under a variety of nutrient and stress conditions (Grossnickle and Folk 2007).

Field performance of somatic plants depends on the *in vitro* conditions under which SEs developed. For example, lengthy contact with ABA during maturation of *Picea abies* SEs and a non-optimal germination treatment reduced height growth during their first two growing seasons in the field (Högberg et al. 2001). By selecting SEs with lateral roots and epicotyls larger than 8mm, taller and more uniform plants were obtained (Högberg et al. 2003). In a 5 year field test of genetically matched Douglas-fir seedlings, rooted cuttings and plants produced by organogenesis, the plants obtained by organogenesis grew slower than seedlings and showed signs of early maturation (Ritchie et al. 1994). Early maturation (premature flowering) was also found in *Picea mariana* emblings (Colas and Lamhamedi 2014). On the other hand, Klimaszewska et al. (2011) observed that primordial shoots of some 10-year-old *Picea glauca* trees obtained by SE were still capable of SE initiation. Such initiation is considered to be a juvenile trait and for this species is normally restricted to zygotic embryos. These trees, therefore, expressed extended juvenility instead of early maturation. It has been suggested that this phenomenon could be due to a suppression of the biotic defense activation (Rutledge et al. 2013). Clones of *Picea glauca* obtained by SE showed, under the same growth conditions, greater variation in growth characteristics within the family than zygotic seedlings within that family (Lamhamedi et al. 2000). In 1999 a clonal test of 70 coastal Douglas fir SE clones was established from two full-sib families with the same female parent on five different sites. After $5^1/_2$ years of testing growth and survival were acceptable across the test sites and stable (Dean 2008). In a subsequent test with 37 SE clones from four full-sib families on five test sites, the SEs grew slower than their zygotic counterparts but 20% of the clones produced 25% greater stem volume after $7^1/_2$ years than the sexually produced seedlings. Clonal stability resulted in little variance due to clone x test interactions (Dean et al. 2009). Wahid et al. (2012) looked at the field performance of *Picea glauca* SE clones on two sites four years after out-planting. Selection of the top 38% of the clones provided a 4% genotypic gain in height and the genotype x site interaction was low. Since juvenile/mature height growth correlations are high for this species one may expect these clones to perform well at a later age (Wahid et al. 2013).

In all the above examples the performance of emblings was compared to that of seedlings of the same family, i.e., no comparison was made between performance of selected superior clones and the family average for seedlings. Data indicating what kind of gain is possible when clones are selected from within the family have, to my knowledge, not yet been published (Adams et al. in this volume). At higher selection intensities a higher genotypic gain can be expected. For example, Park (2002) estimated that a gain of 45% in height growth over that of the average of all clones can be expected at 5 years of age when the 10 best clones are selected from 300 *Picea glauca* SE cell lines.

6. Cost of clonal mass production

There are many potential technical problems involved in mass-clonal propagation that would affect costs of the operation. Aimers-Halliday and Burdon (2003) present a long list of them of which the following are just a few; problems with clonal storage, loss of genetic gain, potentially superior clones may be under-represented because of poor cloning ability of the genotype, somaclonal variation, systemic infections, epigenetic effects, inadequate testing and cultivar decline.

With regard to conifer SE, as was pointed out earlier, initiation rates for several species are low and embryo quality is often poor resulting in poor conversion to plantlet rates and operation costs that are prohibitive. SE requires a substantial input of labor and thus is expensive in comparison to seedling production. Because of the long rotation age of the product, costs must be carried for many years before they can be recovered (Lelu-Walter et al. 2013). This means that the savings obtained by improving productivity by SE should be higher than the extra cost incurred by using emblings instead of sexually produced seedlings. Furthermore, one has to consider whether the extra cost of SE outweighs the cost of gain in production attained by intensive sylvicultural management practices such as weed control, thinning and fertilizer application, inter-planting with nitrogen fixing species, all of which can significantly improve productivity (Binkley and Stape 2004; Sorennson 2006; Gyawali and Burkhart 2015).

An early cost analysis indicated that an increase in genetic gain can make clonal propagation cost effective. This study also suggested that because of the high cost of clonal propagation planting of superior clones should be restricted to high quality sites located close to the mill (Timmis 1985). High productivity on sites close to the mill presumably would result in considerable savings in road building, harvesting and transportation costs which could offset the initial high cost of clonal propagation, in particular of SE. This applies to all forms of intensive plantation management. With intensive management there is less of a need to harvest from less productive natural sites and costs associated with harvesting at these often poorly accessible sites need not to be undertaken. This takes harvesting pressure off these less productive natural sites (Sedjo 2005; Wahid et al. 2013). Other factors that reduce the impact of initial high costs are that with the faster growth obtained by using SE the percentage of logs suitable for saw-timber could rise between 35 to 80% (for *Pinus taeda)* (Sorensson 2006). This latter observation is important because saw logs are of greater value than pulp wood. The financial

gain possible by using selected clones of *Picea sitchensis* obtained by rooting of cuttings instead of sexually produced seedlings at harvest is substantial (Philips and Thompson 2010).

A factor in commercializing clonal propagation is seed productivity and phenology of the species. If seed production is high and occurs early in the life cycle, multiplying the seed in seed orchards for large scale planting is more cost effective than vegetative propagation unless the genetic gain by the latter is large enough to warrant the extra expense (Sutton and Polonenko 1999). For a few species the extra cost has been considered worth taking and large numbers of plantlets obtained either by organogenesis or SE are being produced. For example, Tasman Forestry Ltd by 1993 had developed the capacity to produce 3 million *Pinus radiata* plantlets annually by means of organogenesis from zygotic embryos from seed obtained by controlled pollination. This will result in an approximately 20% increase in yield over that provided by unimproved seed (Nairn 1993). JD Irving Ltd. planted 433,000 *Picea glauca* SE plantlets in 2012, 219,000 in 2013 and 212,000 in 2014. This company recently built a large new facility where they plan to produce 5 million SE plantlets annually (Andrew McCartney, Irving Ltd. Personal communication 2015). CellFor was producing about 2 million SEs annually by 2002 from control-pollinated families of loblolly pine and Douglas fir (Sutton 2002). Even higher numbers are reported for southern pines (Sorensson 2006).

To reduce the cost of producing SEs, efforts are being made to simplify the initiation, maturation, germination and planting protocols and to automate the process. For example, for the production and planting of *Pinus pinaster* SEs it has been possible to initiate them without subculture, to cryopreserve them without the need of a programmable freezer, to mature the embryos without subculture, to improve germination and to eliminate the need for a greenhouse in the acclimatization and planting process (Lelu-Walter et al. 2006).

For a number of non-coniferous species automation has reached a stage of considerable sophistication and effectiveness. For example, *Coffea* spp. SEs have been mass produced in mechanically agitated bioreactors up to the cotyledonary stage, matured in temporary immersion bioreactors and germinated in a raised CO_2 (photoautotrophic) environment. This system can proliferate embryogenic suspensions for about 6 months without causing excessive somaclonal variation (Ducos et al. 2007). To culture plantlets under photoautotrophic conditions and without sucrose in the culture medium was found to be beneficial, production and cost wise, for the conifer *Cunninghamia lanceolata* (Kozai and Xiao 2006). Bioreactors were initially developed for microbial culture and secondary metabolite production but more recently have also found application in plant cell cultures including of woody species (Yoeup and Chakrabarty 2003). An important aspect of automation is the ability to select and remove high quality SEs from a population containing both low and high quality embryos. This can possibly eventually be done with an image analysis sorting system (Ibaraki 1999). As discussed by Ingram and Mavituna (2000) conifer SE cell lines generally will proliferate in liquid medium but SE maturation in most cases requires a solid medium. They tested proliferation and maturation of *Picea sitchensis* in different

types of bioreactors and found bubble reactors to be the most satisfactory. Large scale SE production in liquid medium bioreactors has also been reported for *Pseudotsuga menziesii* (Gupta and Timmis 2005).

Mass production of SEs is only one stage in the commercialization process and perhaps not always the most severe bottleneck. For example, it has been stated that commercialization of SE is not primarily dependent on automation of SE production but on the current lack of reliable delivery systems, i.e., the lack of artificial seed (synseed; encapsulated SEs) that like natural seed can be stored for a long time, that will germinate at high rates and that are compatible with existing commercial propagation systems (Sutton and Polonenko 1999).Unfortunately, in spite of a great deal of effort to develop artificial seed technology into a commercially viable process, it often still does not work well due to often poor survival and germination rates and excessive dehydration of the capsules under field conditions (Sutton and Polonenko 1999; Roy and Tulsiram 2013). As stated by Onishi et al. (1994) the main requirement for synthetic seed to be effective in mass clonal propagation is a high and uniform conversion rate into viable plantlets under practical sowing situations. *Picea glauca engelmannii* somatic embryos require sucrose during germination until they have developed a functioning radicle (Roberts et al. 1995). Unfortunately, sucrose in synseed leads to microbial contamination and this and other problems have so far limited its practical use (Ara et al. 2000, Roy and Tulsiram 2013). The use of conifer synseed has so far been experimental and germination of synseed has been carried out aseptically *in vitro*, for example, with *Pinus radiate* (Aquea et al. 2008).

To improve maturation, germination and survival it has been attempted to load the embryos with storage nutrients to compensate for the absence of a nutritive megagametophyte. By using ABA and instead of sucrose a non-permeating osmoticum, Attree and Fowke (1993) and Attree et al. (1994) obtained *Picea glauca* SEs that contained greatly increased amounts of storage lipids. These SEs could be dehydrated to about 8% moisture content and stored for over one year at minus 20^{o}C, rehydrated and germinated. Embryos such as these are useful in attempts to achieve mechanization of the process (Sutton 2002).

7. DNA and other markers: marker assisted selection (MAS)

Conifers have large genomes and a long breeding cycle which causes problems in traditional breeding programs. These programs may become more effective with the aid of marker assisted selection (MAS) (Ritland et al. 2011, Chhatre et al. 2013). Early efforts in that direction involved the use of quantitative trait loci (QTLs). The use of such loci is based on the likelihood of a quantitative gene occurring near a marker in a particular linkage group. They are helpful in breeding but are not very effective in locating and identifying quantitative genes (van Buijtenen 2001). They are useful only for large families with known relatedness, i.e., full-sib families (Beaulieu et al. 2011, Thavamanikumar et al. 2013) and typically only explain a small proportion of phenotypic variation (Thavamanikumar et al. 2013). Nevertheless, marker assisted selection can be effective in tree breeding. For example, Beaulieu et al. (2011) identified single-

nucleotide polymorphism markers (SNPs) for several wood traits of *Picea glauca* that could be used to speed up future breeding schemes. Lately it was shown that some metabolites could serve as useful markers. For example, levels of inositol in the cambial area of *Pinus densiflora* during the middle of the growing season significantly correlate with stem growth (Kang et al. 2015).

Lately a procedure called genomic selection (GS) has become popular. It increases genetic gain per time unit while maintaining sufficient diversity in breeding schemes and clonal propagation and it predicts at an early age what phenotype will develop (Canales et al. 2013; El-Kassaby and Klápště 2014; Park et al. in this volume). GS predicts phenotype on the basis of the aggregate of whole-genome effects (Grattapaglia 2014). An example of how GS could be implemented in studies of SE or rooting of cuttings has been presented for *Pinus taeda* by Resende et al. (2012).

It is expected that GS will increasingly become more popular as the rapidity at which DNA is sequenced increases and its cost is lowered. As was already pointed out, this may eventually make it possible to optimize genetic gain by SE without the need for cryopreservation. Furthermore, one may expect that application of GS in rooting of cuttings and organogenesis will eventually result in obtaining a similar level of gain for these as is currently possible with SE.

8. Conclusion

Clonal propagation of conifers, primarily by rooting of cuttings, has long been practiced to maximize wood production. However, the development of SE technology, and its use in combination with cryopreservation, has led to genetic gain beyond that obtainable by rooting of cuttings. Because of the enormous genetic variation available in most conifer populations the possibilities of much further genetic gain by this procedure is far from exhausted. However, SE is still not practical for many conifer species. Presumably that problem will be solved for several commercial species eventually as culture protocols improve. In the meantime, rapid advances in genomic selection methods will add further possibilities in obtaining and mass-producing desired genotypes, not only by SE but also by rooted cuttings and organogenesis. Which of these will dominate the future scene will depend on species and local circumstances and combined use of these technologies can be expected to continue where appropriate. Cost of clonal propagation, especially by SE, is still an issue but will likely diminish with expected future automation.

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