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Conifer somatic embryogenesis: II. Applications

Abstract: Somatic embryogenesis (SE) of conifers for clonal propagation has emerged from its earliest beginnings in 1980 to become an integral component of tree improvement strategies. With its capacity for long-term germplasm preservation and scale-up technologies, it is seen to be the preferred avenue to accelerate selection and operational deployment of value-added genotypes. At present, programs for numerous species from the *Pinaceae* family are underway worldwide, with activities ranging from selection trials to pilot production for plantation forestry. This paper will provide a review of current efforts in conifer SE including cryopreservation, commercialization and deployment strategies, and transgenics. A discussion of challenges and issues is directed at genetic fidelity, intellectual property and future needs.

Additional key words: clonal propagation, germplasm preservation, *Pinaceae*, plantation forestry, somatic seedlings.

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Introduction

In many developed countries, forestry is facing reduced production from old-growth stands, increased allocation of forest lands to reserves, and limited opportunity for expansion of plantations outside of the current forest estate (Boyle 1999). For other regions, the impact of exotics on endemic species, competition for arable land and water, and limited opportunities for afforestation are other factors that come into the equation. With respect to conifers, an additional issue is the increased competition from short-rotation hardwoods. As a consequence, tree improvement efforts in conifers have increasingly looked towards the establishment and expansion of high-yield managed plantations. This is viewed to be of paramount importance, as an anticipated 50% increase in demand for forest products by 2010 is expected to require the establishment of 50 to 100 million hectares of new plantations (Pullman et al. 1998; Sedjo 1999; Sutton and Polonenko 1999).

Propagation technologies offer significant opportunities to improve management of breeding populations and accelerate the delivery of improved material to conifer plantations via clonal forestry. In the latter case, the ability to produce and test clones will allow for more effective field testing of family selection, adaptive physiology and the development of marker-aided selection (e.g., QTL). Approaches for clonal forestry include vegetative cuttings, organogenesis (shoot multiplication) and somatic embryogenesis (SE) (Grossnickle et al. 1996; Sutton and Polonenko 1999). In the case of rooted cuttings, not all conifer species are amenable; multiplication rates are low and stock-plants are subject to physiological aging after a few years. By comparison, organogenesis exhibits a greater potential for multiplication; however, it is inherently labor-intensive and to date it has demonstrated a limited capacity for long-term storage of the primary germplasm. Thus, these two approaches have significant restraints with respect to commercialization. By contrast, SE offers significant potential ad-

vantages including effective cryo-storage of juvenile tissue, unlimited multiplication capacity and suitability for automation (Cyr 1999).

Germplasm preservation

The most important role for cryopreservation is within the framework of clonal forestry, particularly as it applies to *in vitro* propagation. In this context, other applications include efforts on ornamental varieties and Christmas trees. For clonal forestry, cryopreservation facilitates the recovery of elite genotypes following long-term selection trials and the subsequent establishment of production clone banks for commercial applications. Thus, the clone bank is effectively the *in vitro* equivalent of a seed orchard. However, the clone bank additionally offers a flexible platform for creating designer deployment populations from within and/or across generations or selections as per end-user needs (Park et al. 1998; Cyr et al. 2001).

In conifer SE, cryopreservation is applied exclusively to the juvenile cultures (i.e. proliferative embryogenic cultures), although there is potential for cryogenic storage of somatic and zygotic embryos (Cyr 1999). These latter approaches, however, are strongly influenced by genotype, and subsequent recovery of embryogenic cultures via secondary embryogenesis has not been demonstrated for most conifer species. By contrast, large-scale applications using embryogenic cultures indicate that the ability to cryopreserve cultures is genotype independent with success primarily being reflective of culture vigor (Cyr et al. 1994; Park et al. 1998).

To date, cryopreservation of embryogenic cultures has been successful for at least 26 species, represented by the genera *Abies* (1), *Larix* (6), *Picea* (8), *Pinus* (10) and *Pseudotsuga* (1). During the early 1990's, several clone bank based programs, driven by commercial opportunities in SE, were initiated. This has led to the establishment of large-scale cryopreservation programs in Canada, France, New Zealand, Sweden and USA (Cyr 1999), with evidence from the literature indicative of additional efforts in Chile, Finland and South Africa. As of 1998, these programs accounted for cryostorage of an estimated 8000 to 10,000 genotypes, predominantly represented by spruce and pine species.

Effective operation of the SE cryopreservation clone bank is critical for the success of long-term clonal programs, particularly with respect to the development of risk management strategies. At the front-end, the use of standard operating procedures and quality control measures are of supreme importance during all tissue culture steps leading up to and including cryopreservation. This is to insure integrity of the genotypes as frozen cultures. A recommended approach for plant production is to restrict tissue maintenance to annual or seasonal cycles, thus avoiding potential problems with tissue culture-induced somaclonal variation. The general strategy for each genotype includes assessment of the number of vials to be stored. This, in turn, is dependent on anticipated short- (research) and long-term (deployment) needs for regeneration. In these scenarios, separate research and production clone banks can be established, leaving the initial clone banks as repositories of the original germplasm. Additional factors for consideration include duplication of storage on- and off-site, inclusive of electronic monitoring and alarm response systems.

Clonal selection

Conifer SE has become important for tree improvement due to its potential for enabling selection and mass propagation of elite lines from a broad genetic base. This is a means of increasing yield and reducing rotation age, thus aiding in the management of forest productivity. Selection targets for SE include those traits that can be captured via traditional tree breeding, namely form, growth, wood quality, and genetic resistance to insects or disease. Operationally, typical clonal selection programs can entail the culture of 200 to 2000 seed explants per family with seed volumes of up to 24,000 per individual initiative (Cyr et al. 2001). In this scenario, the production of 10 to 200 *in vitro* seedlings per clonal line for nursery delivery occurs 12 to 18 months after initial culture, depending on the species, region and product format. SE clonal selection programs currently in place include *Picea abies*, *P. glauca*, *P. glauca x engelmannii*, *P. mariana* and *P. sitchensis*, *Pinus elliottii*, *P. monticola*, *P. patula*, *P. pinaster*, *P. radiata*, *P. strobus*, *P. taeda*, and *Pseudotsuga menziesii* (Table 1). The majority of clonal selection programs are managed within the private sector, represented by forestry companies and third-party providers. Forestry companies known to be active in this area include Bioforest (Chile), Carter-Holt Harvey (New Zealand), International Paper (USA), JD Irving (Canada), Rayonier (New Zealand, USA), Westvaco (USA) and Weyerhaeuser (USA). Other private or related organizations include Afocel (France), Arbogen (USA), CellFor (Canada), GenFor (Chile) and Rubicon (New Zealand). Governmental and academic entities include the Canadian Forest Service (Canada) and Swedish University of Agricultural Sciences (Sweden). Comprehensive publications with respect to clonal selection are few in number, with benchmark reports available for *Picea abies* (Högberg et al. 1998; 2001). *P. glauca* (Park et al. 1993; 1994; 1998), and *P. glauca x engelmannii* (Cyr 1999; Cyr et al. 2001). The preponderance of reports for *Picea* spp. is due to their responsiveness to SE relative to other genera.

Table 1. Integrated and emerging SE programs listed by species and region

Region	Integrated SE cryopreservation and clonal selection programs	Emerging or potential SE programs
Australia		*Pinus elliottii*; *P. pinaster*; *P. radiata*
Argentina		*Pinus taeda*
Brazil		*Pinus elliottii*; *P. taeda*
Canada	*Picea abies*; *P. engelmannii/glauca*; *P. glauca*; *P. mariana*; *P. sitchensis*; *Pinus monticola*; *P. strobus*; *Pseudotsuga menziesii*;	
Chile	*Pinus radiata*	
China		*Pinus elliottii*; *P. radiata*; *P. taeda*
Finland		*Pinus sylvestris*
France	*Pinus pinaster*	*Pseudotsuga menziesii*
Great Britain		*Picea sitchensis*
Ireland		*Picea sitchensis*
New Zealand	*Pinus radiata*	
Portugal & Spain		*Pinus pinaster*; *P. radiata*
South Africa	*Pinus patula*	*Pinus elliottii*; *P. radiata*
Sweden	*Picea abies*	*Pinus sylvestris*
Uruguay		*Pinus elliottii*; *P. taeda*
USA (PNW)	*Pseudotsuga menziesii*	*Picea sitchensis*
USA (SE)	*Pinus elliottii, P. taeda*	

Park et al. (1993) and Sutton et al. (1993) first described the application of SE to tree improvement of spruce. The work by Park et al. (1993; 1994) on *P. glauca* focused on the evaluation of genetic control and genotype x treatment interactions in SE via six parent diallel crosses and reciprocals. Initiation response from mature and immature zygotic embryo explants averaged 26%, with a range of 3 to 55%. This resulted in the establishment of 1700 clones from 30 families, of which 44% were retained for subsequent analysis. For cryopreservation and maturation/germination, 32 and 18% of the original set were tested, respectively. These studies indicated that initiation was under strong additive genetic control, whereas maturation, germination and cryopreservation were much less affected. Embryo and plant production efforts indicated that the largest proportion of clonal variation was observed within families, while based on growth traits, variation was minimal within clones. Collectively, these studies demonstrated the potential for clonal selection using SE. A study of *P. abies* (Högberg et al. 1998; 2001), resulting in the establishment of 317 lines from 17 families, confirmed these observations. They concluded that selection due to SE did not cause directed selection in growth and phenology traits.

In the case of *P. glauca x engelmannii*, an operational program was designed and implemented. Progeny trials of a population of 173 plus-trees were indicative of significant gains in clonal selection for height and weevil resistance (*Pissodes strobi*) (Sutton et al. 1993). The resulting program aimed at the delivery of somatic seedlings for field-testing from 1000 embryogenic lines across 30 to 40 top-families. In actuality, from 1993 to 1996, an excess of 1700 clones representing 21 control-pollinated and 14 open-pollinated top-ranked families were cryopreserved (Grossnickle et al. 1996; Cyr 1999). Initiation frequencies in this work averaged 20 and 44% for the two groups of families, respectively (Cyr 2000). The resultant output encompassed the deployment of 6 to 8 copies per site for each of approximately 1300 clonal lines from 1996 to 1998, using 3 or more sites per year (Cyr et al. 2001). Selection of 30 to 50 clonal lines for weevil resistance, based on 5% intensity, was anticipated to begin 5 years after trial establishment.

Efforts in SE clonal selection of spruce also include weevil resistance in *P. sitchensis* (Cyr et al. 2001). This program resulted in the establishment, from 1997 to 2000, of clonal trials using 378 embryogenic lines representing 25 open-pollinated families. A related study (Klimaszewski et al. 2000) demonstrated the potential for mass screening of putatively resistant SE clones via weevil caging trials.

With respect to other commercial conifer species, clonal selection efforts occur primarily in the private sector and information is available at best from conference proceedings. However, Cyr et al. (2001) documented the trial output of close to 1700 clonal lines representing 68 families from other species than spruce. These, and their primary selection targets (in parentheses), include *Pinus taeda* (yield), *P. radiata* (yield, form and wood traits), *P. elliottii* (fusiform rust resistance) and *P. patula* (yield, blight and *Fusarium* resistance). Additional trials of 455 clonal lines (21 families) encompassing *P. taeda*, *P. elliottii* and *Pseudotsuga menziesii* (yield and form) were projected to occur during 2002 to 2003.

Commercial production

Clonal propagation of conifers via cuttings and/or micropropagation is operational for *Pinus* spp. in several regions, such as Chile, New Zealand and South Africa. Although SE has not yet reached this state, evidence indicates that it is at the semi-operational level. At present, clonal selection trials are in relative infancy, and current production efforts are thus focused on small subsets of clones that are selected on the basis of their performance in the SE and nursery systems. This early phase of commercialization serves to validate the SE process and product through pilot production. With respect to the process, validation is carried out within an organization via technology transfer of the laboratory protocols to pilot production. This activity, in fact, will continue well into the operational phase as process improvements continue to be developed. Ultimately, product validation is customer-driven by results in the nursery and field, whether this is by internal or external parties. Externally, product validation is an important marketing exercise for the emerging technology. The thrust, in this case, includes development of comfort in the forest sector for new SE products and demonstration of the substantive genetic gain that may occur from families using operational plantings (Sutton and Polonenko 1999; Cyr et al. 2001).

The commercialization of SE products, in the long term, is ultimately dependent on the development of a reliable and cost-effective production system. In this circumstance, the management challenges for conifer SE are segmented into biological and mechanical issues. With respect to biological restraints, much of the SE system requires operation under sterile conditions and there is significant variation among genotypes with respect to productivity and response to process improvements (Handley et al. 1995; Timmis 1998). Mechanical challenges include bulk handling and automation for the *in vitro* SE process and nursery delivery. At present, the major gains in cost reduction for conifer SE will come from the circumvention of *in vitro* germination and development of embryo/plant delivery systems. This includes the development of semi-autonomous or autonomous artificial seeds that can be inventoried and are suitable for bare-root and containerized nurseries. Ultimately, cost-effectiveness will be mitigated by the value proposition for SE products, which is determined by supply and demand economics and the incremental value of genetic gain delivered over and above that available from other sources. Factors in this determination include clonal uniformity, improved disease and pest resistance, nutritional improvements, and other genetic traits that can be captured through conventional breeding or genetic engineering. Criteria for successful commercialization also include the development and implementation of manufacturing resources planning (MRP) principles, an approach that includes characterization of productivity and cost inputs, biological and physical constraints, actions and outputs for each step of the SE process (Polonenko 1999).

Historically, conifer SE has been developed using Petri-dish based systems and *in vitro* germination. While these approaches are suitable for the establishment of clonal trials, they are viewed as inadequate for commercial production. Promising developments in the automation of the SE process include liquid maintenance culture, bioreactor maturation, embryo purification and desiccation (Timmis 1998; Cyr et al. 2001). Liquid culture methods, which facilitate rapid bulk-up, uniformity and improved embryo yield, have been developed for *Picea* spp., *Pseudotsuga menziesii* and *Pinus taeda*. Bioreactors, which utilize solid supports and liquid media, can exploit improvements in the developmental modulation of key culture additives such as ABA and osmotically active solutes. To provide a context for scale-up, bioreactors have been considered to be the equivalent of 30 Petri-plates. Relative to the latter approach, in *Pseudotsuga menziesii*, the bioreactor format improved yield, enabled embryo purification and resulted in the desiccation of several thousand embryos on a 5.5-cm filter paper (Cyr et al. 2001). Additional potential developments for large-scale application include image analysis systems for selection of embryogenic cultures and somatic embryos (Hamalainen and Jokinen 1992; Roberts et al. 1995); nevertheless, evidence of their operational use is lacking.

The development of artificial seeds has received significant attention in the agronomic sector since 1973. In general, the desired attributes include storage capacity analogous to that of regular seeds and compatibility with current sowing practices. As conifer seeds sequester the bulk of their storage reserves in the maternal tissue (megagametophyte), then somatic embryos will likely require amendments to function effectively as synthetic seeds. Two approaches have been developed for conifers. Carlson and Hartle (1995) and Timmis (1998) have described a prototype 'manufactured' seed that is comprised of an impermeable seed coat, an artificial megagametophyte, a cotyledon restraint, and a weak spot for root egress. This synthetic seed contains nutrients, antibiotics, plant growth regulators, hydrated gel and oxygen carriers. To date, there is no evidence of its operational application. A second approach, entailing direct sowing of somatic embryos, has been developed (Cyr et al. 2001). This proprietary system, demonstrated on a research scale for *Picea* spp., *Pseudotsuga menziesii*, *Pinus radiata* and *Pinus taeda*, was described to be suitable for use in commercial greenhouses using conventional sowing practices.

As observed with clonal selection, there are a few publications regarding commercial production of conifer somatic seedlings, with the bulk of information focusing on *Picea glauca x engelmannii*. The production of 12,000 interior spruce, first reported in 1993, reached a cumulative total of one million seedlings by 1999. More recently, production volumes from *P. sitchensis* (50,000; 1998), *P. glauca* (1.45 million; 2000), *Pinus taeda* (0.2 million, 1999–01), and *Pseudotsuga menziesii* (0.5 million; 2000–01) have been reported, with a cumulative total of 3.2 million somatic seedlings (Cyr et al. 2001). Pilot production of *Pseudotsuga menziesii* using bioreactors and direct sowing was expected to occur during 2001.

Development of transgenics

Development of SE in conifers has facilitated research on genetic transformation of embryogenic cells and regeneration of transgenic trees (reviewed by Peńa and Séguin 2001). Genetic transformation encompasses controlled introduction and expression of foreign or native genes in plants. This is a rapidly evolving area of forest biotechnology that holds a substantial promise for commercial application, as it has been demonstrated in agriculture. The addition of traits via genetic engineering requires a tissue culture system that enables transformation of individual cells and subsequent regeneration via a reliable plant production system. SE meets these criteria with demonstrable potential for transformation using embryogenic cultures or somatic embryos competent for secondary SE. Potential applications include the introduction of herbicide- and pest-resistant genes; genetic alterations that would provide desired wood characteristics, e.g. altered amount, type and form of cellulose and lignin, and reproductive sterility.

Two methods that are most frequently used to deliver genes to conifer cells are via accelerated particles (microprojectiles) coated with DNA or via cocultivation with *Agrobacterium tumefaciens* that carries the desired genes on a transfer-DNA (T-DNA) plasmid and naturally transfers the T-DNA section of the plasmid to plant cells. Ellis et al. (1993) first achieved stable transformation of embryogenic tissue using particle bombardment with *Picea glauca* whereas Levée et al. (1997) were the first to transform *Larix* hybrid by *Agrobacterium tumefaciens*. Since then, both methods have been successfully applied to other species and have led to the regeneration of transgenics, some of which are being tested for long-term transgene expression in confined field trials in North America (McLean and Charest 2000). These transgenics are *Picea glauca* (expressing the *Bacillus thuringiensis* Cry1A(a) gene in addition to marker and selectable genes), *P. mariana* (expressing marker and selectable genes, B. Rutledge, CFS, personal commu-

nication) and a pine species (expressing marker and selectable genes).

The ease of gene introduction varies with different species and genotypes (Klimaszewska et al. 2001; Tang et al. 2001) and with the method of transformation. Even if genes are delivered efficiently to the plant cells, expression will vary depending on the strength of the transgene promoter, copy number and integration site (Klimaszewska et al. unpublished). Fluctuations of the transgene expression have been observed and may be attributed to the metabolic state of cells and tissues. Moreover, in some transgenics, the silencing of the transgene/s was detected in trees grown over certain period of time. The controlled field trials were designed for studying the issue of stability of transgene expression over a period of several years under variable environmental conditions, which is of paramount importance in these long-lived organisms.

Large-scale release of transgenic trees will require considerable risk assessment investigation, particularly in the forestry context, as the conifers are prolific outbreeders. It may be necessary to ensure containment of certain transgenes (such as those conferring resistance to insect pests and pathogens) to prevent their transfer to wild population. One strategy that has been proposed to address this problem is engineering flower sterility, in addition to the value-added traits, into transgenics. Trees are important in supporting and maintaining microbial diversity in soil, hence analysis of transgenic root-soil interaction will have to be understood before a large-scale deployment of transgenic trees is considered.

Challenges and future

Genetic fidelity

Long-term benefits of propagation of conifers through SE lie in the production of clonally uniform plants. It is well established that plants regenerated in tissue culture may not be genetically identical due to certain stresses imposed by the culture's chemical and physical environment. These changes, referred to as somaclonal variations, may cause unwanted characteristics that might have enormous economic consequences augmented by the trees' long life cycles. Somaclonal variation may be manifested at the morphological, cytological (chromosome number and structure), biochemical (proteins and isozymes) and molecular (nuclear and organellar genomes) level.

To date, there is little evidence of somaclonal variation (Cyr 1999); nonetheless, the issue will persist in the background until trees derived from SE have reached rotation age. Research on spruce somatic seedlings versus conventional seedlings indicates morphological and physiological similarity (Gross-

nickle 1999; Harvengt et al. 2001). Additionally, somatic seedlings exhibit variation in adaptive traits that were reflective of the selection potential of SE (Fan and Grossnickle 1998). Some other groups have identified morphological variants in the population of somatic seedlings of *Picea glauca* and *P. mariana* (Isabel et al. 1996; Tremblay et al. 1999). Thus far the factors identified to contribute to genetic instability are genotype and duration of maintenance culture. Further research is necessary to establish and compare the extent and type of variation arising in field-grown material.

Deployment strategies

Clonal genetic material delivered via conifer SE includes top-ranked families, selected lines and genetically engineered clones (Sutton and Polonenko 1999). The benefits of these approaches, along with associated developments in mapping and marker-aided selection, include increased yield, customized products, delivery to mills of wood with increased uniformity, shorter rotations, pyramiding of value-added traits and earlier selection age (Pullman et al. 1998; Robinson 1999). To effectively achieve these goals will require the development of strategies that address public perception and environmental impact via proper management of genetic diversity and deployment risk.

In general, monoculture or broad deployment of large numbers of clones can both increase risk due to environmental changes, pest infestations or poor growth performance. For each species and region, a balance must be reached between genetic diversity and genetic gain based on the potential gain and effective population. To begin with, breeding strategy factors to be considered in this analysis include family distribution, relatedness among families, clonal lines per family, and the number of copies per clonal line. Additional issues are the use of clonal blocks, clonal mixtures and/or combinations of conventional and clonal orchard material, and the choice of uniform or variable sites (Libby and Rauter 1984; Park et al. 1998). In general, it is expected that selected clones will be deployed on the most highly productive sites and sites for which the material is best adapted.

Sutton and Polonenko (1999) have reviewed genetic diversity and deployment guidelines required for deployment of improved genetic material in British Columbia, Canada. For vegetative propagation, the required minimum number of genotypes was 200 for material derived from untested families and 30 for tested full-sib, open-pollinated and polymix families. For tested clonal material, the number of genotypes required dropped to 20, an approach consistent with risk versus gain assessments by Libby and Rauter (1984) and Roberds and Bishir (1997). Based on these criteria, and being conservative, 130 genotypes

from 13 full-sib families of *Picea glauca x engelmannii* were used for pilot production. Subsequent efforts were directed at operational deployment of 40 to 60 clonal lines representing an effective population of 10 unrelated parents.

Intellectual property

The potential for commercialization of SE in tree species is greatest for coffee, oil palms and conifers. These opportunities have resulted in significant investment by organizations in the development of intellectual property for SE, inclusive of complete recipes, individual process steps, products and delivery systems.

From 1989 on, patenting of conifer SE has increased dramatically, with over 50 patents issued or submitted by 2002. Significant emphasis has been placed on protecting the SE process steps, particularly for initiation from immature and mature explants, proliferation culture and suspension, embryo maturation, and the development of embryo desiccation. This entails the application of specific levels and stage-dependent application of carbohydrates, non-metabolic additives, osmotically active compounds and plant growth regulators. Process patents also include recipe patents; these are aimed at ensuring the freedom to operate an entire SE process that an individual organization has developed. Product patents in conifer SE are few in number and apply for the most part to nursery delivery and synthetic seed applications, which are key steps for commercialization. Conifer SE intellectual property is dominated by a few organizations with Cellfor (and its predecessors) (15%), Westvaco (15%), and Weyerhaeuser (38%) accounting for two-thirds of the current total (Table 2).

Future directions

During the last two decades, substantial progress was achieved in demonstrating the potential for commercialization of conifer SE. However, successful implementation in the long run will depend on numerous factors including public education, integration with nursery and forest practices, and technology management. For the most part, these factors can be managed on a time-scale via gradual scale-up and effective communication among involved parties. In the case of SE technology, a component that poses significant risk, substantive research investment will still be required to achieve the aim of a reliable and cost-effective process.

Conclusion

Current efforts in the international arena indicate that conifer SE is rapidly being accepted as an important component of breeding strategy as an avenue to true clonal forestry. Activities are expected to in-

Table 2. Summary of conifer SE patents

Patent Class	SE Component	Patents (US) Awarded and PCT Published Applications (WO)
Process	Initiation (mature explants)	US5501972(1996), WO99/23874(1999)
Process	Initiation (immature explants)	US4217730(1980)[5], US5310672(1994), US5677185(1997)[4] US5856191(1999)[4]
Process	Maintenance	US4957866(1989)[5], US5563061(1996)[5]
Process	Suspension	US5491090(1996)[4], WO-01/56368(2001)
Process	Maturation	US5034326(1991)[5], US5041382(1991)[5], US5187092(1993), US5236841(1993)[5], US5294549(1994)[5], US5523230(1996)[3], WO98/48279(1998)[5], US6200809(2001)[2], WO01/20972(2001), WO01/64020(2001)
Process	Desiccation/Starvation	US5183757(1993)[2], US6180405(2002)[1]
Process	Germination	WO99/26469(1999)[5]
Process	Recipe (gymnosperms)	US5821126(1998), US6340594 (2001)[2]
Process	Recipe (*Pinus*)	US5413930(1995)[4], US5506136(1996)[4], WO96/370961(1996), US 5731191(1998)[4], US5731203(1998)[4], US5731204 (1998)[4], WO01/56368(2001)
Process	Recipe (*Pseudotsuga*)	US5482857(1996)[5]
Process	Recipe (*Pinus, Pseudotsuga*)	US5565355(1996)[3]
Process	Transformation	US5681730(1997)2, WO97/01641 (1997)[3]
Process	Analytical Tools for SE	US6117678(2000)5, US 6150167(2000)[5]
Process/Product	Bioreactor	WO00/68357(2000)
Process/Product	Synthetic Seed	US5119588(1992)[5], US5236469(1993)[5], US5427593(1995)[5], US5451241(1995)[5], US5486218(1996)[5], US5564224(1996)[5], US5687041(1997)[5], US5701699(1997)[5], WO99/65291(1999)[2], WO99/65293(1999)[2], US6119395(2000)[5], WO00/62599(2000)[2]
Product	Synthetic Seed	US5464769(1995)[2], US5737872(1998)[2]

Notes: [1]Carter Holt Harvey (NZ); [2]Cellfor Inc.(includes BC Research, Pacific Biotechnologies, Silvagen) (Canada); [3]Forest Research (NZ); [4]Westvaco (USA); [5]Weyerhaeuser (USA)

crease in countries currently possessing the technology as well as expanding to new regions in Africa, Asia (e.g., China), and South America. It is expected that, with proper management, higher genetic gain can be delivered in volume to plantations while maintaining appropriate genetic diversity. Certainly, developments during the first decade of the new millennium will be eagerly anticipated. Achievements that can be expected include demonstration of realized gains from clonal trials, implementation of semi-automated production systems and operational demonstration of artificial seed technology.

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