Somatic Embryogenesis Tissue Culture for the Propagation of Conifer Seedlings: A Technology Comes of Age

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Somatic embryogenesis is a tissue culture method of asexual propagation used in horticulture, agriculture, and to some extent in forestry as a means of rapidly multiplying elite varieties or clones. This paper reviews the development of somatic embryogenesis tissue culture procedures for interior spruce— Picea glauca *(Moench) Voss* × Picea engelmannii *Parry ex Engelm. Various components of tissue culture protocols, scale-up technology, nursery production, and field performance arc discussed, as is how this vegetative propagation technology is being integrated within an existing tree improvement program. The somatic embryogenesis tissue culture technology is also compared with the other propagation technologies currently used within forest regeneration programs.* Tree Planters' Notes 47(2):48-57; 1996.

There have been major advances over the past 25 years in the development of operational vegetative propagation systems for conifer species used in plantation forestry programs. These propagation systems provide a means of bringing new genetic material into forestry programs through the capture of a greater proportion of the gain from additive and non-additive genetic components inherent within a selected tree species (Libby and Rauter 1984). Vegetative propagation systems also provide a method for multiplying superior families identified in tree improvement programs (Gupta and Grob 1995; Zobel and Talbert 1984). Vegetative propagation systems being developed for use in forestry utilize the following approaches:

1. Rooted cuttings
2. Micropropagation through organogenesis tissue culture
3. Somatic embryogenesis

Currently, rooted cuttings are the most effective propagation technique that is operationally available to multiply specific individuals that have desirable traits. 1n a recent survey, over 65 million conifer cuttings are produced annually around the world, with this number growing rapidly (Ritchie 1991; Talbert and others 1993). The primary use of rooted cutting technology is for bulk production of genetically improved materials.

Production of rooted cuttings is essentially a 2-step process: the production of cutting-donor plants and the production of rooted cuttings. Donor plants can range from selected trees of wild populations to trees grown from seeds of genetically improved families under an intensive nursery cultural regime. Rooted cuttings of forest tree species are most successfully produced from juvenile portions of donor plants because these juvenile portions of a plant will provide cuttings with the potential for good initiation of root primordia (reviewed by Hackett 1988). Cuttings are placed in a rooting environment (that is, high humidity and soil moisture, warm soils, and moderate light levels), allowed to develop roots, then treated as rising 1-year-old seedlings.

Organogenesis is a tissue culture system that relies on the multiplication of shoots or the *de novo* formation of organs originating from either unorganized callus or preformed shoots or induced buds. Propagules produced through this system are essentially treated as microcuttings. Thus, shoot propagules are placed in an optimal rooting environment and treated in a similar manner as cuttings.

Somatic embryogenesis (SE) is a tissue culture approach where proliferative embryo suspensor masses are established from non-meristematic cells and subsequently cultured to produce organized bipolar structures possessing shoot and root meristems (that is, somatic embryos). The term somatic refers to embryos developing asexually from vegetative (or somatic) tissue. This method has been used-in horticulture, agriculture, and to some extent, in forestry-as a means of rapidly multiplying elite varieties or clones. Through the application of bulk-handling techniques, SE is cost-effective as a propagation approach for conifers (Roberts and others 1995). Distinct from conventional cuttings, SE offers the advantage of long-term storage of germplasm through cryopreservation (Cyr and others 1994). Although considerable research focus and efforts have demonstrated the potential for SE propagation of a large variety of plant species, success has been achieved only at the research scale (Aitken-Christie and others 1995).

Research and development in the area of SE of commercial conifers has been driven by the following 2 factors:

- The multiplication of superior families
- The selection of elite clones from among such families to capture a greater proportion of the genetic gain (Mullin and Park 1992; Park and others 1993)

In conifers, embryogenic cultures can be induced from developing and/or mature zygotic embryos or young germinants, but not from older explants (Attree and Fowke 1991; Tautorus and others 1991; Roberts and others 1993). Thus, SE fulfills a role similar to cuttings with respect to the multiplication of families. The value-added traits that can be captured and propagated through SE are those that can be identified through any tree-breeding program and include yield, wood quality, plus stress, pest and disease resistance. Spruce species appear to be most amenable to SE propagation technology (Hakman and Von Arnold 1988). Interior spruce— a complex of white and Engelmann species *Picea glauca* (Moench) Voss × *Picea engelmannii* Parry ex Engelm.— is a major commercial species in British Columbia, Canada. Within British Columbia since the 1980's, approximately 100 million container interior spruce seedlings have been planted annually in forest regeneration programs. To supply improved genetic material for these programs, a tree improvement program has been developed for large areas of the commercial land base (Kiss 1968). This breeding program affords the opportunity to match significant potential genetic gains with the technical opportunity to deliver value-added material to operational reforestation programs through SE technology. In this paper, we review steps that have been taken in developing SE tissue culture procedures for interior spruce. Various components of tissue culture protocols, scale-up technology and nursery production will be discussed. In addition, SE technology will be compared with other propagation technologies currently used in forest regeneration programs.

Basic Laboratory Protocols for Somatic Embryogenesis

In general, the SE process is divided into several phases (Gupta and Grob 1995):

- Culture **initiation** —induction
- **Proliferation** — maintenance, multiplication, suspension culture
- **Cryopreservation** — germplasm storage
- **Maturation** — somatic embryo production
- **Embryo drying**
- *In vitro* **germination and early growth**

All parts of the laboratory process are performed under-sterile conditions to prevent microbial contamination.

Because technology is most advanced for *Picea* spp., the methodologies discussed in this section are those developed for interior spruce (Cyr and others 1995; Cyr 1996). Apart from the stage of commercial development, the most significant protocol difference among the various conifer species is at the initiation phase. For spruce, stored seed can be used for initiation of SE, whereas for many other conifers, most notably *Pinus* spp. and Douglas-fir, fresh immature cones are required, with initiation success restricted to a 1- to 2-week developmental window (Cyr unpublished data).

Initiation. For spruce, mature zygotic embryos are dissected from the seed and placed onto semi-solid medium containing plant growth regulators (Webster and others 1990). The dissected embryo is referred to as the **primary explant**. The induction of SE is first evidenced by the growth of new tissue, usually at the hypocotyl-cotyledon junction of the explant (figure 1). This process takes approximately 8 to 12 weeks and the new tissue is referred to as "putative" embryogenic tissue or embryonal suspensor mass.

Proliferation. The embryonal suspensor mass is characterized by the presence of early-stage somatic embryo structures that are analogous to those occurring during normal seed development. This tissue is isolated

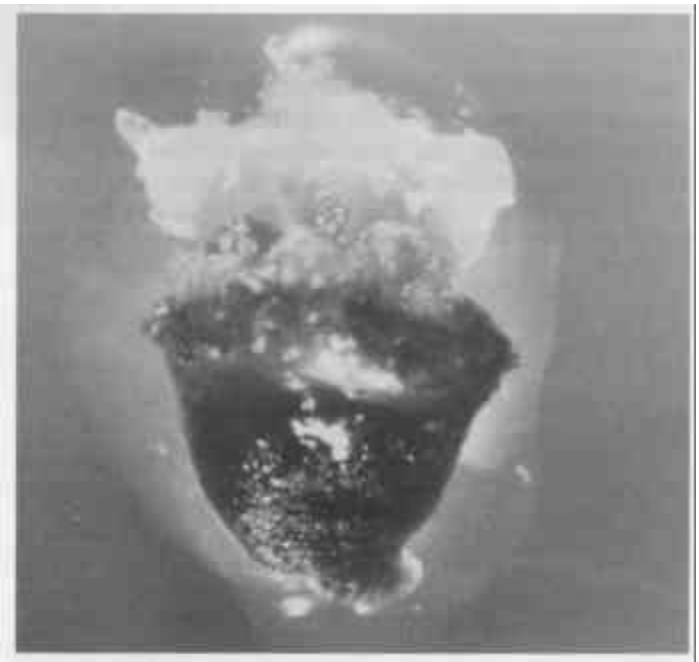

Figure 1—*Initiation of embryogenic tissue from the excised zygotic embryo explant.*

from the primary explant and transferred to fresh medium. Under these conditions, the tissue multiplies and develops as early-stage somatic embryos. Tissue is subdivided (subcultured) and transferred to fresh medium every 7 to 10 days. This material consists of multiple embryos and is often referred to as **embryogenic callus** (figure 2). Sufficient material can be produced for cryopreservation within 4 to 6 weeks.

Cryopreservation. Cryopreservation facilitates long-term storage of the valuable germplasm produced by SE (Cyr and others 1994; Kartha and others 1988). The embryogenic tissue is treated with cryoprotectants, frozen to -35 /C under controlled conditions, and then subsequently stored in liquid nitrogen (-196 /C). Cryopreserved tissue can be regenerated within 1 to 2 weeks after a simple thawing process.

Maturation/embryo drying. To advance the development of somatic embryos, tissue is transferred to a medium containing the phytohormone abscisic acid. Within a period of 4 to 7 weeks, this results in the production of mature somatic embryos that are analogous to their zygotic counterparts (figure 3) (Roberts and others 1993). To complete the process and effect a transition to germination upon exposure to suitable conditions, somatic embryos are harvested and subjected to a drying process (Roberts and others 1993). This step enhances the vigor of the somatic embryo. At this stage, the embryos are often referred to as synthetic seed, or "synseed."

Germination/early growth. Spruce synthetic seed does not require stratification. The embryos are placed on germination media containing no phytohormones and supplemented with a carbohydrate (Roberts and others 1995). *In vitro* germination occurs within 5 to 7 days and proceeds to the development of true needles at 4 to 6 weeks. At this point the plantlets can be transferred to *ex vitro* conditions (figure 4).

Figure 3—*Production of cotyledonary somatic embryos from embryogenic cultures.*

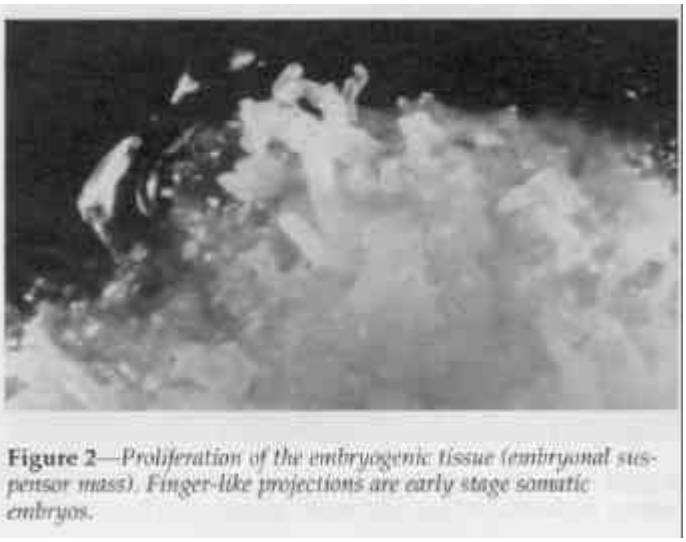

Figure 2—*Proliferation of the embryogenic tissue (embryonal suspensor mass). Finger-like projections are early stage somatic embryos.*

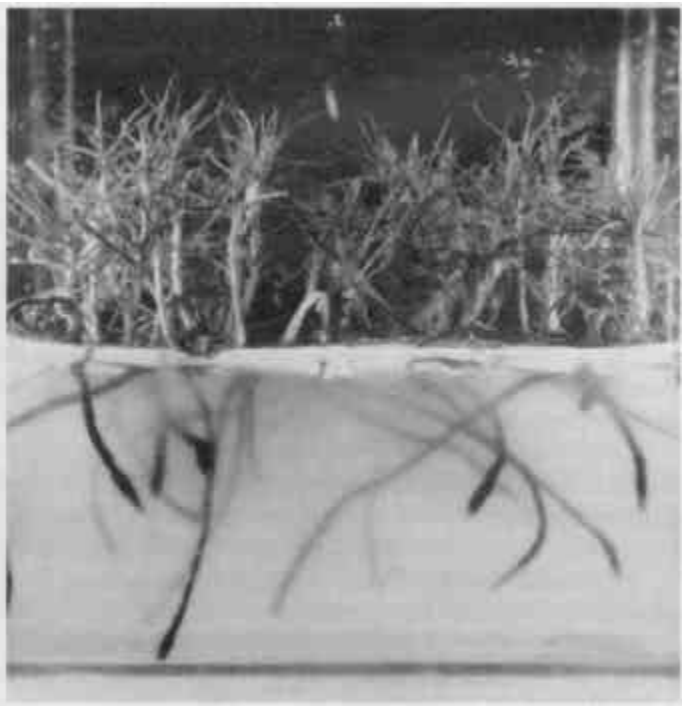

Figure 4—In vitro *germination and early growth of interior spruce somatic seedlings.*

Nursery and Field Performance.

Throughout the 1990's, germinants from SE technology have shown continued improvement in their development into high-quality somatic seedlings in the nursery. In addition, somatic seedling propagation technology has been successfully integrated into the container seedling production system. This containerized delivery system accounts for upwards of 95% of the conifer seedlings grown for reforestation programs in British Columbia, Canada. Through this time period, the performance of somatic seedlings has been compared to zygotic seedlings.

Production of conifer seedlings in container nursery systems is primarily dictated by 2 operational criteria:

- Maximize greenhouse production through the great est number of plantable seedlings per unit area
- Minimize the number of seedlings that do not meet defined morphological standards at the end of the production cycle

The goal of the interior spruce somatic seedling program is to produce seedlings that meet these normal operational criteria and have a high level of seedling quality.

For every nursery production cycle over the past 5 years, survival of somatic seedlings after 1 growing season has consistently averaged 95%. This indicates that germinants are of a high quality coming out of the laboratory. This high survival count is important because it demonstrates that a single sowing of these germinants into container cavities is an effective use of both somatic seedlings and greenhouse production space.

How the germinants initially respond to the nursery environment will have a profound influence on subsequent morphological development. During the first stages of development in the nursery, past studies have found variable success in obtaining normal shoot growth of somatic, compared to zygotic, seedlings (Webster and others 1990, Grossnickle and others 1994). As a result of this variable performance, initial production runs of somatic seedlings resulted in a higher than desired culling rate (up to 20%) based on British Columbia Ministry of Forests shoot-height culling standards (Grossnickle and Major 1994a).

Recent nursery performance studies of somatic seedlings have shown that a proper nursery cultural environment (that is, nutrients, temperature, and moisture) during the initial establishment stage will result in normal morphological development of seedlings. Shoot growth of zygotic and somatic seedlings was similar during first-year development for seedlings grown dur-

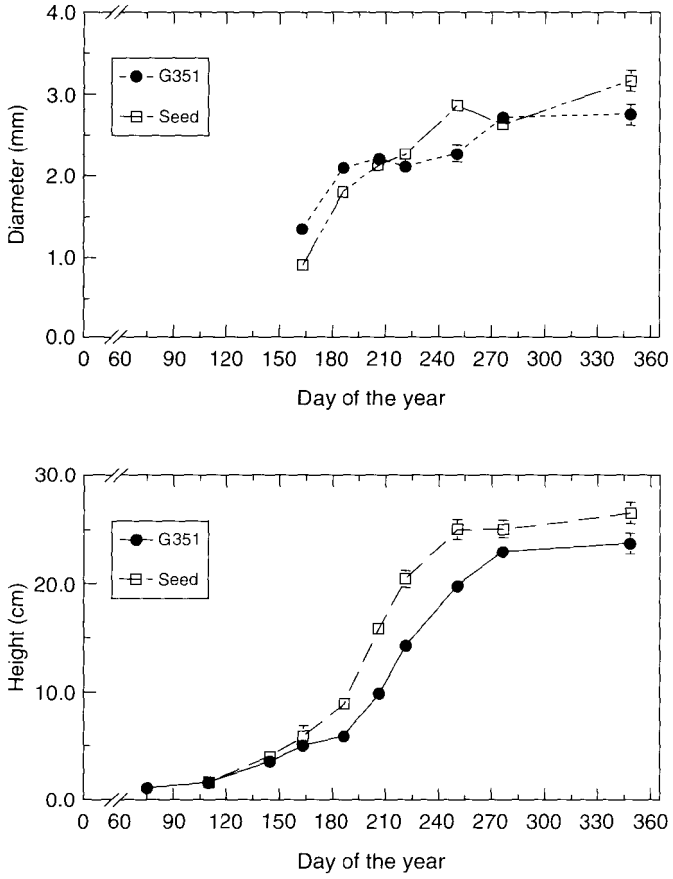

Figure 5 —*Somatic (G351) and zygotic interior spruce seedling height and diameter growth (mean ±SE) of 1 +0 container stock (for 1 representative clone) from one family during the nursery production cycle.*

ing the 1995 nursery production cycle (figure 5). Due to this improved morphological development, culling rates for 1996 have decreased to 5 to 8% (based on British Columbia Ministry of Forests shoot-height culling standards) (Polonenko unpublished data). Thus, currently produced somatic seedlings consistently meet morphological standards required for production of operational containerized interior spruce seedlings (figure 6).

Under normal nursery production procedures for interior spruce, seedlings need to develop the proper level of winter hardiness before they are lifted for storage of the 1+0 frozen-stored stocktype or placed outside of the greenhouse into outdoor holding compounds for the second year of a container production cycle for the 2+0 summer-plant stocktype (Simpson 1990). During fall acclimatization, somatic and zygotic seedlings have similar dormancy, freezing tolerance, and root growth patterns (Grossnickle and others 1994). In addition, somatic and zygotic seedlings have similar performance throughout a normal 5-month period of frozen (-2 /C)

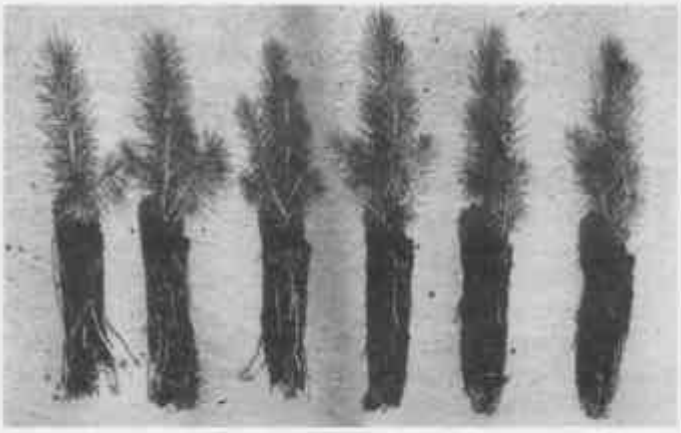

Figure 6—*A comparison of planting stock for conventionally produced interior spruce zygotic (3 on right) and somatic (3 on left) seedlings.*

storage used for holding spring-planted conifer seedlings (Grossnickle and others 1994). This allows somatic seedlings to be treated like normal zygotic interior spruce seedlings during fall and winter nursery cultural practices.

Performance of seedlings on a reforestation site depends upon their inherent growth potential and the degree to which environmental conditions of the field site allow this growth potential to be expressed. To determine a seedling's field performance potential, a stock-quality assessment program needs to use an array of tests that simulate anticipated field environmental conditions (Grossnickle and Folk 1993). This will help forecast seedlings' physiological performance and potential for growth on a reforestation site. Field performance potential testing of somatic and zygotic interior spruce seedlings have found comparable performance capability under both cold (that is, frost and low soil temperature), and drought conditions (Grossnickle and Major 1994a). A similar stock quality assessment program has recently been conducted on the 1996 somatic seedling production run and results are comparable to previous findings (Grossnickle unpublished data). This indicates a consistency in the quality of somatic seedlings to be planted on reforestation sites.

Reforestation site trials have tested the field performance of interior spruce somatic seedlings in comparison to zygotic seedlings (Grossnickle and Major 1994b). These trials have found that somatic and zygotic seedlings have comparable summer seasonal water relation patterns and gas exchange response patterns. In addition, somatic seedlings have comparable or better performance than zygotic seedlings in response to damaging winter conditions. Somatic and zygotic seedlings have corresponding incremental height and diameter growth over at least 2 seasons in the field, resulting in similar new root and shoot development. The survival rates of somatic and zygotic seedlings after 2 growing seasons are comparable, 83 and 77%, respectively. Thus, performance of somatic seedling performance over 2 growing seasons on a reforestation site indicates that they have all of the traits that are desired in container stock for use in forest regeneration programs.

Integration Into a Tree Improvement Program

There is an opportunity with SE technology to not only capture additional gain in height but also take advantage of family differences (Kiss and Yanchuck 1991) and potential clonal differences (Ying 1991) in the resistance to white pine weevil— *Pissodes strobi* Peck— inherent in interior spruce. This clonal strategy involves an "add-on" testing phase to the main breeding program (Sutton and others 1993). The breeding, testing, and selecting of parents for additive gene effects is the main emphasis of the tree improvement program for interior spruce being conducted by the British Columbia Ministry of Forests (Kiss 1968). First-generation clonal seed orchards have been rogued, and genetic gains for improved shoot growth from this orchard were about 11 % based on 10-year height results (Kiss and Yeh 1988). Clonal testing of progeny from first-generation selected parents will capture additional gain for improved shoot growth over and above gains from rogued first-generation seed orchards.

About 1,300 clones have been produced for field trials from 31 full-sib and 14 open-pollinated weevilresistant families of interior spruce using SE technology (Cyr and others 1995; Cyr 1996). Embryogenic callus from all of these clones have been cryopreserved for long-term storage until field selections are made. Parents of the full-sib families were selected, as judged by their progeny, based on 15-year height and 10-year weevil resistance (Kiss and Yanchuck 1991; Sutton and others 1993). The open-pollinated families were selected on the basis of provenance trials (Alfaro 1996). Ramets derived from SE clones are being field-planted to assess for accelerated growth and increased levels of resistance to weevil attacks. Ultimately, a total of 30 to 50 clones will be selected based on mean height growth and weevil damage starting 5 to 6 years after establishment of the trials. The selected clones will be removed from cryostorage and produced as somatic seedlings that will then be deployed operationally to reforestation sites as diverse genetic mixtures. Gains expected from the clonal program will be 22% in height, as well as the deployment of clones with minimal susceptibility to weevil attack.

In parallel with this program, morphological development and physiological performance from a subset of

selected clones is ongoing to define parameters that can be used to profile selected clones. Studies have been conducted to assess the clonal variation in morphological development in the nursery and over the first 2 field growing seasons. In the nursery, there is marked variability in the morphological development that occurs between clones within the same family in response to identical nursery culture conditions (figure 7). This variability in nursery performance will have to be addressed in relation to the current concept of producing morphologically uniform stock for reforestation programs in British Columbia (Scagel and others 1993).

Studies have also examined clonal differences in physiological performance at selected times, and within selected environments, throughout the yearly seasonal cycle. These studies have also found that there is a wide range in physiological performance between clones of interior spruce in relation to potential field site conditions (Grossnickle unpublished data). This information will provide a means of defining clones having desired physiological traits that can tolerate limiting field site conditions. The practical application of this program is to develop a series of measurement parameters that can be used to assist in the profiling of clonal material that has been selected through standard genetic selection field trials and will be deployed within operational reforestation programs.

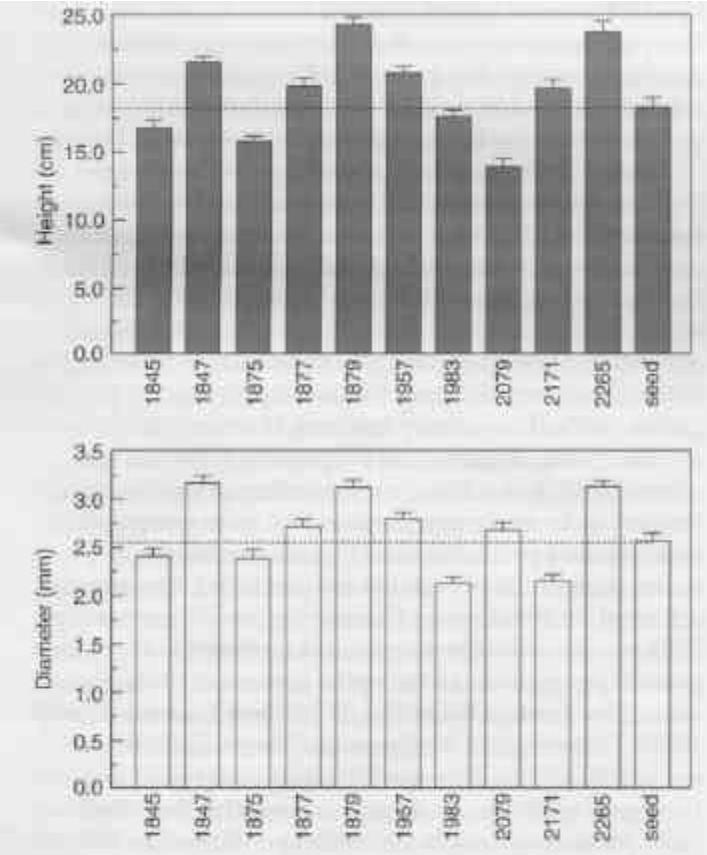

Figure 7—*Somatic and zygotic interior spruce seedling shoot development (height and diameter; mean ± SE) of 1+0 container stock (of 10 representative clones) from 1 family at the end of the nursery production cycle.*

Scale-Up to an Operational SE Production System

The ultimate test for commercial acceptance of a novel technology such as somatic embryogenesis is the ability to develop and implement a successful operational use for the technology. The key components that must be addressed during this stage are

- Development of a cost-effective manufacturing process
- Delivery of high-quality products that provide predictable and reproducible performance
- Technology validation and promotion in the market place

Issues associated with the scale-up of SE production and delivery of SE products will be discussed in this section.

Plant tissue culture processes tend to require significant hands-on manipulation to optimize the quality of plant propagules produced, but the labor costs involved render the products excessively expensive and therefore unacceptable in the marketplace. Attempts to minimize labor costs and improve product quality resulted in the focusing of research priorities during the past decade on automating some or all of the stages required for synseed production and subsequent handling of synseeds and germinated somatic seedlings (Cervelli and Senaratna 1995; Gray and others 1995; Heyerdahl and others 1995; Onishi and others 1994; Walker 1995). However, Cervelli and Senaratna (1995) noted that in spite of all these efforts, there were no successful commercial applications of SE to that date for either angiosperms or gymnosperms.

During the past 18 months, significant progress has been made toward a reliable, high-volume cost-effective SE production system for spruce. The details of the spruce SE process used at the laboratory-scale have been described (Cervelli and Senaratna 1995; Roberts and others 1993, 1995). Rather than trying to develop and optimize automated systems for spruce SE, the major emphasis during this production scale-up period was to overlay the established fundamental principles of manufacturing production planning and control (Wight 1984) on the laboratory-scale SE process. The initial steps required extensive and precise identification and characterization of the inputs, the action steps and related resources and time requirements, and the outputs of each step in the spruce SE process. In addition,

computerized inventory tracking programs were developed and incorporated. The information generated provided a clear identification and understanding of the constraints within the process, and enabled numerous modifications and improvements in SE production planning, scheduling and processes. Furthermore, "standard operating procedures" for each stage of spruce SE production were developed and then strictly followed during every production run. The net effect was that SE productivity increased by more than 300% without any additional labor inputs.

Our recent focus of product development has been assessing the compatibility of various commercial horticulture practices with SE, and then use this information to modify SE production practices to improve quality of somatic embryos at the germination stage and somatic seedlings at the nursery production stage. This market-driven approach— that is, awareness and adoption of existing proven technologies-has also resulted in the elimination of problems in the nursery development (establishment and growth vigor) of somatic seedlings (see "Nursery and Field Performance" section, page 51).

Future of this Technology in Reforestation Programs

The use of tree improvement practices to enhance the genetic characteristics of planted seedlings is a forestry practice that consistently shows a high return on investment by increasing yields obtained from planted forests. The use of improved seed is an effective way of bringing genetic improvement to forest regeneration programs. Seed orchards are currently used to produce seeds in large commercial quantities from trees having desired genetic traits. However, improved seed does not provide a method to multiply specific individuals that have desirable traits. Vegetative propagation techniques provide the best means for multiplying the improved genetic resource developed from tree improvement programs.

Two criteria are considered important for the successful implementation of vegetative propagation systems within an operational forestry program (Carson 1986). First, the propagation system must have the ability to preserve superior candidate clones, without genetic change or further maturation, while genetic selection programs are taking place. The ability to propagate elite clones will require a capacity to maintain individuals in a form capable of regenerating after a minimum period of 5 to 10 years that is required in order to test and select clones in the field. Second, the propagation system has to be able to multiply selected clones in large enough numbers at a reasonable cost. If these 2 criteria can be reasonably met, the selected vegetative propagation systems can be implemented within an operational forestry program.

To have a successful rooted cutting production program, donor material must be maintained in a juvenile state to ensure optimum rooted cutting performance. The phenomenon of maturation in conifers is a natural developmental process; this maturation process is a major impediment in maintaining hedge orchards of elite genetic material for continual production of genetically improved rooted cuttings (Zobel and Talbert 1984). Attempts to propagate mature individuals of northern conifer species by this propagation method has been unsuccessful or severely limited by plagiotrophism or other effects of maturation (Hackett 1988).

An alternative practice for rooted-cutting production currently in use to produce juvenile cuttings is seedling-origin hedges. This practice is currently in operational use by the Weyerhaeuser forest company to bulk-up material from elite Douglas-fir— *Pseudotsuga menziesii* (Mirb.) Franco— seed produced in their tree improvement program (Ritchie 1994). With this program, Weyerhaeuser currently produces 3 million rooted cuttings/year. This same cultural approach has been developed for interior spruce (Russell 1990). A limitation of this propagation approach is that it only provides a means of multiplying specific individuals in 1 production cycle, thus eliminating the long-term capture of a genetic source for future regeneration programs.

Vegetative propagation of conifer seedlings from organogenesis on an operational scale has also been conducted on a limited basis. Large scale production and field establishment trials have been conducted on a number of conifer species since the mid 1980's, for example, loblolly pine – *Pinus taeda* L.— in Amerson and others (1984) and Douglas-fir in Ritchie and Long (1986). The major limiting factor in applying this propagation method in forestry has been the cost relative to the low value of individual propagules (Hasnain and others 1986). In addition, most coniferous species have proven fairly recalcitrant to standard micropropagation methods and problems, usually resulting from early maturation of the plants that are produced. The success achieved with radiata or Monterey pine— *Piftus radiata* D. Don— is a notable exception. An operational organogenesis program on radiata pine is currently being conducted by Tasman Forestry Ltd. in New Zealand (Gleed 1995). This program produces just over 1 million propagules, using elite genetic material selected from their seed orchards, on an annual basis for their high-yield plantation forests. Currently, no more than 20% of their annual plantings are clonal, with genetic diversity broadened by selecting only 2 to 3 clones/non-related family within the mixed family groups of 15 to 20 non-

related families. If there are currently operational limitations to this system, they are the labor-intensive procedures required in handling cultures to produce quality propagules and the limited ability to store (that is, cold-storage for up to 6 years) while field selections are being conducted (Cheliak and Rogers 1990). The same problem of maturation that occurs in rooted cutting programs can also occur in organogenesis tissue culture programs (Gupta and others 1991). Thus, the higher per-unit propagule cost limits the use of this technology to propagate only the very elite genetic material derived from a tree improvement program, whereas limited storability of cultures reduces the time available to make proper genetic selections.

Somatic embryogenesis is the only vegetative propagation technology that provides long-term preservation of the selected genetic component of a conifer species. Embryogenic cultures can be proliferated in a juvenile form for long periods of time to produce unlimited numbers of propagules from the same clone. These cultures can also be frozen-stored indefinitely while genetic selection is being conducted. Somatic embryogenesis protocols have shown no evidence of somaclonal variation (Eastman and others 1991; Cyr and others 1994), thus ensuring the genetic stability of spruce embryogenic clones. This propagation technology also provides a means of incorporating, through genetic engineering, selected traits that will enhance seedling performance, for example, insect resistance (Ellis and others 1993). Thus, SE is a vegetative propagation technology that will allow for the preservation of superior genetic material that can be used within an operational regeneration program.

Once selections of clones having desired genetic traits are made, these clones need to be brought forward to produce propagules that will then be grown under standard nursery cultural practices for the production of somatic seedlings. Somatic embryogenesis as a viable operational propagation technology for conifer species is just coming of age. Forestry companies and government organization around the world are currently working at bringing this technology to a point where it can produce conifer somatic seedlings, on a cost effective basis, with desired genetic characteristics-for example, Douglas-fir (Gupta and others 1994), loblolly pine (Handley and others 1994), and radiata pine (Smith and others 1994). Currently the program at the Forest Biotechnology Centre and Silvagen Inc., BCRI, is the furthest along in the scale-up process of this propagation technology. The production of somatic interior spruce seedlings has grown steadily from an initial production run of 12,000 somatic seedlings in 1993 to 350,000 somatic seedlings in 1996. It is anticipated that 600,000 somatic seedlings will be produced in 1997 with the target of 1,000,000 somatic seedlings for 1998.

Somatic embryogenesis of interior spruce has developed to the point where an operational production program has been initiated. The emphasis of this program in the coming years will be to scale up this propagation technology while reducing the cost per production unit. At the same time, the overall mandate will be to improve the quality of somatic seedlings from a range of genetic sources having desired field performance characteristics. This will allow for the successful implementation of SE technology as a viable vegetative propagation system within operational forestry programs.

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