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Applications of biotechnology for forest regeneration

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Abstract. The Forest Biotechnology Centre is an interdisciplinary research group dedicated to the development and application of advanced technology for the enhancement of forest regeneration. The Centre carries out contracts on behalf of clients in forest-related industries and government agencies. In addition, there are a number of long-term, in-house projects aimed at the development of proprietary technologies in genetics and propagation, and seedling production and establishment. Technical capabilities include: tissue culture, molecular genetics, pathology and microbial inoculants, and ecophysiology. These techniques are also being used to improve nursery culture regimes, disease assessment, planting regimes, and new product development for a variety of conifer species. Additional programs relate population genetics to adaptive traits, and develop clonal testing within elite families from tree-breeding programs.

Introduction

Successful regeneration relies on the application of work from many forestry disciplines toward a common goal. At the center of any successful regeneration program is the production of high-quality seedlings that have good performance on reforestation sites. Performance on a reforestation site depends on seedling growth potential and the degree to which field site conditions allow this potential to be expressed. Seedling growth potential is influenced by the inherent genetic make-up of source material and the culture used during nursery development. If these attributes can be directed toward improving seedling growth on a reforestation site, then the potential productivity of planted forests will be increased. Disciplines that are oriented toward improving these facets of producing high-quality seedlings are the main focus of the Forest Biotechnology Centre (FBC).

The FBC is an interdisciplinary research group dedicated to the development and application of advanced technologies for improved regeneration practices within planted forests (Figure 1). The FBC carries out long-term, in-house projects and contracts on behalf of clients in forestry and related

industries. Technical capabilities applied by the FBC to forest regeneration programs include:

- defining species variation through molecular genetic markers and ecophysiological parameters,
- creating new genotypes through gene transfer technologies,
- developing advanced propagation systems through somatic embryogenesis tissue culture technology,
- improving seedling quality through disease diagnosis, remediation, and incorporation of plant growth-promoting bacteria, and
- applying ecophysiological assessment techniques in support of seedling production, improved quality, and reforestation site performance.

The following sections describe the four major research groups within the FBC – tissue culture, molecular genetics, pathology and microbial inoculants, and ecophysiological assessment. These research groups focus on applying their respective technologies to forest regeneration programs.

Tissue culture

The main emphasis of the tissue culture program is to use somatic embryogenesis to develop propagation systems for conifers. These systems allow the mass propagation of elite families from tree-breeding programs, and, in addition, the selection of superior clones that can be stored and propagated in a sustained manner. Over the last several years, the FBC has made significant advances in the maturation, germination, and acclimatization phases of spruce somatic embryogenesis (Sutton et al. 1993). Progress in producing high-quality somatic embryos and resulting seedlings has enabled the development of an operational production system for spruce. Recently, the FBC has developed protocols to produce and deliver high-quality pine somatic embryos (Cyr 1998). Commercial production has been initiated by an associated company, Silvagen, Inc.

Molecular genetics

The molecular genetics program involves an integrated approach in creating the necessary molecular tools for various short- and longer-term applications to operational forestry. These have ranged from development and use of species-specific probes for analysis of natural hybrids to the development of highly polymorphic DNA markers (DNA fingerprints) for monitoring seed orchards (Sutton et al. 1991a, 1991b, 1994). It is now possible to index clonal material unambiguously, and to monitor pollen contributions, pollen contamination, and in-breeding. In addition, these same tools can be used

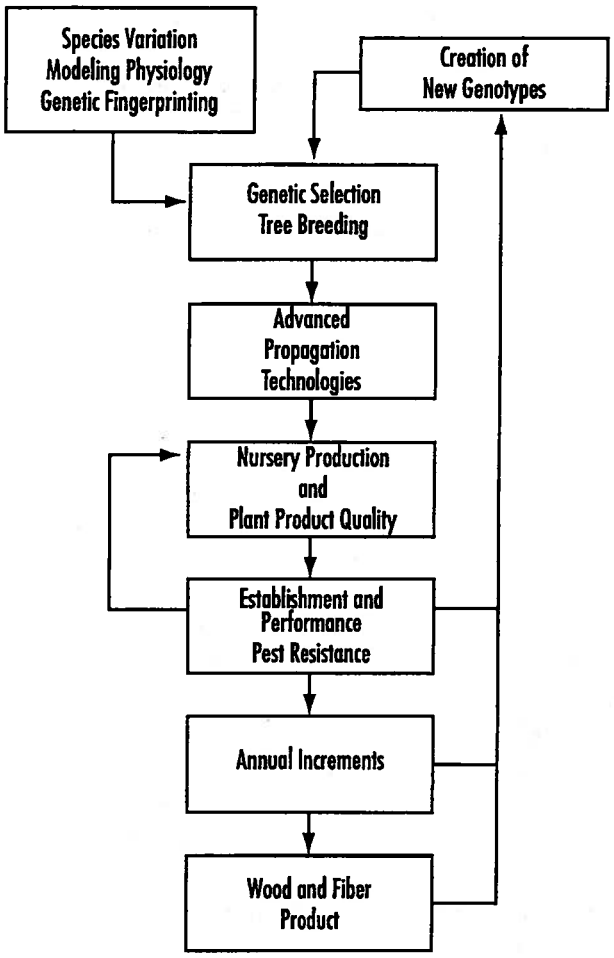

Figure 1. Technology factors influencing plantation forestry.

to characterize natural populations for planning tree-breeding units and seed orchards.

Longer-term work in gene expression and genetic transformation has also been carried out, thus allowing for considerable progress toward the goal of providing routine genetic engineering of improved clonal material for value-added traits. The FBC reported the first successful genetic engineering of spruce carrying spruce budworm resistance (Ellis et al. 1993).

Pathology and microbial inoculants

Fungal disease problems can cause major losses in forest nurseries and on reforestation sites. The FBC has a program to investigate causes of these

problems, and finds means to limit their impact. The approaches taken include both biological and cultural approaches to enhance conifer seedling health and growth. For example, the influence of fungal inoculum sources and nursery cultural practices on root infection of conifer seedlings is evaluated. This has resulted in the development of improved seed treatments that considerably reduce the incidence of *Fusarium* contamination (Axelrood et al. 1995). Through a major in-house research initiative, the Centre has identified a number of microbial strains for biological disease control and growth enhancement of conifer seedlings. These are being developed further for commercial use in association with Agrium Inc.

Ecophysiological assessment

Ecophysiology research projects are carried out on behalf of a variety of clients in the forest and agrochemical sectors. Over the past few years the FBC has developed an assessment program for the laboratory, farm field sites, and reforestation sites that integrates a number of physiological and morphological measurements, including drought and frost tolerance, gas exchange capability, and growth capacity (Grossnickle et al. 1991a, 1991b, 1991c; Grossnickle and Arnott 1992; Major et al. 1994). These techniques have resulted in a stock quality assessment approach that is used to identify optimal nursery culture and planting regimes for a variety of species (Grossnickle and Folk 1993). In addition, these assessment methods are used to describe the effects of various precommercial products on the performance potential of various plant species (e.g., abscisic acid analogs, Grossnickle et al. 1996a).

A methodology is under development for the physiological selection of desirable genotypes from a variety of species. Selection methodology will be generated by collecting data over a wide range of environmental conditions, including light intensity, evaporative demand, and soil temperature and moisture. This assessment procedure is used to forecast genotype performance relative to specific site environmental conditions (e.g., Grossnickle and Fan 1998, Fan and Grossnickle 1998).

Specific examples of FBC programs

The following are two specific examples of how the FBC has integrated various scientific disciplines to produce information or propagation systems that are used to improve forest regeneration programs for planted forests. The examples include the relationship between nuclear DNA markers and physiological parameters for Sitka × interior spruce populations, and somatic embryogenesis in interior spruce.

Introduction to the relationship between nuclear DNA markers and physiological parameters for Sitka × interior spruce populations

Special problems exist for reforestation of introgression zones. One specific area is the Nass Skeena transition, where there is a substantial and increasing demand for successful reforestation. In the Nass Skeena transition, a large introgression zone occurs between Sitka spruce (*Picea sitchensis* [Bong.] Carr.) and interior spruce (*Picea glauca* [Moench] Voss × *Picea engelmannii* Parry ex. Engelm.) (Little 1953; Daubenmire 1968; Roche 1969). Sitka spruce occurs naturally in wet, maritime climates, whereas interior spruce occurs across continental areas which experience summer droughts and severe winters. As a result, there are risks associated with using seed from the Sitka × interior transition zone with unknown genetic characterization. Because the exact extent of this zone is not clear and information on the genotypes represented within it is insufficient, the use of seed orchard seed to reforest areas distant from original parent tree locations has the potential to place progeny off site. The result can be poor survival and/or growth of planted seedlings.

Approach

Research based on chloroplast DNA, to estimate species components of hybrid seedlots, indicates a good agreement between growth patterns and morphological development of nursery grown seedlings (Sutton et al. 1991a). In fact, DNA probes have been developed to analyze species contribution to the mitochondrial and nuclear genomes of hybrids (Sutton et al. 1991b, 1994). Nonetheless, no information has been collected on how DNA analysis relates to seasonal changes in phenology, morphology, and physiology of field-planted Sitka × interior genotypes. By combining ecophysiological and genetic characterization, a better species deployment strategy could be developed for the Sitka × interior spruce transition zone.

Sitka spruce and interior spruce were sampled from a zone of Sitka/interior spruce introgression in British Columbia, Canada. Restriction fragment length polymorphisms of the nuclear ribosomal RNA genes (rDNA) defined species-specific patterns for Sitka and interior spruce populations (Figure 2). Hybridization was estimated from an index based on the relative abundance of polymorphic rDNA bands for each population. The same Sitka and interior spruce populations were assessed for the physiological parameters of drought tolerance, freezing tolerance, and gas exchange (Grossnickle et al. 1996).

Results

Sitka × interior spruce seed sources had an interior spruce (Si) rDNA index ranging from 0.04 (Lower Nass) from the most westerly collected seed

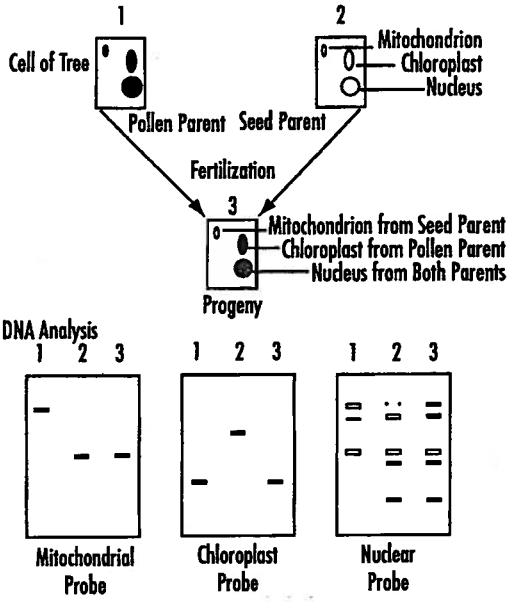

Figure 2. Spruce hybrid analysis using chloroplast, mitochondrial, and nuclear DNA probes. NOTE: Plant cells contain subcellular organelles (chloroplasts and mitochondria) that carry out metabolic functions and also contain genetic material (DNA). Research using probes for each of these organelles in spruce has shown that species-specific patterns can be found for both chloroplasts and mitochondria. By looking at the progeny of crosses between two species it was found that chloroplasts are inherited from the pollen parent and mitochondria are inherited from the seed parent (Sutton et al. 1991b). The nuclear DNA, consisting of two sets of chromosomes, is inherited equally from both parents (one set of chromosomes from each parent). Thus, each organelle represents a maternal or paternal lineage of the ancestors of a tree, whereas the nucleus can display the tree's overall genetic makeup. In the example above, the DNA patterns represent what is seen using the species-specific probes where tree 1 is Sitka spruce and tree 2 is interior spruce. The intermediate pattern displayed by the progeny with the nuclear probe can be quantified to generate and index of hybrid fraction range from 0 (Sitka spruce) to 1 (interior spruce). In this simple case, where the two species have been crossed, the index for the progeny (tree 3) is 0.5.

source to 0.97 from the Bulkely Valley low-elevation, easterly source. Spruce seedling populations exhibited similar Si rDNA index values when compared to mature trees previously surveyed from the same approximate locations (Sutton et al. 1994).

Sitka × interior hybrid seedlings had typical seasonal patterns of shoot water relation parameters (i.e., osmotic potential at saturation, and turgor loss point, relative water content at turgor loss point, and total turgor) (Grossnickle et al. 1996). During shoot elongation in the spring, drought tolerance decreased, whereas in the summer and throughout the fall, after budset, drought tolerance increased in a manner that is very typical of spruce species

(Grossnickle 1989). During all times of the year, there were linear relationships between the Si rDNA index and shoot water relations parameters; as Si rDNA index increased, drought tolerance, represented by osmotic potential at turgor loss point (Ψ_{tlp}) just after summer budset, increased in a predictable manner (Figure 3A).

Gas exchange patterns measured just after summer budset, under optimum conditions, indicated that Sitka, compared to interior spruce populations, has higher gas exchange capability, represented by net photosynthesis, P_n (Figure 3B; Fan et al. 1997). When seedlings were soil droughted (predawn shoot water potential = −1.5 MPa), P_n rates declined overall but interior spruce populations maintained higher P_n rates relative to coastal populations. Thus, under optimum conditions, populations with a greater Sitka spruce component had greater P_n capability. On the other hand, during drought conditions, populations with a greater interior spruce component had greater P_n capability, which was related to their greater drought tolerance (shown in Figure 3A).

During the fall, Sitka × interior hybrid seedlings had a seasonal increase in freezing tolerance that was dependent upon species hybridization (Grossnickle et al. 1996). Seedlings with a higher Si rDNA index had greater freezing tolerance, represented by the lower lethal temperatures that caused 50 percent needle electrolyte leakage (LT_{50}) throughout the fall (Figure 3C, measurements taken on November 10th). Populations with a greater interior spruce component had greater development of freezing tolerance during fall acclimation.

Conclusions

The following conclusions can be drawn from this program. First, DNA analysis is a useful tool for accurate identification of natural Sitka and interior spruce hybrids in the Nass Skeena Transition Zone. Second, seed source Si rDNA index was directly related to the degree of drought tolerance throughout most of the year, to freezing tolerance during fall acclimation, and to gas exchange patterns during the summer season. Third, incorporation of genetic marker procedures, when correlated with ecophysiological patterns, should improve the seed transfer guidelines of Sitka and interior spruce populations for forest regeneration programs.

Introduction to somatic embryogenesis in interior spruce

Somatic embryogenesis is a tissue culture method for asexual propagation. The term "somatic" refers to the fact that embryos develop asexually from vegetative (somatic) tissue rather than as a result of fertilization. This propagation method allows for the multiplication of superior families, as identified

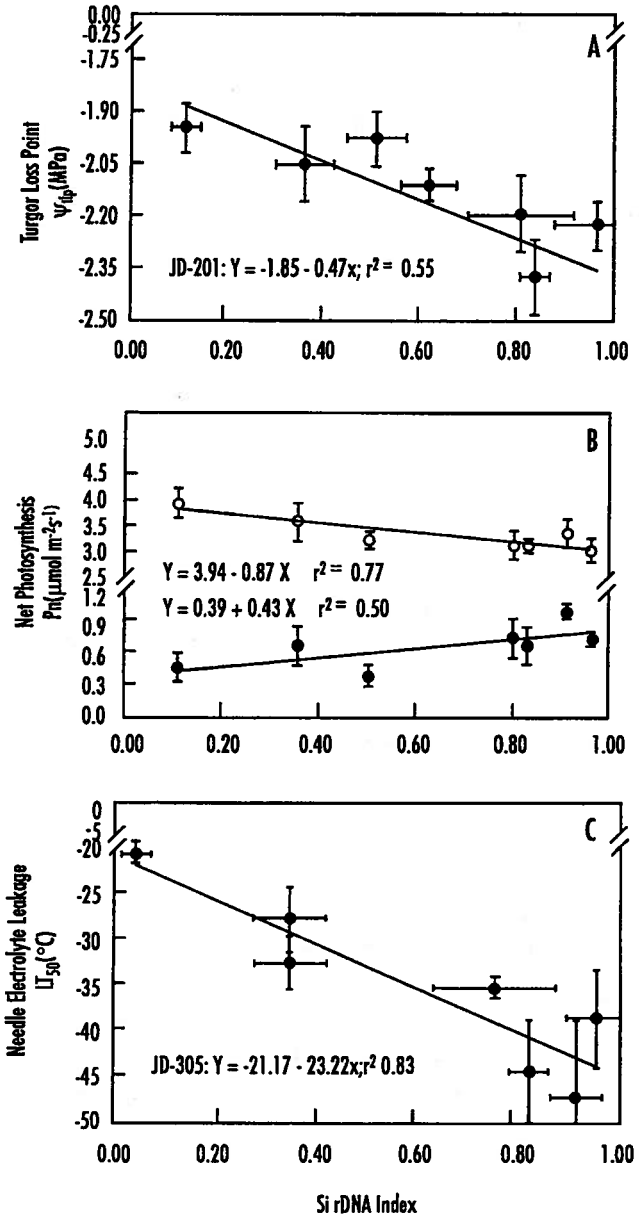

Figure 3. Relationship between Sitka × interior spruce seed sources in relation to their Si rDNA index (mean ± SE) and: (A) drought tolerance, osmotic potential at turgor loss point, Ψ_{tlp} (mean ± SE), of seedlings just after summer budset (Julian Day 201); (B) net photosynthesis, P_n, under both optimum (open symbol) and drought (filled symbol) soil moisture conditions of seedlings just after summer budset; and (C) freezing temperature resulting in 50 percent needle electrolyte leakage, LT_{50} (mean ± SE), measured on November 10th (Julian Day 305).

in tree improvement programs, and the selection of elite clones to capture a greater portion of the gain from additive, dominance, and epistatic variation (Libby and Rauter 1984; Mullin and Park 1992). In addition, somatic embryogenesis is a propagation technique that allows for cryopreservation of propagated clones, thus allowing for maintenance of culture lines while testing is ongoing for selection of elite clones to be used within a deployment strategy.

Value-added traits that could be captured and propagated through somatic embryogenesis include yield, wood quality, and pest and disease resistance. Particular opportunities also exist where seed supply or germination is inherently poor. The somatic embryogenesis propagation system is also ideal for genetic engineering of improved clonal material with value-added traits.

Propagation technique

Somatic embryogenesis is a successfully implemented tissue culture method for the asexual propagation of interior spruce (*Picea glauca* × *Picea engelmannii*). Somatic embryos are derived from excised seed embryos which are placed on the proper medium to produce a culture composed of many proembryos (i.e., early stage somatic embryos), similar in appearance to zygotic embryos soon after fertilization (Hakman and von Arnold 1985; Webb et al. 1989). Each culture can produce essentially an unlimited number of proembryos, each proembryo being a clone of the original explant. In order to produce plants, cultures are placed on a different medium where proembryos stop proliferating and proceed through more advanced stages of embryogenesis, this resulting in the formation of cotyledonary embryos similar to a mature seed (Roberts et al. 1990a; Flinn et al. 1991). Somatic embryos are germinated in enclosed containers to produce somatic seedlings which resemble young seedlings (Roberts et al. 1990b; Cyr et al. 1991). Somatic seedlings are transferred to styrofoam blocks, acclimatized to *ex vitro* conditions, and placed in the nursery (Webster et al. 1990).

Protocols for cryopreservation of interior spruce suspension cultures have been developed (Kartha et al. 1988), and interior spruce cultures from a range of genotypes are currently being preserved for long-term storage. In addition, applying somatic embryogenesis to clonal propagation requires genetic stability. An examination with isozyme patterns and culture morphology of the genetic stability of these embryos has revealed no evidence of somaclonal variation (Eastman et al. 1991).

Operational testing

Somatic seedlings have been tested during all phases of an operational forest regeneration program. Somatic seedlings from nine clones representing three families were germinated *in vitro* concomitantly with the germination of

genetically related seed in the nursery. Somatic seedlings were grown alongside control seedlings in an operational nursery and overwintered in frozen storage. Somatic and regular seedlings were tested with a comprehensive stock quality assessment approach just prior to planting, and spring planted on a reforestation site in the interior of British Columbia (52°28′ N, 122°41′ W) with field performance monitored over a 2-year period. Results from the operational testing are briefly described below with details of the program reported elsewhere (Grossnickle and Major 1994a, 1994b; Grossnickle et al. 1994).

Somatic, compared to regular, seedlings have slower growth during the initial phase of growth in the nursery. Somatic seedlings initially require higher humidity and lower light levels during acclimation in the nursery. Thereafter, height and root growth are similar between somatic and regular seedlings throughout the growing season. Recent nursery performance of somatic seedlings have shown that a proper nursery cultural environment during the initial establishment stage results in normal morphological development of seedlings (Grossnickle et al. 1996b). During fall acclimatization, somatic and regular seedlings have similar dormancy, freezing tolerance, and root growth patterns, and meet all standards for the successful lifting and storage of interior spruce.

Just prior to planting, somatic and regular seedlings were assessed for stock quality. Somatic seedlings met all testing standards for interior spruce seedlings used in operational regeneration programs in British Columbia. In addition, both stock types were tested for physiological performance and morphological development under environmental conditions that simulated potential reforestation site conditions. Somatic and regular seedlings performed similarly in simulated low temperature and drought conditions. This indicated that somatic seedlings have good field performance potential.

On a reforestation site, somatic and regular seedlings had comparable patterns of summer seasonal water relations and gas exchange responses, and were comparable in response to damaging winter conditions. Somatic and regular seedlings had a similar rate of incremental height and diameter growth across two growing seasons. Regular seedlings had larger shoot systems and root development at the end of the first growing season, and this reflects their larger shoot and root systems at the time of planting (Figure 4). At the end of the second growing season, somatic and regular seedlings had comparable root development. Although regular seedlings had larger shoots than somatic seedlings at the end of the second growing season, they had a similar shoot-to-root balance. Similar morphological balance was reflected in their comparable gas exchange and water relations patterns. At the end of the second growing season, survival was 83 percent for somatic seedlings

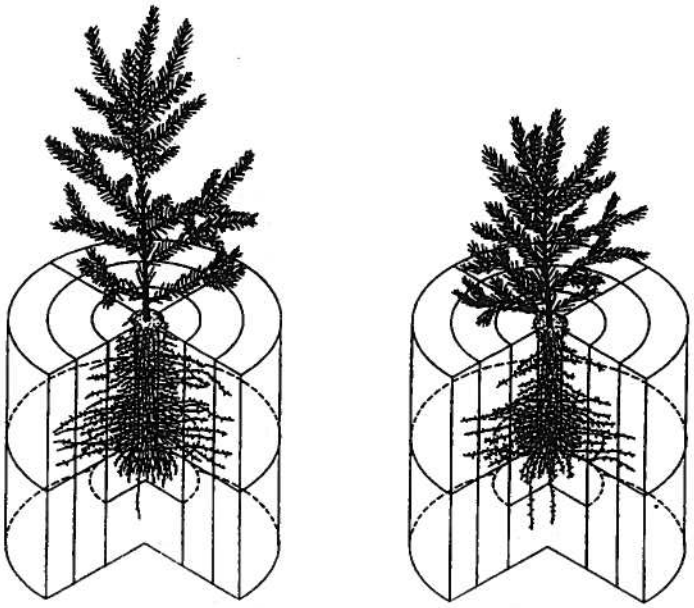

Figure 4. Diagrammatic representation of morphological development after one growing season on a reforestation site for regular and somatic interior spruce seedlings.

and 77 percent for regular seedlings. Long-term (i.e. seven years) field trials indicate that somatic seedlings are capable of sustaining good shoot development when grown under plantation conditions (Grossnickle 1998). Somatic seedlings had all of the traits desired of stock used in successful forest regeneration programs.

Program development

The FBC has successfully implemented a somatic embryogenesis tissue culture production system with the following elements:

- Establishment of a diverse array of embryogenic culture lines (1,400 to date) from superior seed families.
- Application of a reliable long-term capability to store cultures in liquid nitrogen.
- Rapid bulk up of embryogenic cultures followed by bioreactor-based bulk embryo production.
- Drying of embryos in a manner suitable for subsequent storage or germination.
- Capacity for mass germination.

- Semiautomatic planting of germinants for growing in the nursery.
- Development of nursery cultural protocols to produce quality somatic seedlings.
- Development of early clonal selection capability.

To date approximately 100,000 somatic seedlings have been produced for trials. Commercial production has begun through an associated company, Silvagen, Inc., which delivered over 300,000 interior spruce somatic seedlings for commercial nursery production in 1997 and 1998. The major emphasis of this commercial program is the delivery of genetic material carrying insect resistance and increased growth rate.

Initiatives are underway in the following areas to improve the somatic seedling program. First, scaling-up of production capability to 1,000,000 over the next 3 years. Second, further establishing a diverse array of embryogenic culture lines from superior seed families. These lines are now undergoing field performance trials that will select elite lines for deployment in reforestation programs. Third, improving the cultural protocols for nursery production of somatic seedlings from a wide array of clonal lines. Fourth, developing early selection capability to identify superior families and lines. This early selection capability will be used to develop profiles of lines that will be deployed in reforestation programs.

Conclusion

The FBC is an interdisciplinary research group that develops and applies advanced technologies to enhance forest regeneration programs. The FBC has developed major research groups in the areas of tissue culture, molecular genetics, pathology and microbial inoculants, and ecophysiological assessment. These research groups work together to apply various disciplines toward a common goal of developing high-quality seedlings. By producing high-quality seedlings, improved seedling growth on reforestation sites will increase the productivity of planted forests.

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