Tissue Culture of Conifer Seedlings—20 Years On: Viewed Through the Lens of Seedling Quality

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Abstract: Operational vegetative propagation systems provide a means of bringing new genetic material into forestry programs through the capture of a greater proportion of the genetic gain inherent within a selected tree species. Vegetative propagation systems also provide a method for multiplying superior varieties and/or families identified in tree improvement programs. Twenty years ago, a program at the Forest Biotechnology Centre, BC Research Incorporated (Vancouver, British Columbia, Canada), was initiated to apply somatic embryogenesis technology to conifer species with the intent of creating a commercially viable vegetative propagation system that could produce large numbers of somatic seedlings (then called emblings).

As this program was being initiated at the Forest Biotechnology Centre in the early 1990s, there was a perception that seedlings produced through somatic embryogenesis technology might have attributes unsuitable for large scale reforestation programs. To overcome this skepticism, a comprehensive seedling quality assessment program was designed to assess the performance of somatic seedlings. As the somatic embryogenesis technology for conifer seedlings improved, the application of quality practices to the production of somatic seedlings evolved into an approach that is comparable to the International Organization for Standardization (ISO) quality assurance programs now being applied across many industries. The following is a brief history of the evolution of this seedling quality approach (from the author's perspective) applied to conifer seedlings produced with the somatic embryogenesis technology.

Keywords: zygotic embryos, somatic embryos, seedling quality

Somatic Embryogenesis Tissue Culture Process

Somatic embryogenesis tissue culture technology is the most recent vegetative propagation system to be implemented on an operational scale. In this tissue culture approach, proliferative embryo suspensor masses are established from non-meristematic cells and subsequently cultured to produce organized somatic embryos possessing a shoot and root meristem. The term somatic refers to embryos developing asexually from vegetative (or somatic) tissue. Somatic embryogenesis technology was developed for conifer tree species in the late 1980s, originally on spruce species (*Picea* spp.) (Hakmann and von Arnold 1988; Webb and others 1989). Since then, somatic embryogenesis of tree species has expanded to encompass both conifer and hardwood species. Detailed examples of the application of somatic embryogenesis in woody plants can be found in Jain and others (1999).

Laboratory Steps

In general, the somatic embryogenesis process is divided into several laboratory steps that are performed under sterile conditions to prevent microbial contamination.

Culture Initiation—Mature zygotic embryos are dissected from seeds and placed onto semi-solid medium containing plant growth regulators.

Proliferation—Maintenance of 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 is followed by a multiplication step in which the tissue multiplies and develops as early-stage somatic embryos. Embryogenic cultures can be proliferated in a juvenile form for long periods of time to produce unlimited numbers of propagules from the same variety. Tissue can then be allowed to continue to grow, or it can be placed into long-term storage.

Cryopreservation—Cryopreservation is a means whereby germplasm can be stored. The embryogenic tissue is treated with cryoprotectants, frozen to -35 °C (-31 °F) under controlled freezing rate, and subsequently stored in liquid nitrogen (-196 °C [-321 °F]). Cryopreserved tissue can be stored indefinitely, and regenerated within a few weeks after a simple thawing process. This long-term storage option offers a distinct advantage of somatic embryogenesis tissue culture over rooted cuttings and organogenesis tissue culture.

Maturation—Maturation advances the development of somatic embryos by exposing tissue to phyto-hormones and controlled environmental conditions. Within a period of a few months, they are transformed into mature somatic embryos that are analogous to zygotic embryos.

***In vitro* Germination**—The final lab step, *in vitro* germination, takes place when embryos are placed on germination medium under controlled environmental conditions. *In vitro* germination occurs within a week and proceeds to the development of true needles. Somatic germinants can then be transferred to *ex vitro* nursery conditions.

Nursery Production

After somatic germinants are transferred to the nursery, they can be treated with cultural practices that are comparable to rooted cuttings (Dole and Gibson 2006). Once somatic germinants become established as young somatic seedlings, they are grown under standard seedling practices used by the forest seedling nursery industry.

Seedling Quality and Somatic Seedling Stocktypes

Beginning in the early 1990s, it was important to show the forest industry that somatic embryogenesis technology could produce high-quality somatic seedlings in the nursery that would become established and grow rapidly after outplanting. As with any new technology, forest practitioners were skeptical that this tissue culture technology would provide seedlings with the desired benefits reported by the biotechnology industry. New seedling quality approaches were therefore, developed to provide foresters with enough information to understand how tissue culture technology could produce quality seedlings for outplanting. A program was initiated at the Forest Biotechnology Centre to develop a comprehensive seedling quality assessment procedure that “Measured the Product” to show the forest industry that seedlings produced from tissue culture practices would be a positive improvement for their reforestation programs.

Measuring the Product

Seedling quality assessment is based on the need for a better understanding of performance capabilities of nursery seedlings for outplanting on reforestation sites. Wakeley (1954) is usually recognized as the first person to identify the importance of morphological and physiological grading of seedlings prior to outplanting. As this concept began to take hold in the forest industry, seedling quality was defined as “fitness for purpose” (Lavender and others 1980) as it relates to achieving specific silvicultural objectives. Throughout the 1980s and 1990s, seedling quality assessment evolved to include both morphological and physiological tests (for example, Sutton 1979; Ritchie 1984; Duryea 1985a; Glerum 1988; Lavender 1988; Johnson and Cline 1991; Puttonen 1997). These seedling quality assessment procedures encompass both nursery development (“Monitor the Process”), and testing immediately before outplanting to determine probable field survival and/or field performance (“Measure the Product”).

Conceptual Approach—Seedling performance on a reforestation site depends on the inherent growth potential and the degree to which environmental conditions of the field site allow growth potential to be expressed (Grossnickle 2000). The degree to which a seedling can adapt to site conditions immediately after outplanting influences its growth on the reforestation site (Burdett 1983). To determine the field performance potential, seedlings should be assessed in relation to anticipated environmental conditions at the site (Sutton 1982, 1988; Duryea 1985b; Grossnickle and others 1988, 1991a; Puttonen 1989; Hawkins and Binder 1990). Determination of seedling quality combines measurements of seedling properties that have been defined as material (that is, measure of a seedling subsystem) and performance (that is, subjecting whole seedlings to test conditions) attributes (Ritchie 1984). An array of morphological and physiological tests that examine factors important for determining field performance potential is required because seedling quality reflects the expression of a multitude of physiological and morphological attributes. Results from testing programs could be integrated to develop a means of expressing the overall physiological and morphological quality of seedlings (Grossnickle and others 1991b). An array of tests that simulate anticipated field environmental conditions would help forecast seedling physiological performance and potential for survival and growth on a reforestation site (Grossnickle and Folk 1993; Folk and Grossnickle 1997).

The Stocktype—An assessment of seedling performance was conducted in the early years of somatic embryogenesis technology for interior spruce (*Picea glauca* (Moench) Voss X *Picea engelmannii* Parry ex. Engelm.). This work indicated that somatic seedlings were smaller than zygotic seedlings, and this was related to the timing of integrating the tissue culture germinants into the nursery (Grossnickle and others 1994). The seedling quality testing program found that zygotic seedlings (because of their overall larger size) had better performance potential if they were to be outplanted on reforestation sites having optimum environmental conditions (Figure 1). On the other hand, zygotic and somatic seedlings had comparable performance potential if they were to be outplanted on sites under limiting cold (Figure 1) or drought conditions (Grossnickle and Major 1994a).

A subsequent field trial found that somatic seedlings could become successfully established on boreal reforestation sites (Grossnickle and Major 1994b), and had comparable physiological performance to zygotic seedlings in relation to reforestation site environmental conditions (that is, in both summer and winter). Both of these stocktypes had comparable growth over two field seasons, although the larger initial size of zygotic seedlings was still evident after 2 years. This trial work showed that somatic seedlings had comparable performance to zygotic seedlings at the nursery, in a seedling quality testing program, and in the field. It was felt that as long as the issue surrounding the timing of nursery planting could be rectified, this technology had the capability to produce high quality somatic seedlings that met the required standards of an operationally acceptable seedling.

During the mid 1990s, somatic embryogenesis technology improved and the Forest Biotechnology Centre developed the capability to produce somatic seedling crops of 350,000 seedlings (Grossnickle and others 1996). Trials conducted on somatic seedlings by other research groups during this timeframe found no major differences in either physiological

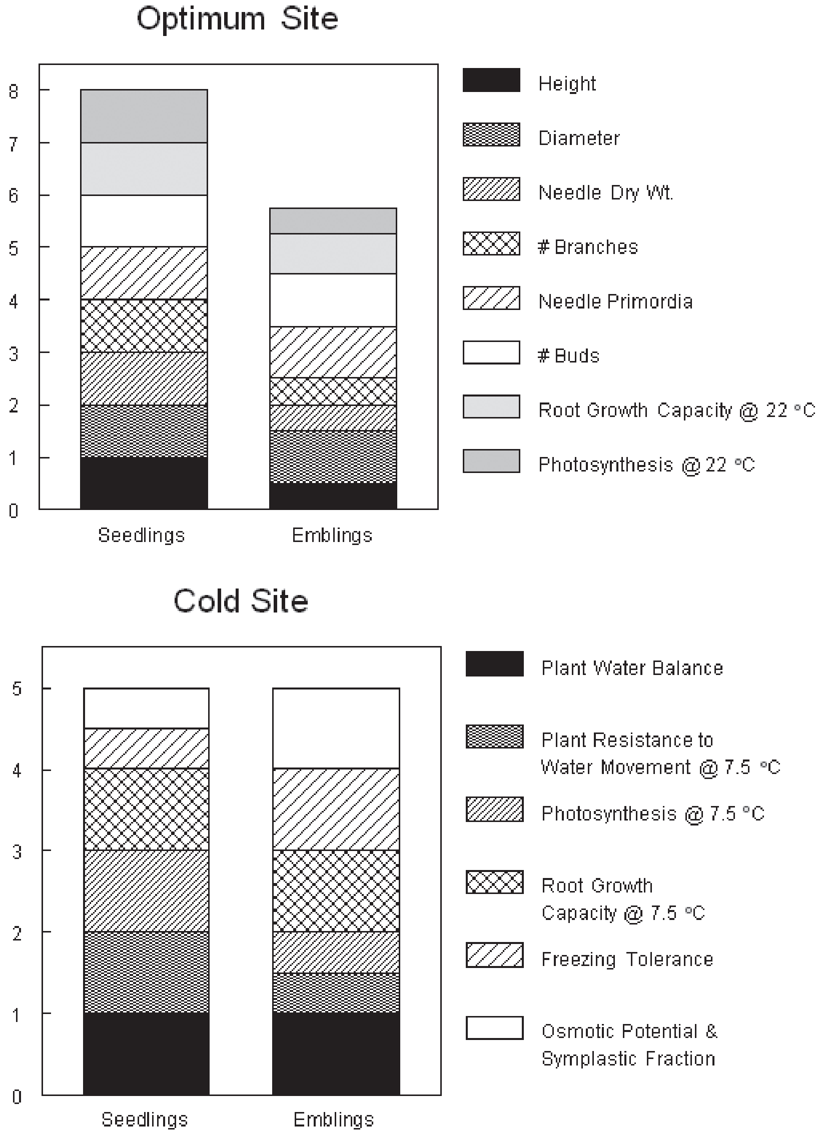

Figure 1. Performance potential index of interior spruce seedlings and emblings under optimum and cold reforestation site environmental conditions (from Grossnickle and Major 1994a).

or morphological attributes of spruce stocktypes produced through somatic embryogenesis technology compared to zygotic seedlings (Nsangou and Greenwood 1998; Lamhamedi and others 2000). Further testing of somatic spruce seedlings from the Forest Biotechnology Centre found that these seedlings met all of the criteria for an operationally acceptable seedling in British Columbia (Figure 2). This step was critical for the acceptance of somatic seedlings into plantation forestry programs requiring comparable performance to zygotic seedlings of similar genetic quality (Menzies and Aimers-Halliday 2004).

Varietal Performance—Testing field performance potential at the varietal level is required to determine varietal differences in somatic crops. This propagation procedure may lend itself to the selection of varieties with desired growth and stress resistance traits (Lamhamedi and others 2000). As with stocktype testing, this type of seedling quality testing was conducted with reference to environmental conditions of the outplanting site to get a more representative understanding of field performance capability (Grossnickle and Folk 1993; Folk and Grossnickle 1997).

A seedling quality program was conducted to determine the field performance potential for an array of varieties that made up an interior spruce somatic seedlot (Grossnickle and Folk 2007). The somatic seedlot was composed of 34 varieties from 12 full-sib families (one to six varieties per family). This somatic seedlot was the first of its kind to be registered for deployment in the Prince George seed zone in British Columbia during spring 1999 (seedlot number V4023), and to meet vegetative deployment guidelines regulated by the British Columbia Ministry of Forests. The somatic seedlot tested in this seedling quality assessment program met the operational criteria for a viable interior spruce seedlot that could be outplanted in reforestation programs in British Columbia (Grossnickle and Folk 2005). Field performance potential testing indicated that the 34 varieties comprising the somatic seedlot had a wide range of performance for measured parameters under optimum, nutrient-poor, cold, or drought conditions that simulated reforestation site conditions (Grossnickle and Folk 2007). Examples of the range of varietal performance are shown in Figure 3 in the shoot-growth potential under spring environmental conditions, as well as the varietal response of new shoots to a spring frost. This breadth of varietal performance is valuable to forest managers involved with varietal deployment and reforestation planning for two reasons: 1) it allows for the development of somatic seedlots with attributes for potential site conditions, with the possibility of field testing to verify findings from seedling quality tests; and 2) it ensures that somatic seedlots can be developed with a wide enough genetic base to minimize the vulnerability of plantations to environmental stress. This somatic seedlot approach for varietal deployment would allow for resilience to environmental stress and yet still confer benefits of clonal forestry.

Measuring the Process

During the past decade, significant progress has been made towards developing reliable, high-volume, cost-effective somatic embryogenesis production systems that can produce millions of seedlings. CellFor has begun to commercialize the production of loblolly pine (*Pinus taeda*) somatic seedlings (Grossnickle and Pait 2007). This has led to somatic seedling propagation technology being successfully integrated into both bareroot and container seedling production systems (Figure 4). This integration into standard nursery production systems has resulted in somatic seedlings consistently meeting required morphological standards for

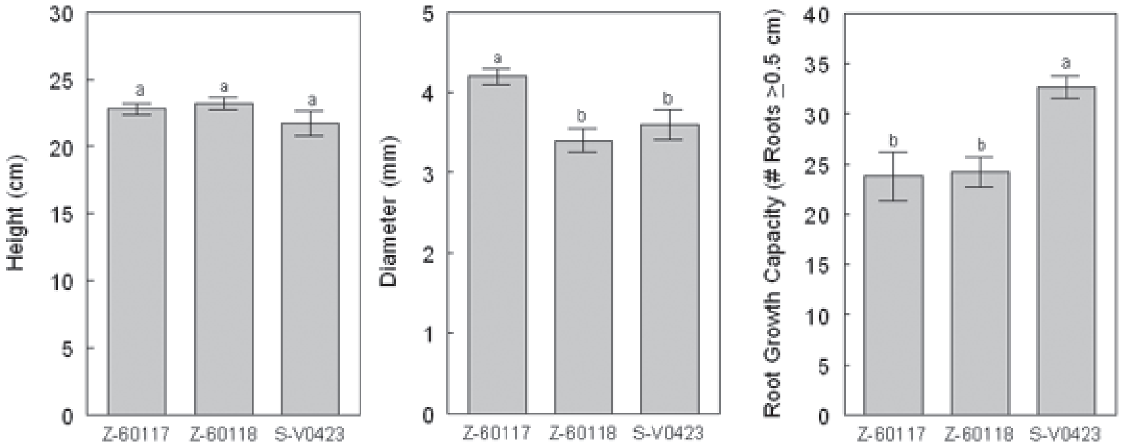

Figure 2. Height, diameter, and root growth capacity of somatic (S) and zygotic (Z) seedlots of interior white spruce (1 cm = 10 mm = 0.4 in). Columns topped with a different letter indicate significantly different seedlots; errors bars indicate ± 1 standard error (Grossnickle and Folk 2005).

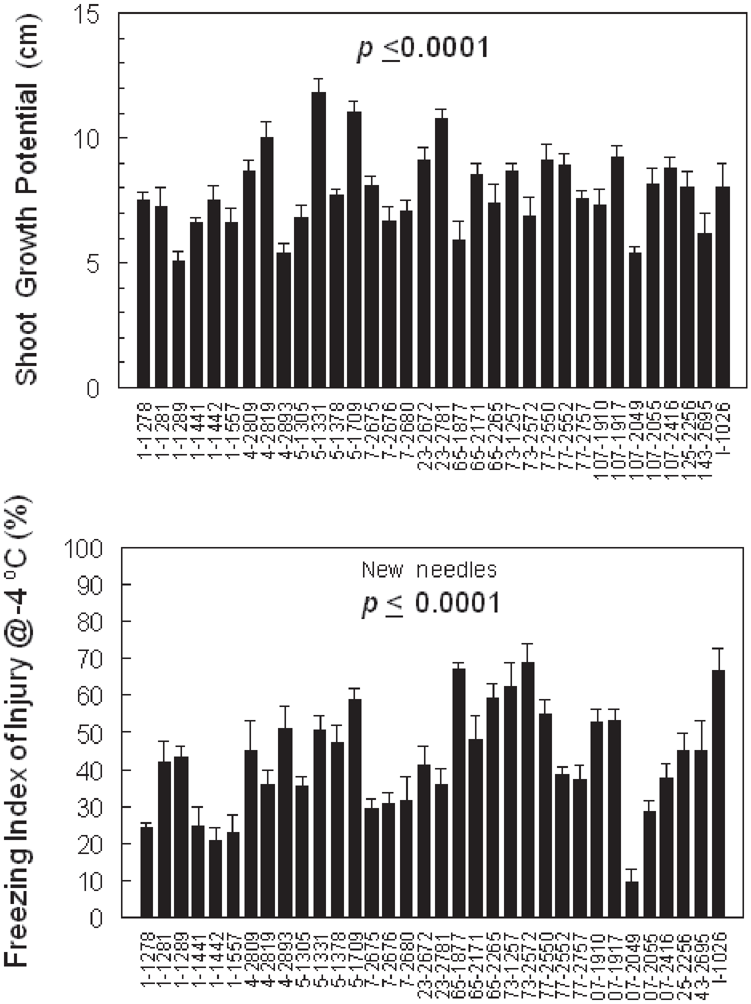

Figure 3. Varietal performance of a somatic seedlot (seedlot number V4023) to low soil temperatures in spring (that is, 10 °C [50 °F]) and frost events (new shoots exposed to -4 °C [25 °F]) that can typically occur on boreal reforestation sites (Grossnickle 2000). Measurements shown are shoot growth capacity (1 cm = 0.4 in) and freezing tolerance, with *P*-value for the performance difference between varieties (from Grossnickle and Folk 2007).

seedling production. In addition, reforestation site trials have tested the field performance of loblolly pine somatic seedlings and found these seedlings to have all of the traits that are desired in seedlings for use in forest regeneration programs (Figure 5).

Application of Plant Quality Control in Industry

Commercial implementation of a novel technology, such as somatic embryogenesis, requires the ability to develop and implement a successful operational nursery production program. In addition, the quality of somatic seedlings produced during the nursery program needs to be monitored to ensure that they meet the required standards for a shippable seedling.

Understand Species Performance Capabilities

Each tree species has its own unique pattern of physiological response to environmental conditions. It must be recognized that environmental conditions change daily, seasonally, and yearly. Each species shows a specific physiological response pattern to these changing atmospheric (light, humidity, temperature) and edaphic (temperature, water, fertility) conditions throughout the year. Their physiological performance in response to the environment ultimately determines subsequent performance under nursery conditions. To develop an effective seedling monitoring program, the nursery needs to understand how species respond to cultural conditions. If growers understand the physiological response of a species to environmental conditions, they can create cultural

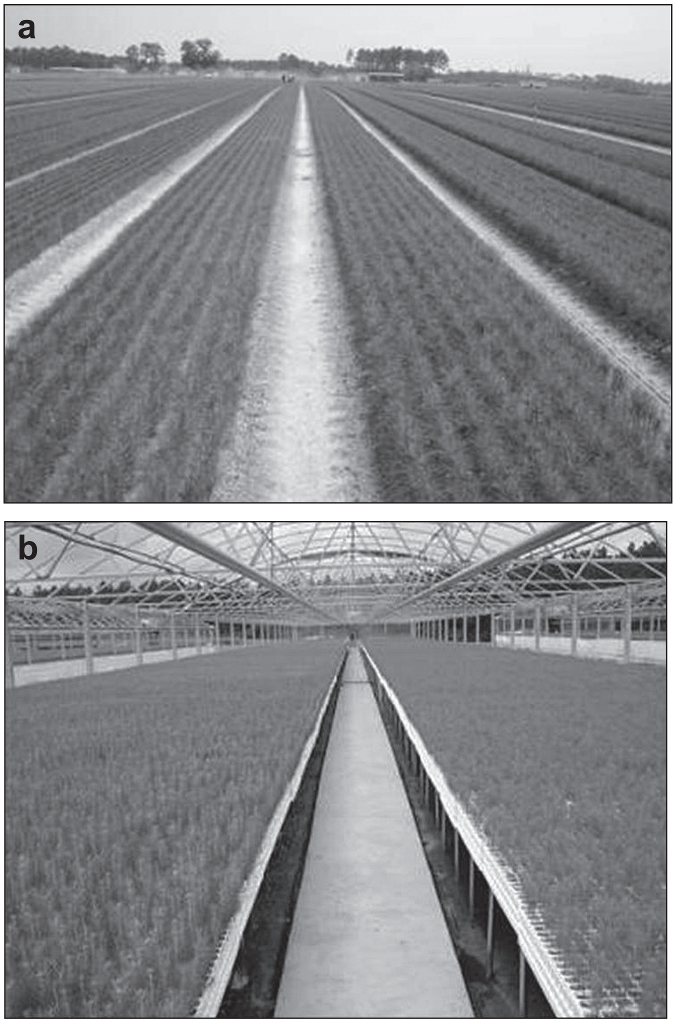

Figure 4. Loblolly pine somatic seedling nursery production of CellFor Incorporated: a) bareroot seedlings (Plum Creek Nursery, Jesup, GA; b) container seedlings (International Forest Company, Moultrie, GA).

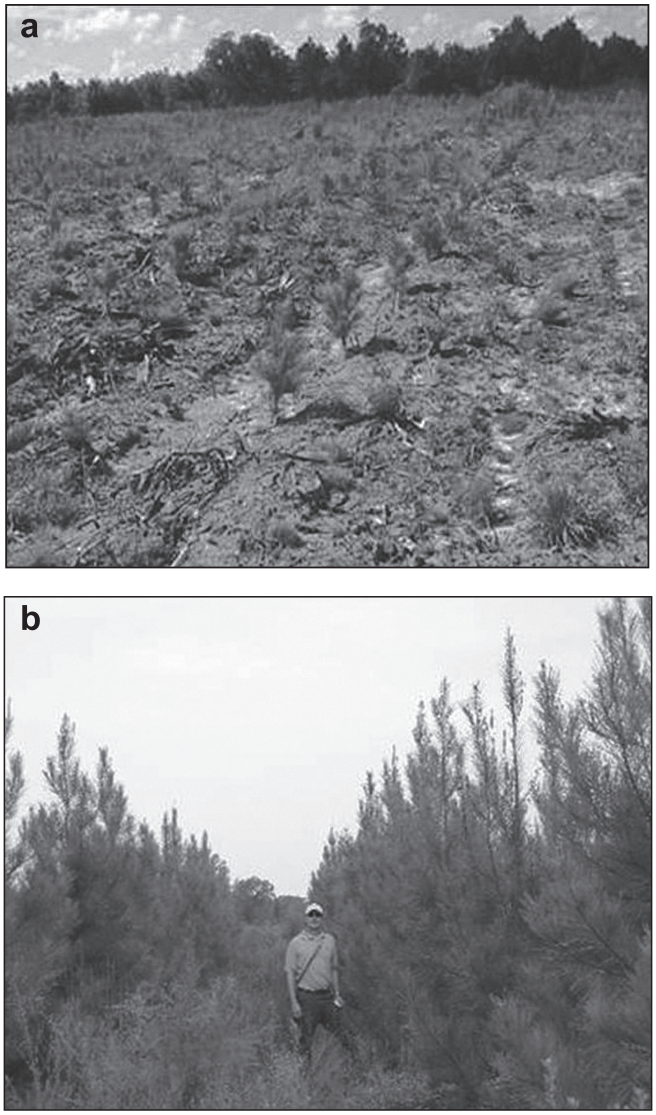

Figure 5. Field performance of CellFor Incorporated bareroot loblolly pine seedlings growing on a reforestation site: a) 7 months following outplanting; b) 21 months following outplanting.

guidelines, or standard operating procedures, that will be the cultural plan of how to grow a quality seedling crop.

Define the Process

In growing a quality seedling crop, each step of the growing process must be defined and cultural guidelines developed. The use of these guidelines to define the cultural process in detail ensures that the growing of a quality crop can be repeated with each production season. This approach fits into the International Organization for Standardization (ISO) Quality Assurance program that is built on the principle of a controlled and consistent approach for the production of a product to ensure the effective operation of a program (Anonymous 2002). Within a nursery program, this means defining cultural practices related to planting, growing, integrated pest management, consolidation, hardening, storage, handling, and shipping in an easy-to-use format that can be quickly read and applied by growers and all other critical nursery personnel.

Monitor the Process

Once the cultural plan has been developed, it is important to track the crop to ensure the agreed-upon cultural guidelines are followed and a quality crop is grown. Monitoring the cultural process is necessary to ensure the crop is growing according to the crop plan. The ISO Quality Assurance program requires monitoring the production process to ensure achievement of the planned results (Anonymous 2002). The process for monitoring the production of seedling crops falls into three major areas of activity.

Tracking the Crop Environment—Both optimum and limiting environmental conditions for crop performance need to be defined, and methods need to be developed to track the environment in real time. The capability to synthesize environmental information also has to be present so seasonal changes can be easily tracked and any deviations from the recommended environmental conditions defined in the cultural guidelines.

Tracking the Plant Performance—Important points in the development process of the crop must be defined. It is also important to select critical morphological and physiological parameters that give the grower a good understanding of the crop performance. Tools must be built that allow one to easily follow the plant performance throughout the entire crop cycle. An end-of-crop assessment needs to be conducted so shippable seedlings consistently meet morphological standards required for production of operational bareroot or container seedlings (that is, a simplified, one-time event of "Measuring the Product").

Crop Diary—A crop diary is required that defines operational and cultural adjustments to the crop plan. As with any plan to grow seedlings, the combination of seasonal environmental conditions, equipment capabilities and breakdowns, plus the "human factor" can result in deviations from the crop plan. These deviations need to be recorded so that when a crop review is conducted, one can understand where adjustments to cultural practices need to be refined to improve performance in future crop production cycles.

Read and Respond to Signs

Information on crop performance will not help in producing a quality crop unless there is a system in place to respond when the crop begins to deviate from the crop plan. To avoid deviation that can result in crop losses, the quality monitoring program needs to follow a number of simple steps: 1) weekly benchmarks for data collection must be established; 2) data must be tracked continuously and synthesized rapidly in an easy to read format; and 3) there needs to be a system for transferring incident reports into operational action plans.

Learn from The Past

One must continue to learn from the past to ensure that the quality of seedlings being produced improves with each production cycle. The quality system needs to track crop cycles and define the good, bad, and ugly patterns of crop performance. This information then needs to be synthesized from across crops and seasons to define poor crop performance patterns that need to be eliminated. In addition, there needs to be an integration of beneficial cultural practices into the cultural guidelines. In this way, the quality assurance program becomes a positive system of change and continued improvement in the cultural practices used to produce seedlings.

Conclusions

In the early years of seedling quality programs designed to measure the performance of seedlings produced from somatic embryogenesis programs, the focus was on measuring the final product of the crop production cycle. This approach was important when validating a "Proof of Concept" for this new seedling product that needed to gain acceptance within the forest industry. Seedling quality systems have evolved as high-volume, cost-effective somatic embryogenesis production systems and have grown to the point of producing millions of somatic seedlings. Within a large scale production setting, a seedling quality program must focus on three central themes: 1) understand the species performance capabilities; 2) define the process to successfully grow plant material; and 3) monitor the process to ensure that quality somatic seedlings are being produced within every production cycle. This approach is applicable to the production of all commercial seedling crops, and it ensures that a nursery produces the best quality crop that meets the objective of quality seedlings for forest regeneration programs.

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