



# Somatic embryogenesis for conifer seedling production – The biology of scaling

Plamen Denchev<sup>1</sup>, Steven C Grossnickle<sup>2</sup>

<sup>1</sup>Tissue Culture Solutions, Canada

<sup>2</sup>NurseryToForest Solutions, Canada

✉ [p.denchev@hotmail.com](mailto:p.denchev@hotmail.com)

## Abstract

Conifer somatic embryogenesis has long been recognized as an advanced vegetative propagation technology. Somatic embryogenesis provides a platform for capturing and long-term preservation of elite genotypes and developing commercial scale-up systems for mass production of plants. Although, significant success has been reported in improving conifer somatic embryogenic protocols, little has been presented to describe the complexity of integrating *in vitro* (laboratory) and *ex-vitro* (greenhouse) programs for developing a commercial production system capable of delivering tens of millions of conifer somatic seedlings. This integration requires both programs to run in concert and produce propagules capable of surviving and growing under greenhouse conditions at very early stages. It also requires the integration of seedling development events to ensure the production of quality seedlings that meet needs of the forest restoration program.

This paper describes the importance of protocol optimization for scaling-up the *in vitro* and *ex vitro* programs. The 'biology of scaling' is discussed in view of plant cell, embryo, germinant and somatic seedling requirements throughout the program. Logistical issues related to protocol optimization and scale-up are addressed. Specific control points for monitoring and controlling the commercial process are presented. The importance of developing standard operating procedures, media batch records, and quality control systems are discussed. Ultimately, a fully integrated system capable of producing tens of millions of conifer somatic seedlings is presented.

## Keywords

Conifer somatic embryogenesis; *in vitro* (laboratory) program; *ex-vitro* (greenhouse) program; Total program integration; Biology of scaling

## Contents

1	Introduction	110
2	Laboratory <i>in vitro</i> scale-up operations	112
2.1	Initiations and culture establishment of somatic embryos	112
2.2	Cryo-preservation and cryo-bank creation	113
2.3	Culture maintenance of conifer somatic embryos	114
2.4	Culture bulk-up of somatic embryos	117
2.5	Pre-maturation of conifer somatic embryos	119
2.6	Maturation of somatic embryos	119
2.6.1.	Commercial maturation logistical issues	120
2.7	Scale-up of media production	121

## ARTICLE INFO

### Citation:

Denchev P, Grossnickle CS (2019) Somatic embryogenesis for conifer seedling production. *Reforesta* 7:109-137.

DOI: <http://dx.doi.org/10.21750/REFOR.7.08.70>

Editor: Vladan Ivetić, Serbia

Received: 2019-04-14

Accepted: 2019-06-02

Published: 2019-06-28



Copyright: © 2019 Denchev Plamen, Grossnickle Steven. This work is licensed under a [Creative Commons Attribution 4.0 International Public License](https://creativecommons.org/licenses/by/4.0/).



2.8	<i>In vitro</i> Germination of somatic embryos	122
3	Nursery scale-up operations	124
3.1	Miniplug seedling production	124
3.1.1.	Germinant quality	124
3.1.2.	Miniaturizing the container system	126
3.1.3.	Germinant nursery establishment environment	128
3.1.3.1.	Photothermal grows models	129
3.1.3.2.	Hardening and holding	131
3.2	Finished seedling production	131
3.3	Seedling quality and nursery/field performance	133
4	Conclusion	134
5	Acknowledgement	134
6	References	134

## 1 Introduction

Somatic embryogenesis is the preferred *in vitro* process for the large-scale commercial production of conifer seedlings. The major advantage of somatic embryogenesis is the long-term preservation of elite genetic resources and the ability for scale-up of each program step. However, a major challenge for commercializing this technology has been the higher cost per plant produced compared to the average zygotic seedling cost (Lelu-Walter et al. 2013; Bonga 2015). In general terms, the *in vitro* process is considered expensive, takes a long time and is limited by the number of genotypes that can be produced within a production year.

Major challenges in commercializing this technology include:

- a) Low and inconsistent initiation and multiplication rates delivering variable quantities and quality of somatic embryos,
- b) Losses throughout the program associated with cryopreservation and variable quality of the material removed from cryopreservation,
- c) Low number and poor quality of mature somatic embryos due to insufficient protocol optimization,
- d) Losses due to contamination and poor laboratory practices,
- e) Low conversion rates in nursery due to poor germinant quality attributable to the absence of *in vitro* germinant selection criteria,
- f) Disconnect between the lab *in vitro* program phase and the *ex vitro* nursery program phase in planning, execution and managing somatic seedlings production,
- g) Loss of seedlings due to inability of the *in vitro* system to meet the nursery production window resulting in the planting of under spec germinants,
- h) Requirement of expensive high-quality greenhouse facilities to maintain precise environmental conditions during the rooting/establishment phase,
- i) Improper timing of lab and nursery program steps leading to finished seedlings not meeting specifications of a quality seedling at time of shipping.

Although, significant improvements have been achieved in commercializing spruce (*Picea* spp.) (Egertsdotter 2018) somatic embryogenesis programs, no such success has been reported for pines (*Pinus* spp.), where the programs have been characterized as providing low and inconsistent yields at all somatic embryogenesis stages. One reason is that most research has been focused on a particular program step. There has been lack of understanding that somatic embryogenesis is a complex

multistep program where all steps are interconnected, and they influence each other as well as the final product. Figure 1 represents steps of a commercial somatic embryogenesis tissue culture program for large scale conifer seedlings production.

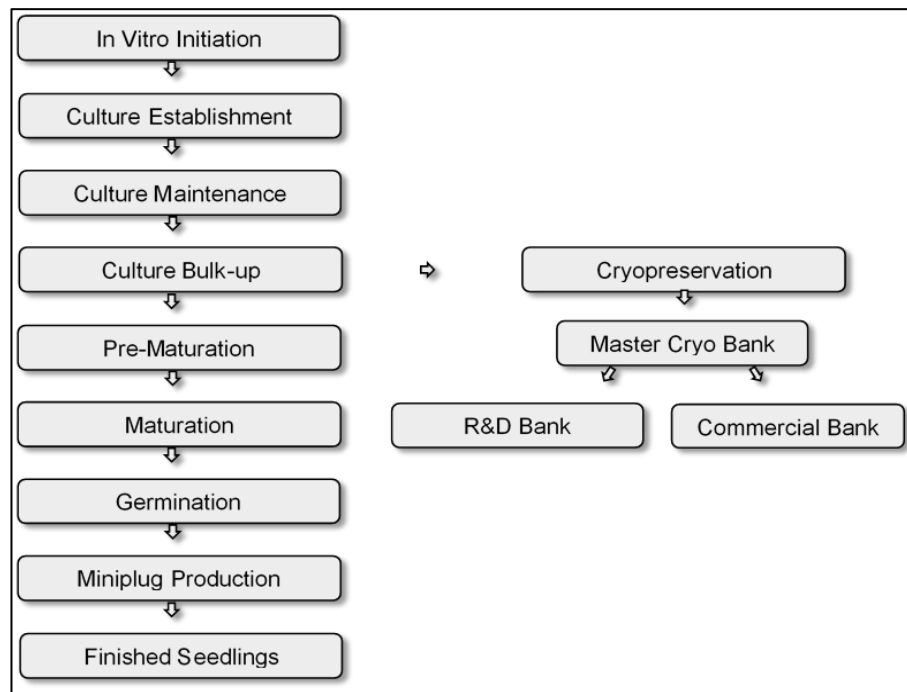


Figure 1. A commercial program for conifer seedlings production utilizing somatic embryogenesis tissue culture procedures.

Each program step consists of a biological component, environmental component, and a technical component. The biological component is associated with plant tissue, embryo, germinant, and seedling requirements for growth and development. The environmental component represents conditions plant materials are grown under (e.g., temperature, light, water, nutrients, relative humidity, atmospheric gases). The technical component represents the culture vessel/container type the tissue, embryos, germinants, and seedlings are grown in, sophisticated equipment and facilities, and the people's expertise and ability to establish linkages between each step, thereby handle and process the plant material across all program steps.

The idea behind scaling-up of a biological process is to enhance the outcome from each step without compromising product quality. Several key questions must be answered before investing in scale-up activities:

- a) Does the *in vitro* protocol provide necessary conditions for genetic material to fully express its potential?
- b) Can the *in vitro* protocol provide a common approach across all genotypes for all technicalities involved in the scale-up process (e.g., changes in container type, automation, and storage requirements)?
- c) Is there a nursery program in place to address all environmental requirements for germinants produced *in vitro* to survive and further develop when entering the *ex vitro* environment at very early stages?

d) Does the nursery program have the capability to take young established tissue culture produced seedlings through to a finished seedling that meet quality standards required for successful field performance on a forest restoration site?

Material chosen to be produced *in vitro* normally consists of different genotypes. Each genotype will perform differently under the same protocol. Often superior performance in the field does not compare with a similar performance *in vitro*. In addition, *in vitro* performance does not always match *ex vitro* development of various genotypes. Therefore, protocol optimization must precede scale-up activities to maximize the *in vitro* and initial *ex vitro* performance for each genotype. This paper addresses both - process optimization and scale-up requirements that have to be met in order to create a commercial system for conifer somatic seedling production.

Developing a scale-up process cannot be successfully achieved without addressing the biology of scaling. Simply, increasing the initiation amount of plant material per program step without adjusting other parameters will not increase the yield at the end of each biological stage and often, causes negative results. Understanding plant cell, embryo, germinant and seedling requirements at every step of the *in vitro* and *ex vitro* process is the key for achieving success. Generally speaking, plants require two things - ideal edaphic and atmospheric environmental conditions to survive and grow (Larcher 1995; Pallardy 2008). Whether one is addressing the plant material in the *in vitro* or *ex vitro* program phase, the creation of optimum environmental conditions is critical for plant cell, embryo, germinant and seedling development during the scale-up of any tissue culture program.

This paper examines issues that need to be considered during the scale-up of a somatic embryogenesis tissue culture program. During the time we were at the company called CellFor Inc. a somatic embryogenesis tissue culture program for loblolly pine (*Pinus taeda* L.) was scaled into the 10s of millions of seedlings. During this program development the 'biology of scaling' issue became a reoccurring theme as one tried to create a successful program. This paper presents some of the critical program steps that need to be considered if one is going to create a successful and cost efficient process for the large-scale commercial production of somatic seedlings from a somatic embryogenesis tissue culture program.

## 2 Laboratory *in vitro* scale-up operations

### 2.1 Initiation and culture establishment of somatic embryos

Initiation of conifer somatic embryogenesis *in vitro* is a program step that has been well described in the scientific literature. Immature zygotic embryos are usually used to start cultures. (Becwar et al. 1990; Gupta and Grob 1995; Becwar et al. 1995; Li and Huang 1996). Cones are staged to determine the level of embryo maturity and the induction process is initiated when embryos are at the optimal stage of development. As described by Pullman and Johnson (2002) initiation rates, reported in the literature are low and are limited in some genotypes. The inability to capture a wide array of valuable genotypes is considered a major reason preventing the commercialization of conifer somatic embryogenesis. A way to overcome this challenge is to introduce a battery of media for induction and genotype capturing in addition to improving *in vitro* environmental conditions. This allows the capture of a larger genetic pool and further

introduces genotypes into field testing programs that eventually contribute towards a higher genetic gain in clonal forestry.

## 2.2 Cryo-preservation and cryo-bank creation

Once genotypes have been initiated and established, they can be cryo-preserved and stored in liquid nitrogen. This provides for a long-term storage and field testing of newly induced material for evaluation. As results from field trials are available, the best genotypes can be selected for protocol optimization and future production.

A master bank is created after each induction event. This bank represents the history of induction events and is the core of germplasm collection of every conifer biotechnology company. Out of this bank two additional cryo-banks are created (i.e. research and development (R&D) and commercial). Developing a R&D cryo-bank is important for screening the productivity of newly induced genotypes and optimizing *in vitro* cultural protocols. The size of this bank can vary, but it should reflect the needs for testing and developing protocols. A commercial bank is created once newly induced genotypes are evaluated, protocols are optimized, and field test results are assessed. This bank contains material that is strictly related to production and the bank size should reflect genotype productivity by stage, and future production targets. When commercial production is initiated, the amount of cryo-preserved embryogenic tissue for selected genotypes becomes a critical program decision. Increasing the cryo-bank size and creating a commercial cryo-bank requires a cryopreservation scale-up process. Thus, it will impact the initial amount of tissue entering into the cryo process, the size of cryo vials, the amount of tissue dispensed in each vial, as well as cryo tank size and the cryo facility. A robust cryopreservation process as well as corresponding technical staff must be in place to handle these increased program requirements.

In order to keep the process consistent, certain programs should be developed along with cryo-banks creation. The first program is targeting genotype screening and genotype productivity. The reason for these programs is two-fold. First, is to screen newly induced genotypes for productivity. Second, is to screen genotypes in master banks that were created in previous induction years. As research protocols are constantly evolving and become more efficient to satisfy genotype requirements, it is important to screen genotypes that were cryopreserved from previous induction events. Many initially low-productive genotypes can meet production requirements when updated R&D protocols are used for screening.

Often, variability among vials is observed (Figure 2a). Therefore, a cryo-vial improvement program is required. This program identifies the most productive vials by running the embryogenic tissue through updated R&D protocols. Thus, an improved master bank can be created (Figure 2b) by re-cryoing embryogenic tissue from these 'improved' genotypes and they are used for developing a commercial bank (Figure 2c). Therefore, genotype screening and cryo-vial improvement programs become a critical tool for increasing the genotype portfolio size of the company.

An additional program is required after creation of the commercial bank to deal with bank integrity and bank certification. A randomly selected, statistically meaningful, number of vials from the commercial cryo-bank are grown following commercial protocols. Periodically, throughout the year, the tissue is transitioned from multiplication growth to standard maturation, germination and miniplug production in order to observe performance with increased generations. These tests confirm stability

characteristics over time with respect to embryo yield, germination and conversion. This program also defines productivity windows for safe and stable production from the bank and confirms relative vial-to-vial uniformity of the bank.



Figure 2. Commercial bank consistency as a result of a Cryo-Vial Improvement program. Productivity of the cryo vials in original banks (a); Improved master banks created from the most productive vials (circled in red) of the original cryo banks (b); Productivity of the cryo vials in generated commercial banks (c). ME -mature somatic embryos; FP – filter paper (Denchev unpublished data).

### 2.3 Culture maintenance of conifer somatic embryos

Although, this stage has not received enough attention in the scientific literature (Pullman and Johnson 2002), maintenance and bulking up of embryogenic tissue on solid medium is the very first step in increasing the embryogenic tissue amount

for the production program. Commercial production of conifers requires a constant supply of embryogenic tissue throughout the cycle. The quantity and consistency in quality of the embryogenic tissue influences every program step following solid bulk-up (SBU) up until seedling production. When scaling-up to produce millions of seedlings, optimizing the quality and maximizing the amount of embryogenic tissue on solid medium becomes very important. Producing embryos to ultimately grow millions of seedlings requires multiple maturation events throughout the production cycle. Therefore, the productivity window for the production genotypes during SBU must be strictly determined and closely maintained.

Different media formulations (Litvay et al. 1985; Teasdale et al. 1986) have been used and further modified (Klimaszewska et al. 2000; Pullman et al. 2014) to assure growth and multiplication of conifer embryogenic cultures. However, every conifer genotype possesses a different growth pattern and produces different quality of tissue during the SBU process. The tissue represents a mix of embryogenic, non-embryogenic tissue and single cells. Embryogenic tissue consists of embryos at different early developmental stages, some of having abnormal growth. Growth rates of non-embryogenic tissue often exceeds that of embryogenic tissue. Further multiplication of this mix increases tissue heterogeneity and decreases the number of somatic embryos produced throughout the next program steps.

When scaling-up the entire protocol, it is essential to increase the amount of “productive” tissue in the SBU stage to meet requirements for the following program steps. Therefore, the SBU protocol needs to be optimized accordingly. A procedure for physically selecting the embryogenic tissue (e.g. micro-selection) should be developed as a standard operating procedure (SOP) for SBU. The procedure requires microscopic examination of every culture and selecting embryogenic tissue consisting of stage 1 and stage 2 embryos (Figure 3a). Selected tissue is then transferred to fresh maintenance medium and is normally maintained as clumps (Figure 3b, c).

Each clump is about 3mm in diameter and about 3mm high at the start of the SBU stage. The clump size as well as SBU stage duration have a major effect on culture quality and productivity.

Maintaining tissue in larger clumps leads to low growth and less embryogenic tissue. Often, this results in development of necrotic tissue areas, tissue browning and tissue death.

In order to standardize consistency for all SBU cultures, a template should be developed for the clump size and clump arrangement within a plate (Figure 3d).

A total amount of embryogenic tissue per plate, the number of clumps per plate, and a transferring time per cycle should be determined and constantly maintained for all production genotypes.

The effect of micro-selection should be verified by comparing the yield of plantable germinants produced when micro-selected and non-micro-selected embryogenic tissue is run through the *in vitro* cultural program (Figure 4). As can be seen from this example all genotypes benefited from micro-selection and resulted in producing a significantly higher number of plantable germinants.

An additional resource for helping to meet scale-up requirements for the SBU process is further improvement of solid multiplication rates and tissue quality by optimizing SBU medium. This can be achieved by adjusting concentrations of plant growth regulators (PGR), and by additions of a mix of lactose and sucrose to SBU medium as carbon and energy source for cultures (Denchev et al. 2011).

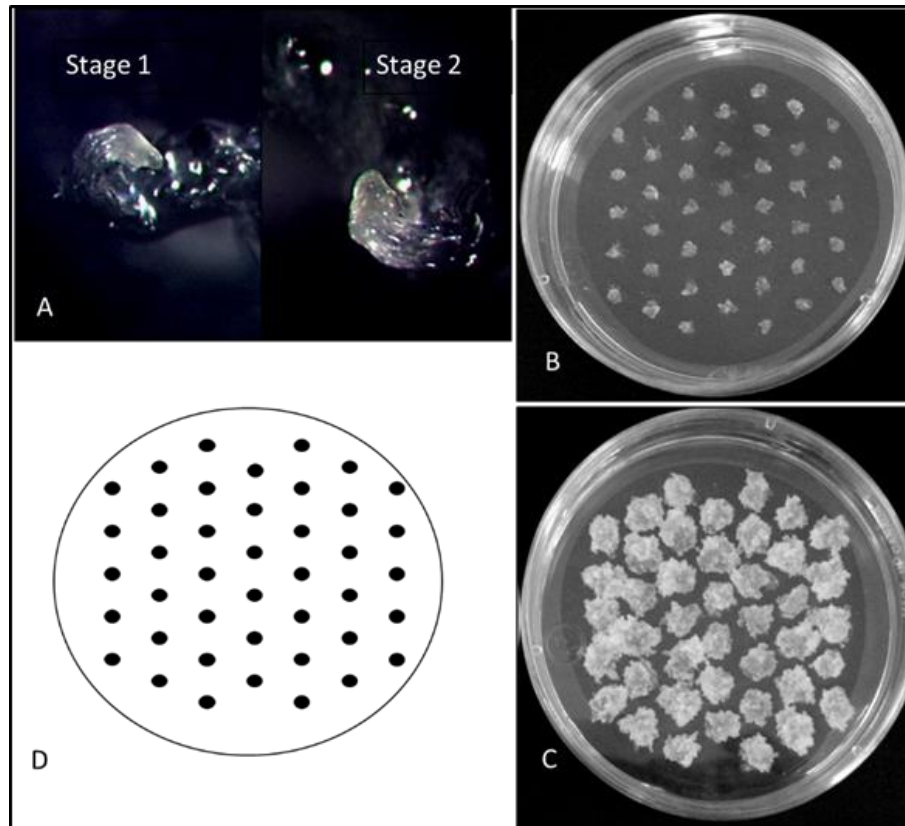


Figure 3. Micro-selection of loblolly pine embryogenic tissue (Denchev unpublished data). (A) Stage 1 and stage 2 somatic embryos. (B) Embryonic tissue arranged as micro-clumps at the start of SBU stage. (C) Micro-clumps appearance at the end of SBU stage. (D) Micro-clump layout for placement of embryogenic tissue on a petri plate.

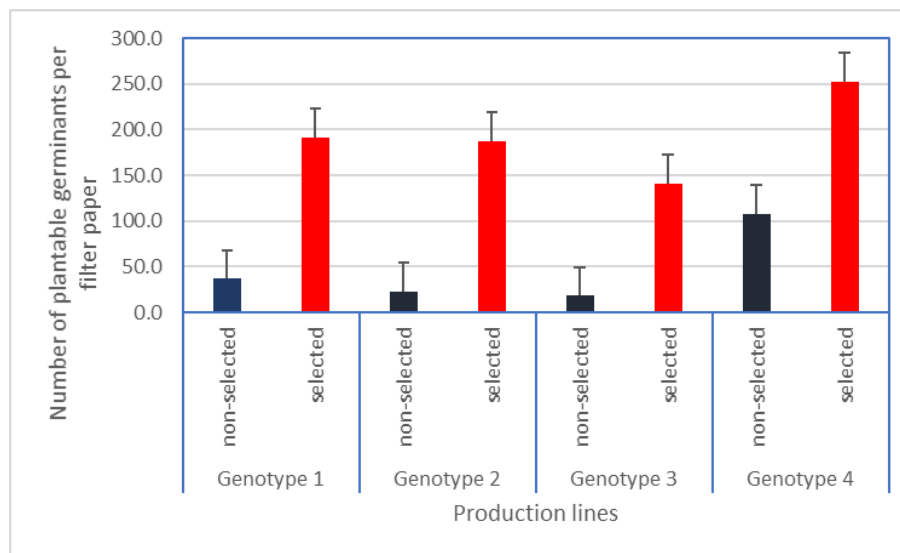


Figure 4. Effect of micro-selection of plantable germinant yields for production genotypes (Denchev unpublished data).

In our experience, enriching the tissue growing environment with additional oxygen and CO<sub>2</sub> led to a significant improvement in embryo uniformity and supported healthy tissue growth and consistency per SBU cycle for up to 3 months.



## 2.4 Culture bulk-up of somatic embryos

Producing high quality embryogenic tissue that generates a substantial amount of high-quality somatic embryos and plantable germinants is a major requirement for every production program. Tissue can be multiplied on solid medium when the volume is not an issue. However, when the tissue amount is required for meeting production targets of millions of plants, multiplication must be performed in a liquid environment. This scale-up step can be achieved in flasks. Liquid medium provides better nutrient access and allows for stricter control over media osmolarity, dissolved oxygen and head space gases. Thus, liquid multiplication is recognized to deliver better quality embryogenic tissue and higher tissue yield at the end of the multiplication stage. Growing cultures in flasks, containing liquid medium is done in 7- or 14-day cycles. Agitation is required to maintain tissue in a suspension state and to ensure a constant supply of dissolved oxygen.

When scaling-up liquid multiplication it is important to understand that certain media volume supports growth of a fixed maximum amount of inoculum. The inoculum amount is unique for every genotype and the starting density for individual genotypes can vary and must be determined. In order to keep consistency in cultures' growth and multiplication rates, the inoculum amount at the start of each multiplication cycle must be strictly controlled.

Increasing flask size requires a higher amount of inoculum, and therefore, an additional amount of oxygen in the culture vessel head space. Proper flask size and flask closure must be used to allow for a proper gas exchange. Scaling-up liquid multiplication process stage for producing millions of seedlings requires a vessel such as a large fernbach flask, containing one liter of embryogenic suspension. Flasks are incubated in the dark in a controlled gas environment, where oxygen and CO<sub>2</sub> concentrations are slightly elevated and humidity is strictly controlled to prevent evaporation of water from medium, thereby avoiding changes in medium nutrient concentration.

As for every step of the somatic embryogenesis program, media composition plays a key role in tissue multiplication. Multiplication medium contains a combination of an auxin and cytokinin. When scaling-up multiplication one must strictly control the concentrations of these PGR. Overdosing the culture with PGR to achieve a fast growth or higher yield is always detrimental for culture and final product quality.

Success of liquid multiplication scale-up depends on embryogenic tissue uniformity. Generally, conifer embryogenic suspensions cultured in medium containing sucrose consist of a mixture of embryos at early developmental stages and non embryogenic tissue. Studies have shown that using other carbohydrates in addition to or instead of sucrose can improve the morphology of early stage somatic embryos and can increase mature embryo numbers during later program stages (Gupta 1996; Denchev et al. 2011). For example, supplementing multiplication medium with lactose or galactose improves embryo morphology. Lactose alone or in combination with sucrose, promotes differentiation of immature embryos in proliferating cultures. These embryos when subsequently transferred to developmental medium show reduced growth of suspensor tissue, improved embryo quality and improved yields of mature embryos. In addition, this treatment results in higher numbers of genotypes that can be successfully cultured, and significantly increases the capability to produce large numbers of somatic seedlings.

Generating large amounts of embryogenic tissue for producing millions of somatic seedlings requires a number of multiplication cycles. However, each genotype, becomes unstable and productivity declines overtime, even under favorable conditions. Genotype stability reflects on the genotype’s ability to produce embryogenic tissue consistently during several production cycles without compromising embryo quality and decreasing embryo yield. The stability window is determined by monitoring culture growth and viability, and both the embryo yield and conversion to plants during the culture cycling time. The first indication of genotype instability is a sudden increase in growth and reduction of culture viability, followed by reduction in both, embryo yield produced during the maturation stage and subsequent conversion of these embryos into plants. Figure 5 shows an example of differences in stability window between two genotypes. Genotype 63 maintained stable growth and somatic embryo production for 6 LBU cycles, where genotype 68 showed a less stable pattern in growth and embryo production with a decrease in embryo production after cycle 2 and low productivity after cycle 5 where a sudden increase in growth was registered. Therefore, prior to scaling-up the liquid multiplication stage, genotype stability must be determined and strictly maintained.

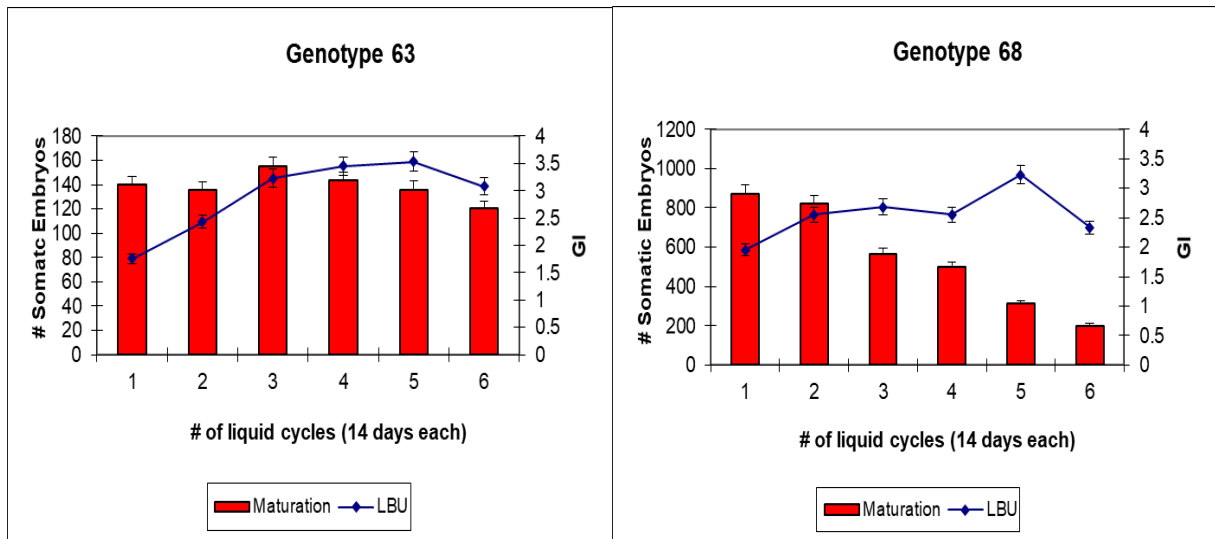


Figure 5. Genotype stability during LBU cycling and its effect on growth and embryo productivity for each LBU cycle and subsequent maturation event (Denchev unpublished data).

It should be noted that under standard nutrient and environmental conditions different genotypes grow differently within the same multiplication cycle. Some genotypes multiply faster than others. These fast-growing genotypes tend to deplete culture medium nutrients sooner than slow growing genotypes. This results in nutrient deficiency towards the end of the cycle, production of an excessive amount of CO<sub>2</sub> and ethylene, causing abnormal embryo development and unexpected changes in the growth pattern during the following multiplication cycles. Controlling growth during the multiplication stage can be done by monitoring culture fresh weight (FW) or dry weight (DW) at the end of the cycle. However, one can also consider using the growth index (GI) as a reliable parameter for monitoring growth. GI is calculated by dividing the final FW by the initial FW. Critical range for GI for individual genotypes must be established

and used as a “go” or “no go” decision for the culture at the end of each multiplication cycle.

Developing proper SOPs and master media batch records (MBR) for the multiplication process is required for program scale-up and commercialization. Organizing genotype requirements and corresponding protocols into production genotype profile documents is a required practice. The genotype profile consists of information related to nutritional requirements, inoculum amounts, vessel types, environmental conditions, stability/operational window, cycling time, and productivity. It is an important tool used for planning and scheduling of production runs.

## 2.5 Pre-maturation of conifer somatic embryos

In general, during early embryo development in or on medium containing sucrose, conifer embryogenic tissue is not uniform and a significant amount of disorganized growth is present. Such cultures do not readily undergo further development when exposed to abscisic acid (ABA). As previously described by Cyr and Klimaszewska (2002) a short 3 to 7 days pretreatment of embryogenic tissue on/in medium without PGR containing activated charcoal, prior to maturation, has improved embryo quality and the ability of embryos to germinate. Including lactose or galactose at the pre-maturation step has the benefit of reducing disorganized growth, and leads to the production of well-organized immature embryos with enlarged embryonal regions (Denchev et al. 2011). When transferred to maturation medium these lactose/galactose embryos have a greater propensity to develop to mature embryo stages than those grown on media containing other sugars, including maltose, while inhibiting disorganized suspensor tissue proliferation. The result is that mature embryos are produced in higher yields and they have greater uniformity. These embryos are of higher quality and lead to greater number of plants produced at the end of the program.

## 2.6 Maturation of somatic embryos

Maturation is a program stage that has been intensively researched with the aim to improve embryo quality and enhance process scalability. Research has examined the effect of available media formulations and developed specific media that support embryo development and maturation for conifers (Coke 1996; Pullman et al. 2003b). Despite differences in media formulations, in all cases, media were designed to contain ABA, and an osmotic agent controlling water potential. The latter was achieved by either increasing sucrose concentrations (Cheliak and Klimaszewska 1991; Klimaszewska et al. 1997; Lelu-Walter et al. 2013), supplementing the medium with manitol (Roberts et al. 1990), adding non-permeating agents like polyethylene glycol (PEG) (Attree et al. 1991; Pullman et al. 2003a; Pullman et al. 2003b), or by reduction of water availability to the tissue by supplementing media with high gellan gum concentrations (Klimaszewska et al. 2000).

Development of a commercial maturation media procedure for conifer production followed results reported by Klimaszewska (2000). Phytigel was chosen as the solidifying agent along with a higher sucrose concentration were important additions to the maturation media. These maturation media modifications contributed to achieving a high quality and large numbers of mature somatic embryos capable of germination and subsequent conversion to fully functional plants.

It was determined that conifer somatic embryos can mature on medium supplemented with S(+)-ABA alone (Kong et al. 2011). Results found a significant increase in maturation, and germination yields for a number of valuable genotypes including genotypes that were previously considered to be less productive. Once the optimum concentration of S(+)-ABA and maturation duration are determined, maturation of different genotypes is achieved without additional transfers to fresh S(+)-ABA medium. These findings are important in light of commercial production of conifers, where the cost per plant is critical. First, replacing racemic ABA with S(+)-ABA reduces the cost for raw materials since S(+)-ABA is considerably less expensive than racemic ABA. Second, this eliminates the need for multiple transfers during maturation, thus, eliminates the need for multiple media batches and new maturation containers. Finally, it eliminates the extra work for operators moving containers in and out of maturation facility, aseptically transferring tissue to fresh media, labeling containers, and recording all activities.

Environmental conditions including temperature and head space gas composition during embryo development and maturation played an important role in process efficiency. Temperatures in the range of 21° to 24°C were found optimal for embryo maturation. In addition, reducing the temperature to 4°C at the end of the maturation process significantly improved embryo quality. It also provided environmental conditions that allowed for the holding of containers of mature embryos for up to several months prior to germination. Additionally, further improvements to the maturation containers provided gas exchange and embryo culturing in fully controlled gas environment where CO<sub>2</sub> concentration was maintained at an optimum level.

### 2.6.1 Commercial maturation logistical issues

A commercial production of clonal forestry involves working with multiple genotypes. However, genotypes differ in their requirements for nutrients, plating density, and maturation duration. Therefore, one needs to develop specific protocols for individual genotypes or groups of genotypes with similar requirements. Efficiencies of these protocols must be evaluated, against the genotypes field value before making a decision which genotypes should be included in the production portfolio.

As mentioned, improvements throughout the entire program are necessary in order to produce high quality germinants and somatic seedlings. The ability of somatic embryos to convert into plantable germinants is one of the most critical parameters for testing the process and assessing maturation improvements. An example is presented in Figure 6 where three different maturation media were tested for their ability to meet nutrient requirements of somatic embryos to mature and convert into plantable germinants. Although, the highest yield of mature somatic embryos was produced on D maturation medium, the highest conversion rate of mature embryos to category #1 germinants was observed on maturation medium A. Therefore, maturation medium A was selected because of its ability to produce a higher number of quality germinants. This example shows the complexity of the decision making when developing a commercial process. Considering both, treatment efficiency and effect on the next program step must be taken into account.

This challenge becomes even greater, when the production target is to annually produce tens of millions of seedlings. Producing seedlings on this large of a scale in one

production run is a difficult task. It requires a year-round production program and the production of the same genotype several times during the production cycle. Year-round production eliminates logistical challenges of once-a-year germination, provides flexibility to nursery operations, allows for accurate delivery projections, provides continuous feedback to the lab program and permits for the smooth introduction of improvements. A careful planning of maturation events requires verification and validation of protocols, process step duration, yield of mature embryos per container, and conversion rate of mature embryos to plantable germinants. All parameters must be incorporated into a production database. Accordingly, an adequate maturation facility must be available that is capable of handling multiple events throughout the year.

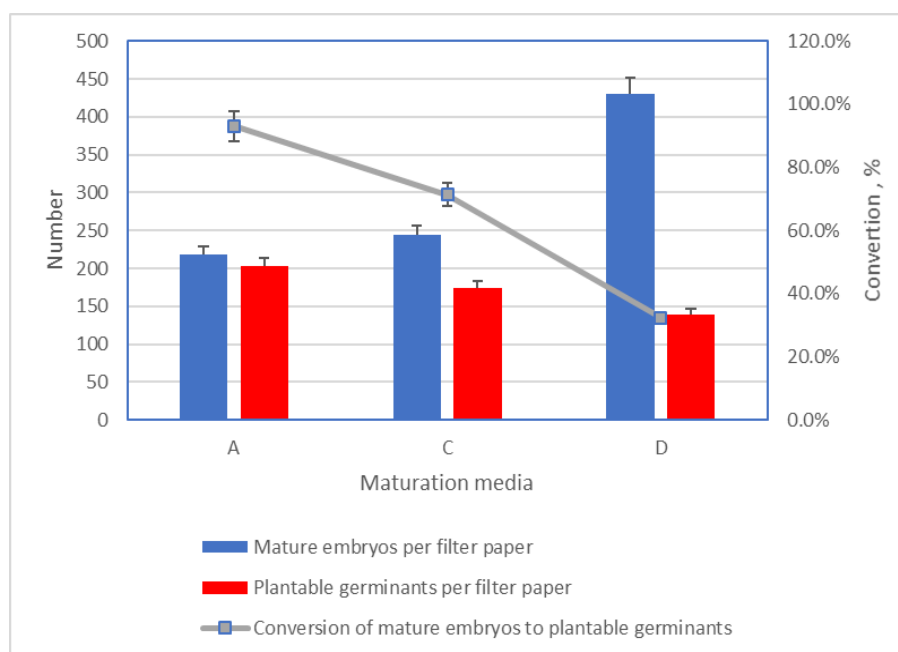


Figure 6. Effect of maturation media on embryo yield and conversion of mature embryos into plantable germinants (Denchev unpublished data).

## 2.7 Scale-up of media production

Media is central to every *in vitro* process. Scaling-up to produce tens of millions of seedlings requires the production of large media volumes. Satisfying this requirement entails operational and logistical challenges in how media is produced, stored and distributed. One must perform a comprehensive analysis of media types and media volumes required for the entire production program. Vendors for all required raw materials must be identified and secured, programs for testing and verifying material quality and batch to batch consistency must be developed, along with constructing a storage facility for all chemicals and materials. In addition, it should be recognized, that media requirements for a multimillion plant crop production program requires strictly defined media formulations in far larger amounts than R&D media needs. Therefore, preparing media for a commercial production requires a separate facility equipped with all the machinery capable for mixing, dispensing and storing large media volumes.

In general, media can be prepared by using pre-made powder/liquid media, available through different commercial companies, or by preparing media in house.

While, ordering media from a third party is often less expensive, due to the large volumes ordered, it limits any further process optimization, as pre-made media mixture cannot be modified. Since the commercial program is under constant improvements for reducing cost and increasing productivity, it is logical to prepare media in house.

Media batches for commercial production have to be large enough to satisfy requirements for every program step. Larger batches reduce errors and variability, normally recorded for smaller multiple batches. Making large media batches requires developing comprehensive, professionally rendered SOP and MBR; creating quality programs for raw materials sampling, testing, labeling and inventory. In addition, quality control tools for testing of raw materials, controlling media hardness, plate out, environmental conditions and microbial contamination are required.

## 2.8 *In vitro* germination of somatic embryos

The final product of the lab portion of the somatic embryogenesis program is to produce plantable germinants that are capable of transitioning into the nursery environment and growing into quality seedlings. After the maturation stage, somatic embryos are germinated under sterile conditions on tissue culture medium. The duration of *in vitro* germination can have a significant impact on both germinant morphology and germinants ability to tolerate *ex vitro* greenhouse environmental conditions. In general, *in vitro* germination can take up to 12 weeks before germinants are fully developed and ready to be sent to nursery (Figure 7). It is largely accepted that greater germinant development at the end of the *in vitro* process results in a higher conversion rate in the greenhouse environment. Delivering fully grown *in vitro* germinants to nursery is a well-established process for many tissue culture companies (Grossnickle et al. 1996; Sutton et al. 2004). However, the duration of *in vitro* germination directly impacts the facility capacity to handle multiple rounds of germination within a production year. As the program scales in size, one major program limitation is having enough facilities with controlled environmental conditions to meet the standard 12 weeks required for full germinant development. The only economical way to address this space issue is to reduce the *in vitro* germination time without compromising germinant quality that affects the nursery conversion rate. The logical approach is to understand biological requirements of both mature embryos and germinants to complete *in vitro* germination in a shorter timeframe. In practical sense this means creating much smaller germinants that are sufficiently physiologically and morphologically developed to survive transition to the nursery environment.

It was hypothesized that there is a point in germinant development where they transition from a heterotrophic to an autotrophic organism. If this transition point can be defined, then theoretically germinants acquiring the autotrophic state can be transitioned into the greenhouse. This can result in minimizing the time required for *in vitro* germination, thereby reducing the need for costly controlled environment germination facilities.

In an attempt to reduce germination time mature somatic embryos were imbibed on germination medium for one week in dark and further exposed to three weeks light under *in vitro* conditions until these germinants reached a minimum morphological size (Figure 7). Studies determined when these germinants became autotrophic organisms. Findings showed that as germinants were exposed to light their photosynthetic activity reached near maximum level by week four in the *in vitro*

germination environment (Figure 8). At this point germinants could be transferred into a greenhouse under strictly controlled environmental conditions for further development. The level of photosynthetic capability was maintained throughout the *in vitro* germination phase and into the greenhouse rooting phase with net photosynthesis continuing to increase during the rooting phase as young germinants began to fully express shoot growth.

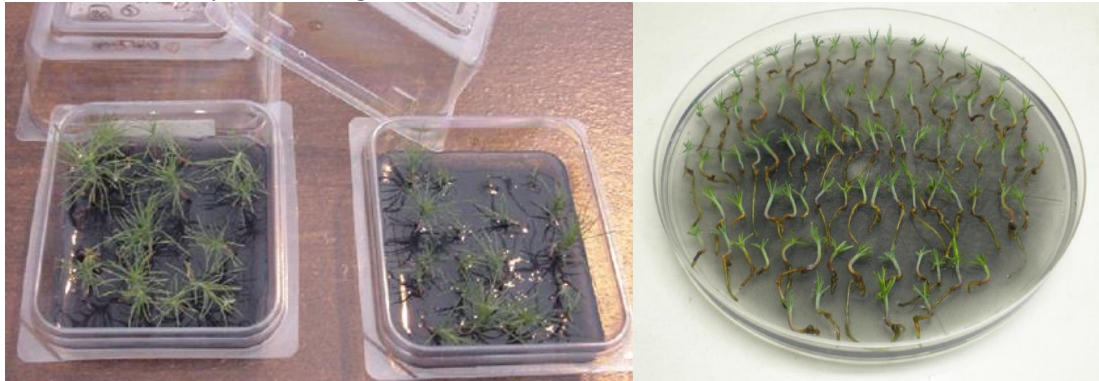


Figure 7. Examples of development for germinants grown in standard phytatrays for twelve weeks (left) and sorted germinants grown for four weeks in Petri dishes (right) in an *in vitro* germination environment that are ready to be transitioned to the greenhouse rooting environment (Denchev and Grossnickle unpublished data).

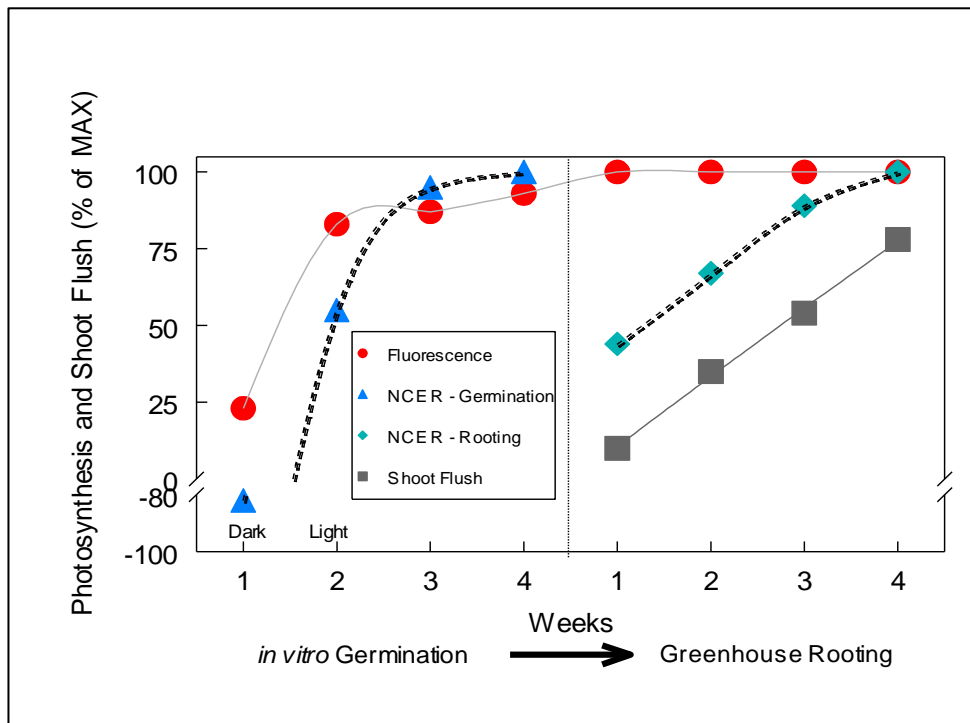


Figure 8. Photosynthetic process (i.e. defined as percent of maximum response for chlorophyll fluorescence [ $F_v/F_m$ ] and the net carbon exchange rate [ $\mu\text{mol CO}_2 \text{ cm}^{-2} \text{ sec}^{-1}$ ]) of germinants during four weeks of *in vitro* germination (i.e. first week in the dark followed by three weeks in the light) and during the rooting phase in the greenhouse. Note: chlorophyll fluorescence across the germination and root periods was compared to the final value at the end of the rooting phase, while the net carbon exchange rate was defined separately for the *in vitro* and rooting phases because photosynthetic rates increased when germinants expressed shoot growth. Germinant establishment in the greenhouse was determined by measuring the expression of shoot flush during the rooting phase (Grossnickle unpublished data).

Thus, germination time was reduced from twelve weeks to four weeks. Further increases in germination efficiency and improvement in germinant quality were achieved by optimization of germination media formulations and germination conditions. A medium for germination of Loblolly pine somatic embryos was optimized (Kong 2011) and the germination process was further enhanced by introducing liquid germination media overlay at the point of transition from imbibition to germination. Adding media overlay throughout germination process contributed to increasing nutrient access and delivering nutrients required for somatic embryos to germinate and achieve the required morphological and physiological development so germinants could be transferred out of the *in vitro* environmental conditions. Further optimization of germination program occurred by converting the germination facility lights from horizontal to a vertical position.

This allowed containers to be stacked on top of each other in columns instead of sitting next to each other across a bench. This increased the facility capacity to handle tens of millions of germinating embryos. There are no published reports documenting this unique *in vitro* germination approach to produce pine germinants at this early stage of germinant development that are capable of becoming established and rooting at a high conversion rate under controlled greenhouse environmental conditions.

### 3 Nursery scale-up operations

Transition from the laboratory program, where germinants are produced *in vitro*, to the *ex vitro* nursery program, where these germinants are turned into seedlings, is a critical step in the success of any operational somatic embryogenesis tissue culture program. If transition is done properly, there is a good chance for program success. To ensure success, both program steps need to understand conditions used in the final steps of *in vitro* germinant development and transition into the *ex vitro* rooting and establishment phases in the greenhouse environment. This understanding by both programs ensures that there is a proper 'hand off' of the plant material as it continues through establishment and growth to a finished seedling that can be delivered to the customer.

As one scales this vegetative propagation system, there are unique issues that occur at seedling development phases that need to be considered. The following discussion describes a series of program steps that were addressed in the nursery process for miniplug and finished seedling production during the scaling to tens of millions of loblolly pine seedlings produced through the somatic embryogenesis tissue program.

#### 3.1 Miniplug seedling production

##### 3.1.1 Germinant quality

Plant quality is a critical factor in the success of any nursery and forest restoration program. There is an extensive body of evidence that planting seedlings with desirable morphological and physiological attributes is beneficial to the success of seedling establishment and growth after outplanting (Grossnickle and McDonald 2018a). Historical (Grossnickle et al. 1996; Park 2002; Sutton et al. 2004) and recent (e.g. Egertsdotter 2018; Tikkinen et al. 2018) work reviewing the commercialization of somatic embryogenesis technology does not discuss whether germinant selection is



critical for the survival and growth of germinants produced via somatic tissue culture propagation procedures after they are planted in the nursery. As one scales this propagation technology, understanding how germinant quality affects the nursery conversion rate and the speed with which young germinants develop into a desirable miniplug seedling crop is critical to maximize program efficiency and reduce nursery production costs.

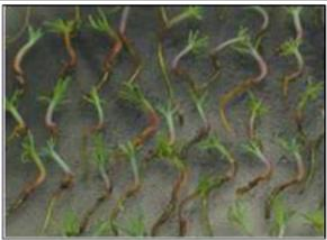
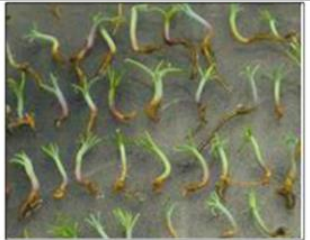
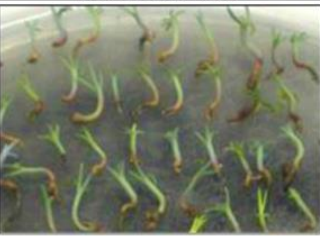
Assessment Criteria	Category 1 (High converting germinants)	Category 2 (Good converting germinants)	Category 3 (Variable converting germinants)
<b>Germinant Form</b>	Hypocotyl and root are straight with bend <45° (i.e., looks like a seedling)	Hypocotyl and root can be straight but typically has a bend between 15° and 60°	Hypocotyl and root can be straight but typically has a bend between 15° and 60°
<b>Germinant Deformity</b>	No Deformity	Deformities Present - though with SOP criteria	Usually needles, stem or root has some type of atypical form
<b>Germinant Size</b>	Total Length: >1 cm & <3 cm	Total Length: >1.2 cm & <2 cm	Total Length: 0.8 to 1.2 cm or >3 cm
<b>Needle Number</b>	Normally ≥ 3, though 2 allowed if epicotyl is present	Developed needles (≥ 3)	Under developed needles (≥ 3)
<b>Needle Color</b>	Green needles	Light green needles	Light green to yellow-green needles
<b>Needle Display</b>	Displayed in an open form	Displayed in an open to slightly-open form	Displayed in a closed to slightly-open form
<b>Needle Quality</b>	Well developed - thick needles	Developed - Medium needle thickness	Poorly developed and/or very short or very long
<b>Epicotyl Development</b>	Normally a presence of Epicotyl (genotype specific - Q-3802 normally not present)	Sometimes a presence of Epicotyl	No Epicotyl development
<b>Stem Size</b>	Thickness in the stem	Tall & skinny stem	Skinny stem
<b>Stem Color</b>	Green & sometimes pink	Green & sometimes pink	Green to light green
<b>Root</b>	White root and/or suberized root tip	White root and/or suberized root tip	Suberized root tip or questionable presence of a root tip
<b>Example of PGs</b>			

Figure 9. Selection criteria for somatic embryogenesis tissue culture loblolly pine germinants into three conversion categories having a range of nursery performance (Grossnickle unpublished data).

As a somatic embryogenesis program on loblolly pine increased production numbers into tens of millions of young seedlings, it was recognized that not all ‘plantable germinants’ are alike. As a result, work was conducted to identify various categories of plantable germinants that defined the range of germinant quality that was coming from the laboratory production program. Through a series of trials, a set of morphological criteria were defined that allowed for the separation of plantable germinants into three categories (Figure 9). Plantable germinants from these three categories were found to have variable conversion rates (i.e. defined by survival, and expression of shoot and root growth four weeks after planting) (Figure 10). In addition, germinants of variable quality, across all genotypes, grew at different rates with up to eight weeks difference in the time required for each category of plantable germinants to grow to an acceptable size for use in the finished seedling program (i.e. category #1 the fastest and category #3 the slowest). Trials were conducted across five genotypes, and though the overall conversion rate between genotypes was variable, differences in conversion rate between germinant quality categories remained the same. This confirmed the requirement for a universal level of germinant quality across genotypes.

This allowed for the creation of a simplified set of germinant selection criteria that were used by germinant sorters in an operational program (i.e. stem and needle length, number of needles and form, needle color, stem straightness, presence of an active root tip). In addition, these findings generated the need for further improvements in the *in vitro* part of the program to maximize the amount of category one germinants. Additionally, this germinant selection procedure helped address logistical issues in managing greenhouse space and germinant processing throughout the miniplug stage.

Maximization of the nursery conversion rate is critical to the commercial success of any vegetation propagation program, because empty container cavities increase shippable seedling costs. In addition, with variable germinant quality affecting establishment of planted germinants and growth of young seedlings, there are two additional operational steps that need to be included in the nursery production program. First, additional consolidation steps are required to remove dead or poor performing planted germinants. Second, germinant quality variability results in a range in seedling development requiring grouping of seedlings of similar growth patterns to enable the production of a uniform crop for transfer into the finished seedling program. As the crop size increases this can become a crop management problem necessitating operating procedures to address this variability in crop development.

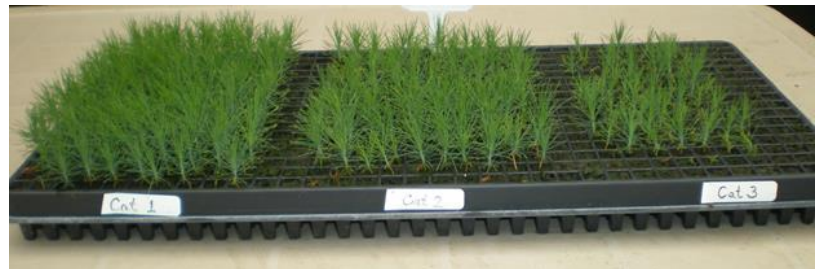
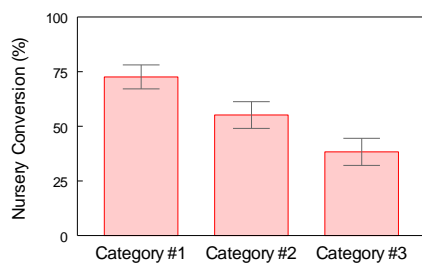


Figure 10. Nursery conversion of selected somatic embryogenesis tissue culture loblolly pine germinants based on selection criteria defined in Figure 9 (i.e. combined tests from five genotypes). Note: conversion rate was defined by the planted germinant being alive with the presence of an actively growing shoot and root after four weeks in the greenhouse. Photographic representation of seedling development, after ten weeks, of loblolly pine miniplug seedlings from selected somatic embryogenesis tissue culture germinants based on selection criteria defined in Table 1 (Grossnickle unpublished data).

### 3.1.2 Miniaturizing container system

To achieve both biological optimization and operational efficiencies a new container system is required as a program scales to produce tens of millions of seedlings. One must consider both biological and operational issues for maximizing conversion of somatic germinants as it miniaturizes the growing system at the initial nursery program stage. In addition, one needs to consider ramifications that miniaturization has on their integration into a finished seedling program. All of these factors need to be addressed when creating a miniaturized tray and media system that fits the entire seedling production program.

It is important to provide both an optimum shoot and root environment for the planted germinants. Edaphic conditions in the rooting environment are critical for

establishment of plants in a vegetative propagation system. To successfully root a vegetative propagule, rooting media has to provide a proper balance of water and air (Hartmann et al. 2002; Fonteno and Dole 2006). During very early stages of rooting, vegetative propagules do not have a high level of transpiration (e.g. Loach 1988). Thus, the media needs to have a 'dry-down potential' to avoid an extended timeframe of an excessively wet rooting environment. The container cavity volume has to be of a desired size containing media with large macropores that provide readily available water, though drainage of excessive water to ensure adequate media aeration (Styer and Koranski 1998). In addition, the media needs to retain its structure and not change through successive watering cycles over the timeframe required to produce a spec size seedling in a miniaturized tray and media system. Trials found that media with polymers or glues to ensure plug integrity were required to meet biological needs of the germinant establishment process.

In addition, both the tray and media had to optimize the nursery growing space and be sturdy enough to withstand all handling steps. Trial work resulted in tray miniaturization to allow growing of 3,814 seedlings  $m^{-1}$  (Figure 11). Operational assessment found that the rigid structure of Styrofoam trays was best suited for all operational program handling steps.

The result was the creation of miniplug plugs and trays that were strong enough to withstand rigors of consolidation and handling in the miniplug nursery, and then transplanting in the finished seedling nursery. Plug integrity during every handling step is critical, because damaged plugs cause root damage and ultimately lost inventory. Thus, the selected miniplug tray and media system needed to meet durability standards that allowed the rapid transfer and handling of millions of seedlings in an efficient manner while retaining their media integrity and plant quality. When developing this container and plug system for the establishment of somatic germinants, one had to be cognizant of the program used to grow these miniplug seedlings into finished seedlings. This means that miniplugs also need to meet size specifications for both the transplanting process and the desired final planting density in the finished seedling nursery. Thus, careful consideration was required to create a miniplug container system and growing media that meets all of these necessary program requirements.



Figure 11. Left) Miniplug seedling containers of loblolly pine somatic seedlings, at six weeks, grown from somatic embryogenesis tissue culture germinants. Right) Full size miniplug seedling grown in stabilized peat media that provides beneficial edaphic conditions and ease of handling during operational practices (Grossnickle unpublished data).

### 3.1.3 Germinant nursery establishment environment

In creating a nursery cultural environment to maximize the conversion of somatic germinants into established seedlings, one needs to ensure that all environmental parameters are addressed. These include all important atmospheric (i.e. temperature, humidity, vapor pressure deficit, light intensity, photoperiod) and edaphic (i.e. water, temperature, nutrients) conditions as well as including a program addressing integrated pest management practices. This cultural program needs to be specific to each development phase as somatic germinants grow into miniplug seedlings while they go through rooting, growth, hardening and holding phases. Each species has specific needs within each of the development phases. However, there are many similar cultural practices required for any successful vegetative propagation system and a number of excellent texts have been produced to define these required cultural conditions (e.g. Landis et al. 1999; Hartmann et al. 2002; Dole and Gibson 2006).

When scaling a somatic embryogenesis tissue culture program to produce tens of millions of seedlings some unique operational issues come into play which can affect how one grows quality miniplug seedlings for the finished seedling program. As has been discussed, germinants that come from the lab in a high-volume production program are typically quite small (see *In Vitro* Germination of Somatic Embryos and Germinant Quality sections) and are planted into a miniplug system (see Miniaturizing the Container System section) that allows one to maximize greenhouse production space. Though environmental conditions to establish vegetative propagules can be well defined, as the propagule being planted in the nursery decrease in size and are less well developed morphologically and physiologically, the need for precision to maintain proper cultural conditions during the rooting phase increases. Therefore, the variation in environmental conditions around defined cultural parameters needs to be reduced and deviations outside of this defined environmental range need to be avoided. Any deviations outside of this defined environmental range critical for optimum germinant performance during the rooting phase typically ends up reducing the program conversion rate. Other work has also found that tight control of the environment during the initial period of time outside *in vitro* culture conditions is important for the germinant establishment under greenhouse conditions (Egertsdotter 2018). Thus, maintaining the initial rooting environment is critical and requires an investment in infrastructure to maximize control of the greenhouse environment.

When producing tens of millions of seedlings, the programs lab phase has a production schedule to produce germinants that come to the nursery on a year-round basis. Consequently, the miniplug phase of the greenhouse program needs to be able to receive and continually grow germinants into finished miniplug seedlings. These miniplug seedlings are ultimately scheduled to go to the finished seedling nursery for transplanting and growing into full-size finished seedlings. Typically finished loblolly pine seedlings are grown into full-sized bareroot or container-grown seedlings that are ready to transfer to the forest restoration site in one growing season. This means the transplanting of miniplug seedlings into the finished seedling nurseries occurs from March through May for completion of the finished seedling crop by late fall. As a result, a year-round miniplug production program has to create unique cultural practices that can address integrating a year-round germinant production cycle with the miniplug production cycle that produces spec size miniplug seedlings to fit within the very short late winter and spring planting window of the finished seedling program. Thus, the

miniplug nursery program requires the development of unique cultural practices that are not standard within the nursery industry. The following are two examples of cultural activities that were unique to the year-round miniplug production cycle of loblolly pine somatic seedlings.

### 3.1.3.1 Photothermal growth models

Two of the main atmospheric variables that drive plant growth are light and air temperature (Larcher 1995). Light is the energy source for photosynthesis, while temperature is important because of its effect on all metabolic processes which drive plant growth. Understanding the interaction of these two atmospheric variables provides a means to model loblolly pine seedling nursery growth. This allows one to look at energy inputs (i.e. lights & heat) and define schedules for year-round production based on historic light levels that occur across the year, and required temperature requirements, at North American regional greenhouse locations.

The heat summation approach has been used for over two centuries as a method for studying plant-temperature relationships (Wang 1960). This concept is based on the method of describing plant-temperature relationships through the accumulation of daily temperatures above a certain threshold temperature (i.e., base temperature) during the growing season. This practice of heat summation has been used to forecast plant development and thus scheduling (i.e., rate of growth versus projected timeframe of crop completion) of commercial agricultural crops (e.g. Boswell 1929; Magoon and Culpepper 1932; Madariaga and Knott 1951; Perry et al. 1986). To create a comprehensive atmospheric model for environmental to plant response, temperature and light can be combined for a total energy unit approach (Liu and Heins 1997, 2002).

In developing the model for loblolly pine, temperature and light were reduced to a common unit and each weighted equally. Modeled temperature and light ranges were based on reported work on the physiological performance and morphological development of loblolly pine to these atmospheric conditions. These ranges were defined as: 1) temperature range of 4 to 48°C and 2) light levels up to full sunlight (i.e. 0 to 2,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). For the temperature model, findings from a series of scientific papers were normalized and combined for growth in relation to temperature (Barney 1951; Kramer 1957; Teskey et al. 1987; Teskey and Will 1999; Sword-Sayer et al. 2005). The photosynthetic response curve to light from Teskey et al. (1986) was used to model plant growth response of loblolly pine because seasonal net assimilation rates (capturing light intensity and duration responses of photosynthesis) are correlated with seedling growth (Ledig and Perry 1969). For both temperature and light the plant response to these environmental parameters were calibrated as net growth and net photosynthesis, respectively, as a percentage of maximum response. This allowed for the generation of a common value unit that combined temperature and light response models to create a single photothermal unit for each hour during the day (i.e. photothermal hour or PT Hour). This photothermal model was used in the production of both miniplug and finished seedlings. For miniplug seedlings the greenhouse temperature control allowed one to maintain the optimum temperature for growth (i.e. 24 to 26°C) meaning the temperature parameter was set to 1 in the model, thereby allowing one to define the response to seasonal shift in light for plants grown in a greenhouse. This photothermal model was also used for the growing of finished

seedlings in outdoor compounds and provided a means to define delivery dates for finished seedling stocktypes (Grossnickle and El-Kassaby 2016). Findings showed that this photothermal growth model projected miniplug seedling growth. A pooled growth model for five genotypes indicates that miniplug seedlings need ~300 PT Hours to root and initiate shoot flush followed by an additional number of PT Hours to reach height specifications for the operational program (Figure 12). A number of height criteria were defined based on operational deliverable parameters of minimum shoot height (2 cm achieved in 480 PT Hours), target height (4 cm achieved in 645 PT Hours), and maximum height (8 cm in 800 PT Hours). Modeling of PT Hours requirement for growing loblolly pine miniplug seedlings to size required for the operational program was done for every week during the year for various nursery locations (Figure 12).

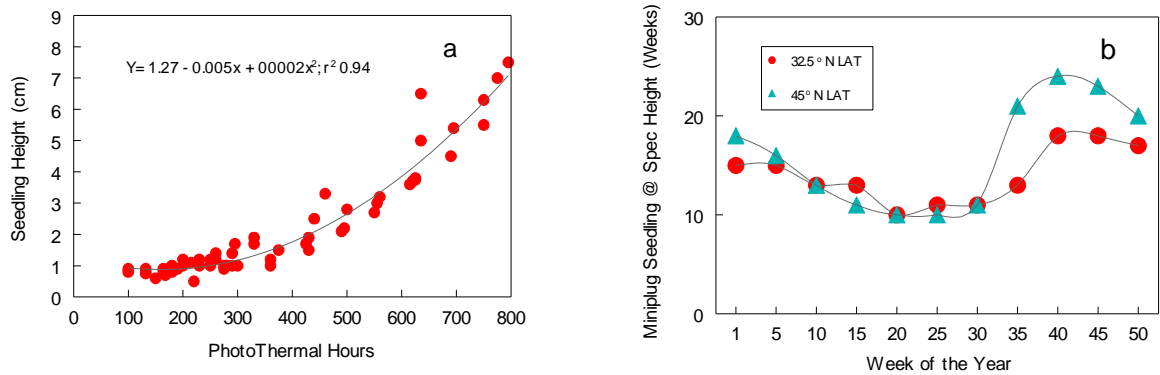


Figure 12. a) Loblolly pine germinant to miniplug seedling height growth in relation to photothermal hours. b) The number of photothermal hours to grow a loblolly pine germinant into a target height miniplug seedling in the greenhouse at nurseries located at two different latitudes (Grossnickle unpublished data).

This allowed lab and nursery production cycles to become synced. It allowed the lab to know how many plantable germinants were required at each nursery during every week of the year, because the nursery could determine how fast recently planted germinants were moving through the rooting greenhouse, thereby freeing up space to allow for more germinants to be planted. These models also allowed the nursery to manage the production cycle to have an efficient flow of germinants turning into seedlings through various cultural zones. The miniplug production program was conducted at a number of locations in North America that had different seasonal light regimes (Fisher and Runkle 2004). This meant that the seasonal cycle for the production of target height miniplug seedlings varied depending upon latitude of the nursery location (Figure 12). The photothermal growth model enabled operations of the lab and nursery production cycles to be matched by understanding the timeframe it took to produce a target miniplug seedling at different regional nursery locations throughout the year. This ensured an even flow of plant material through the production system.

### 3.1.3.2 Hardening and holding

By producing miniplug seedlings in a year-round production cycle, sometimes miniplug seedlings were at their target height months before they were to be transplanted into the finished seedling program. This resulted in the need to develop hardening and holding cultural regimes that stop growth, but maintain the physiological quality of miniplug seedlings. Nursery cultural programs have been devised to harden and hold seedlings. These cultural regimes typically apply an array of environmental limitations on tree seedling crops (e.g. reduced fertility and water; Landis et al. 1989, 1999) which slow and then stop seedling growth in the late summer; with the fall change of decreasing seasonal photoperiod and temperatures used as an environmental cues to stop growth, develop stress resistance and allow for long term holding to retain seedling quality (Grossnickle and South 2014).

Within this year-round miniplug production cycle, a portion of miniplug seedlings would meet spec by June and had to be held until the following spring. This required the application of a combination of the above environmental cues; with the applied level of stress beyond what is normally used in a cultural regime. This hardening cultural regime took approximately six weeks to apply (i.e., creating water stress by stepping down the watering regime and flushing all nutrients from the plug with minimal fertilizer applications at every other watering). The result was to reduce the nitrogen level and maximize drought resistance, creating a hardened seedling with complete cessation of shoot and root growth (Figure 13). Miniplug seedlings could then be held in an outdoor compound for eight to fourteen weeks without any growth. Thereafter, as fall temperatures declined seasonally low temperatures were used to limit growth and develop stress resistance (i.e. freezing tolerance) (Figure 13), with a fall watering and fertilization regime applied to create a desirable seedling nutritional status required for future seedling growth. This cultural regime provided a means to hold spec miniplug seedlings that were ready for transplanting in June until February, while retaining their plant quality.

### 3.2 Finished seedling production

The primary objective of a somatic embryogenesis tissue culture program is to ultimately produce a plant propagule that feeds into the standard operational finished seedling production program. Once this objective is achieved somatic loblolly seedlings can be grown for one nursery growing season using standard cultural practices to produce bareroot (Mexal and South 1990) or container-grown (Brissette et al. 1990) seedlings. Thus, the last operational step to achieve this objective is to transplant miniplug seedlings into the finished seedling nursery. To achieve this operational step a transplanter was designed to plant miniplug seedlings at the proper density and depth, and at a planting rate for millions of miniplug seedlings within the desired March to May planting window (Figure 14). This transplanter was able to achieve over a 95% transplanting efficiency and resulted in the desired establishment and growth of somatic seedlings.

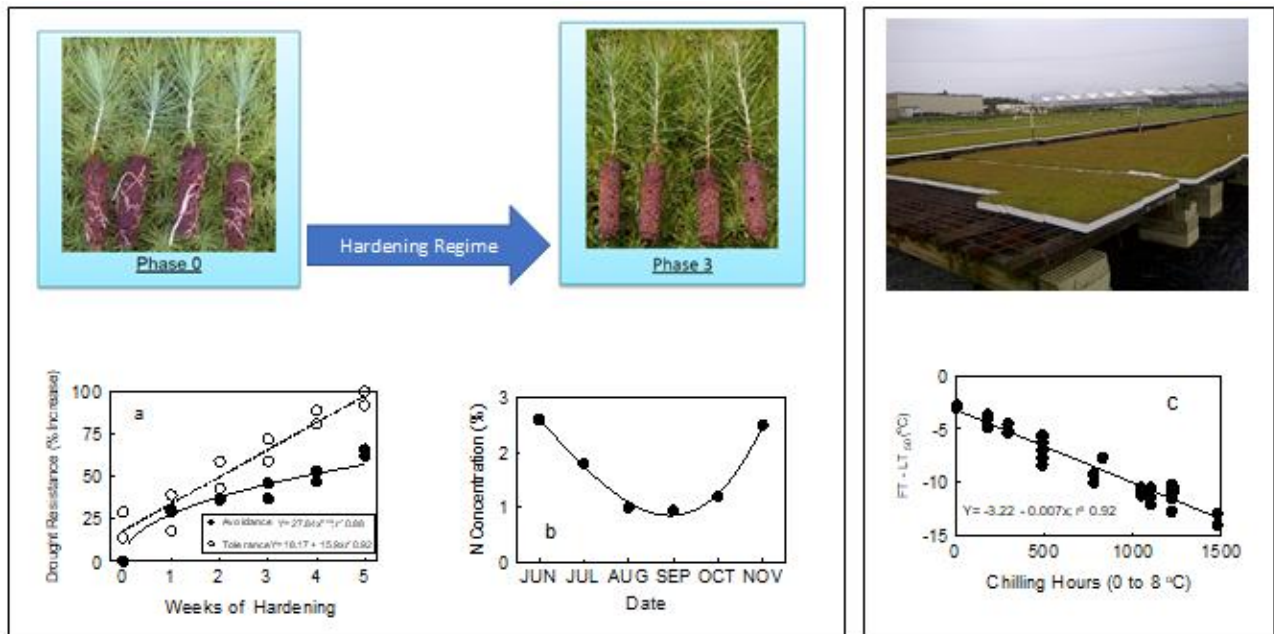


Figure 13. Left Panel - Hardening regime for holding loblolly pine miniplug seedlings for up to fourteen weeks during the summer in an outdoor compound. This regime utilized a combination of reduced watering and low fertilization to stop seedling growth (pictures at top – phase 0 shows actively growing seedlings and phase 3 shows fully hardened seedlings) and develop drought resistance (a - The shift in drought resistance as measured by drought avoidance (cuticular transpiration that declined from 3.8 to 2.3 percent water loss  $h^{-1}$  after stomatal closure) and drought tolerance (osmotic potential at turgor loss point that declined from -1.0 to -2.0 MPa) (Grossnickle and South 2017) during nursery hardening (i.e. reduced fertilization and watering in an outdoor compound) and a very low nitrogen concentration (b) followed by an increase in the fall seedling nitrogen concentration (Grossnickle unpublished data) (figures at bottom). Right Panel – Acclimation of crop in an outdoor compound (note needle color change with chlorophyll breakdown for the genotype in the foreground) as freezing tolerance (c) developed during the fall (i.e. FT; temperature causing 50 percent electrolyte leakage from needles) responds to increased chilling hours (0 to 8 °C) (Grossnickle and South 2017).



Figure 14. Transplanter designed to plant miniplug loblolly pine seedlings (left) into bareroot nursery beds (insert) and established miniplug seedlings in the bareroot nursery (right) (Grossnickle unpublished data).



### 3.3 Seedling quality and nursery/field performance

A quality monitoring program was developed to ensure that the production of somatic seedlings met a high standard of seedling quality. This program monitored the process, meaning that a system was created to monitor nursery cultural practices and crop development that allowed one to track miniplug and finished seedling growth to desired specifications. Proper application of nursery practices was a key component of the program for miniplug (Grossnickle 2011), bareroot (Mexal and South 1991) and container-grown (Brissette et al. 1990) loblolly pine seedlings. To develop an effective seedling quality program that monitors the process, one needs to understand how the crop responds to cultural conditions. A crop's physiological response to the environment and its subsequent morphological development response ultimately determines its growth performance in the nursery (Grossnickle 2016). Understanding of loblolly pine physiological capability and morphological development in relation to environmental conditions was used to create detailed cultural practices that became SOPs for both the miniplug and finished seedling programs. Seedling SOPs were then used to develop crop plans to consistently producing high quality miniplug (Grossnickle 2011), bareroot (Wakeley 1954; May 1985; Duryea 1984) and container-grown (Tinus and McDonald 1979; Landis et al. 1999) seedlings. Once the crop plan with SOPs was developed, a quality monitoring system was instituted to track both the nursery environment and crop performance (Grossnickle 2011).

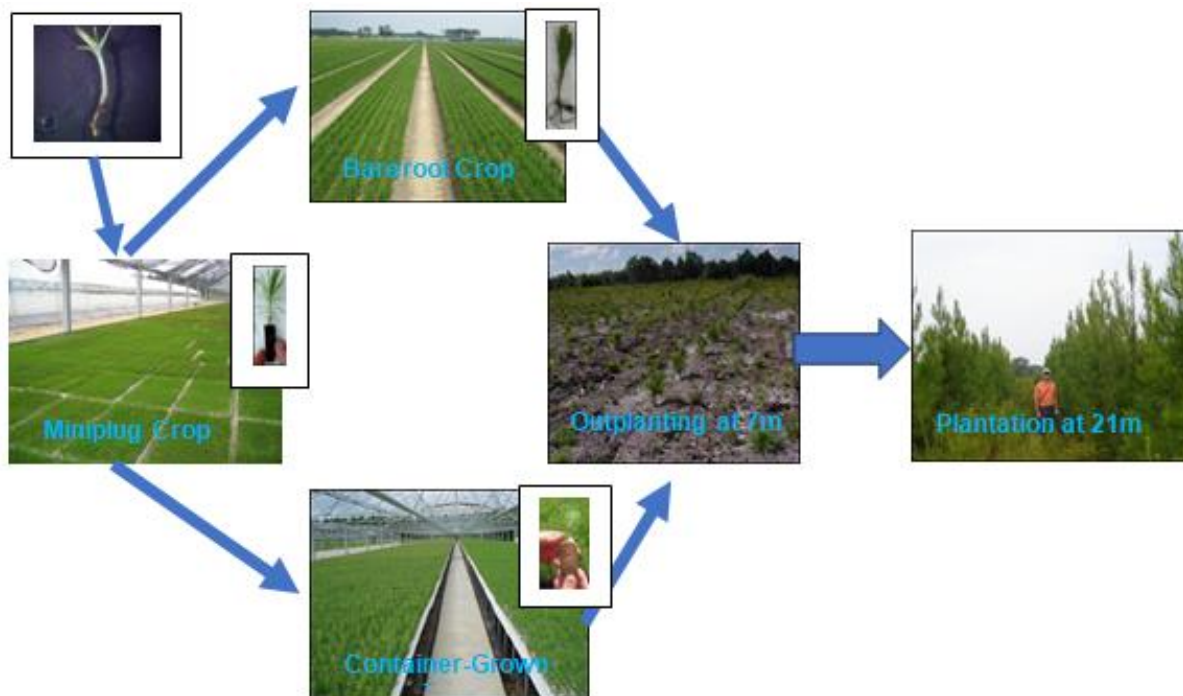


Figure 15. Somatic loblolly pine plantable germinants (insert) were grown into miniplug seedlings that were transplanted into bareroot (top) and container-grown (bottom) nurseries and grown into finished seedlings. These finished seedlings were then outplanted onto forest restoration sites where they became established (at 7 months) and then grew into forest plantations (at 21 months), (Grossnickle unpublished data).

This approach is part of the “target seedling concept” where attention to crop plan are important to achieve the desired seedling product (Rose et al. 1990; Landis et al. 1995). This approach is also similar to ISO Quality Assurance programs that monitor any manufacturing program to ensure achievement of planned results (Stebbing 1993; Anon. 2000). This quality assurance program was designed and operated at ten miniplug and finished seedling nurseries across North America (Figure 15) that produced tens of millions of high-quality somatic loblolly pine seedlings (Grossnickle 2011). A quality assessment program for monitoring the final product was conducted at the end of the finished seedling program. This created an information database that allowed dialogue between the company producing somatic seedlings and forestry clients on seedling performance capabilities. This quality program measured an array of seedling attributes that assured the customer they were receiving seedlings that met quality standards for both good survival (Grossnickle 2012) and growth (Grossnickle and MacDonald 2018b) after outplanting into forest plantations. The result of this quality assurance program was that somatic loblolly pine seedlings that were planted throughout the Southeastern United States became established and grew into well-established forest plantations (Figure 15).

## 4 Conclusion

The utilization of somatic embryogenesis tissue culture protocols to produce somatic seedlings for operational forestry programs is a multi-step program. This forest regeneration approach is unique because it combines an *in vitro* laboratory program with an *ex vitro* nursery program to produce seedlings. Each program step has to not only maximize process efficiencies within that step, but also must ensure compatibility with the next program steps all the way through to the final finished seedlings. This linkage between each step needs to be reassessed every time the program grows in size. Ultimately it is understanding and addressing the biology of scaling at each program step and how all steps fit together that results in the creation of a fully integrated somatic embryogenesis tissue culture program that can produce tens of millions of seedlings for forest regeneration programs.

## 5 Acknowledgement

The authors would like to thank all of the dedicated men and women who were part of the Research and Operational Teams that achieved the biological success of creating a system that could produce tens of millions of loblolly pine somatic seedlings.

## 6 References

- Anonymous. ISO 9000:2000 (2000) Quality management systems – Fundamentals and vocabulary, 2nd ed.; International Organization for Standardization, Geneva, Switzerland.
- Attree SM, Moore D, Sawhney D, Fowke LC (1991) Enhanced maturation and desiccation tolerance of white spruce (*Picea glauca* [Moench] Voss) somatic embryos: effects of a non-plasmolysing water stress and abscisic acid. *Ann Bot* 68: 519-525.  
<https://doi.org/10.1093/oxfordjournals.aob.a088291>
- Barney CW (1951) Effects of soil temperature and light intensity on root growth of loblolly pine seedlings. *Plant Physiol* 26:146-163. <https://doi.org/10.1104/pp.26.1.146>

- Becwar MR, Chesick EE, Handley III L, Rutter MR (1995) USA Patent No. 5413930.
- Becwar M, Nagmani R, Wann SR (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinustaeda*). *Can J For Res* 20(6): 810-817. <https://doi.org/10.1139/x90-107>
- Bonga JM (2015) A comparative evaluation of the application of somatic embryogenesis, rooting of cuttings, and organozonesis of conifers. *Can J For Res* 45: 379-383. <https://doi.org/10.1139/cjfr-2014-0360>
- Boswell VR (1929) Factors influencing yield and quality of peas. Maryland Univ. Bulletin 306.
- Brissette JC, Barnett JP, Landis TD (1991) Container seedlings. In: Duryea ML, Dougherty PM (eds) *Forest regeneration manual*. Kluwer, Netherlands, pp 117–141. [https://doi.org/10.1007/978-94-011-3800-0\\_7](https://doi.org/10.1007/978-94-011-3800-0_7)
- Cheliak WM, Klimaszewska K (1991) Genetic variation in somatic embryogenic response in open-pollinated families of black spruce. *Theor Appl Genet* 82(2):185-190. <https://doi.org/10.1007/BF00226211>
- Coke JE (1996) *Patent No. 5,534,433*.
- Cyr D, Klimaszewska K (2002) Conifer somatic embryogenesis: I. Development. *Dendrobiology* 48: 31-39.
- Denchev P, Attree, SM, Kong L, Tsai C-J, Radley RA, Lobatcheva II (2011) USA Patent No. US 7,906,334 B2. <https://doi.org/10.1126/science.334.6058.906-c>
- Dole JM, Gibson JL (eds) (2006) *Cutting propagation: A guide to propagating and producing floriculture crops*. Ball Publishing, Batavia IL
- Duryea ML (1984) Nursery cultural practices: impacts on seedling quality. In: *Forest nursery manual: production of bareroot seedlings*, Duryea ML, Landis TD eds.; Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, The Netherlands, pp. 143–164. [https://doi.org/10.1007/978-94-009-6110-4\\_15](https://doi.org/10.1007/978-94-009-6110-4_15)
- Egertsdotter U (2018) Plant physiological and genetical aspects of the somatic embryogenesis process in conifers. *Scand J Forest Res* <https://doi.org/10.1080/02827581.2018.1441433>
- Fisher P, Runkle E eds. (2004) *Lighting up profits: understanding greenhouse lighting*. Meister Media Worldwide.
- Fonteno WC, Dole JM (2006) Media. In: Dole JM, Gibson JL (eds) *Cutting propagation: A guide to propagating and producing floriculture crops*. Ball Publishing, Batavia IL, pp 39-50.
- Grossnickle SC (2011) Tissue culture of conifer seedlings—twenty years on: viewed through the lens of seedling quality. In: *National proceedings of the Forest and Conservation Nursery Associations—2010*; Riley LE, Haase DL, Pinto JR (Technical Coordinators); U.S. Department of Agriculture, Forest Service, Fort Collins, CO, U.S.A., RMRS-P-65, pp. 139-146.
- Grossnickle SC (2012) Why seedlings survive: importance of plant attributes. *New Forest* 43:711-738. <https://doi.org/10.1007/s11056-012-9336-6>
- Grossnickle SC (2016) Restoration silviculture: an ecophysiological perspective - lessons learned across 40 years, *Reforesta* 1:1-36. <https://doi.org/10.21750/REFOR.1.02.2>
- Grossnickle SC, El-Kassaby YA (2016) Bareroot versus container stock types: a performance comparison. *New Forest* 47:1-51. <https://doi.org/10.1007/s11056-015-9476-6>
- Grossnickle SC, MacDonald JE (2018a) Seedling Quality: History, Application, and Plant Attributes. *Forests* 9:283; doi:10.3390/f9050283. <https://doi.org/10.3390/f9050283>
- Grossnickle SC, MacDonald JE (2018b) Why seedlings grow: influence of plant attributes. *New Forest* 49: 1-34. <https://doi.org/10.1007/s11056-017-9606-4>
- Grossnickle SC, South DB (2014) Fall acclimation and the lift/store pathway: effect on reforestation. *The Open Forest Science Journal* 7:1-20. <https://doi.org/10.2174/1874398601407010001>
- Grossnickle SC, South DB (2017) Seedling quality of southern pines: Influence of plant attributes. *Tree Planters' Notes* 60:29–40
- Grossnickle SC, Cyr D, Polonenko DR (1996) Somatic embryogenesis tissue culture for the propagation of conifer seedlings: A technology comes of age. *Tree Planters' Notes* 47: 48–57
- Gupta PK, Grob J (1995) Somatic embryogenesis in conifers. In GP Jain SM (ed), *Somatic embryogenesis in woody plants* (pp. 81-98). Dordrecht, the Netherlands: Kluwer Academic Publishers.

- Hartmann HT, Kester DE, Davis Jr FT, Geneve RL (2002) Plant Propagation: Principles and practices, 7<sup>th</sup> Edition. Prentice Hill Upper Saddle River, NJ.
- Klimaszewska K, Bernier-Cardou KM, Cyr DR, Sutton BC (2000) Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus Strobus* L. In Vitro Cell Dev Biol Plant 36: 279-286. <https://doi.org/10.1007/s11627-000-0051-1>
- Klimaszewska K, Devantier Y, Lachance D, Lelu MA, Charest PJ (1997) Larix laricina (tamarack): somatic embryogenesis and genetic transformation. Can J For Res 27: 538-555. <https://doi.org/10.1139/x96-208>
- Kong L, Denchev P, Radley R, Lobatcheva II, Attree SM (2011) USA Patent No. 7,951,594 B2.
- Kramer P (1957) Some effects of various combinations of day and night temperatures and photoperiod on the height growth of loblolly pine seedlings. Forest Sci 3: 45-55.
- Landis TD, Tinus RW, McDonald SE, Barnett JP (1989) Seedling nutrition and irrigation. The container tree nursery manual, Volume 4, U.S. Department of Agriculture, Forest Service, Washington, DC, U.S.A., Agriculture Handbook 674.
- Landis TD, Tinus RW, McDonald SE, Barnett JP (1995) The container tree nursery manual, Nursery planning, development, and management. Volume 1, U.S. Department of Agriculture, Forest Service, Washington, DC, U.S.A., Agriculture Handbook 674.
- Landis TD, Tinus RW, Barnett JP (1999) Seedling propagation. The container tree nursery manual, Volume 6, U.S. Department of Agriculture, Forest Service, Washington, DC, U.S.A., Agriculture Handbook 674.
- Larcher W (1995) Physiological plant ecology: Ecophysiology and stress physiology of functional groups. 3rd Edition, Springer, Berlin.
- Ledig FT, Perry TO (1969) Net assimilation rate and growth in loblolly pine seedlings. Forest Sci 15: 431-438.
- Lelu-Walter M-A, Thompson D, Harvengt L, Sanchez L, Toribio M (2013) Somatic embryogenesis in forestry with a focus on Europe: State-of-the-art, benefits, challenges and future direction. Tree genet Genomes (9) : 883-899. <https://doi.org/10.1007/s11295-013-0620-1>
- Li XY, Huang FH (1996) Induction of somatic embryogenesis in loblolly pine (*Pinus taeda* L.). In Vitro Cellular & Developmental Biology - Plant 32 (3): 129-135. <https://doi.org/10.1007/BF02822755>
- Litvay JD, Verma DC, Johnson MA (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep 4: 325-328. <https://doi.org/10.1007/BF00269890>
- Liu B, Heins RD (1997) Modeling poinsettia vegetative growth and development: The response to the ratio of radiant to thermal energy. II Modelling Plant Growth, Environmental Control and Farm Management in Protected Cultivation 456, pp.133-142. <https://doi.org/10.17660/ActaHortic.1998.456.15>
- Liu B, Heins RD (2002) Photothermal Ratio Affects Plant Quality in: Freedom Poinsettia. J Am Soc Hortic Sci 127: 20-26. <https://doi.org/10.21273/JASHS.127.1.20>
- Loach K (1988) Water relations and adventitious rooting. In: Davis TD, Hassig BE, Snakala N (eds) Adventitious root formation in cuttings. Adv Plant Sci Series Vol 2., Dioscorides Press, Portland OR, pp 102-116.
- Madariaga FJ, Knott JE (1951) Temperature summations in relation to lettuce growth. Proceedings American Society Horticulture Science 58: 47-152.
- May JT (1985) Seedling growth and development. In Southern pine nursery handbook, Chapter 7; Lantz, C.W., Ed.; U.S. Department of Agriculture, Forest Service, Southern Region, pp. 7-1-7-18.
- Mexal JG, South DB (1991) Bareroot seedling culture. In: Duryea, M.L.; Dougherty, P.M., eds. Forest Regeneration Manual. Dordrecht, The Netherlands: Kluwer Academic Publishers: 89-115. [https://doi.org/10.1007/978-94-011-3800-0\\_6](https://doi.org/10.1007/978-94-011-3800-0_6)
- Magoon CA, Culpepper, CW (1932) Response of sweet corn to varying temperatures from time of planting to canning maturity (No. 04; USDA, FOLLETO 2500.). US Department of Agriculture.
- Pallardy SG (2008) Physiology of Woody Plants, 3rd edition. Academic Press, New York.

- Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. *Ann Forest Sci* 59: 651-656. <https://doi.org/10.1051/forest:2002051>
- Perry KB, Wehner TC, Johnson GL (1986) Comparison of 14 methods to determine heat unit requirements for cucumber harvest. *Hortscience* 21: 419-423.
- Pullman GS, Bucalo K (2014) Pine somatic embryogenesis: analyses of seed tissue and medium to improve protocol development. *New Forest* 45: 353-377. <https://doi.org/10.1007/s11056-014-9407-y>
- Pullman GS, Johnson S, Peter G, Cairney J, Xu N (2003a) Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. *Plant Cell Rep* 21(8): 747-58.
- Pullman G, Johnson S (2002) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): Improving culture initiation rates. *Ann Forest Sci* 59(5-6): 663-668. <https://doi.org/10.1051/forest:2002053>
- Pullman G, Montello P, Cairney J, Xu N, Feng X (2003b) Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: maturation improvements by metal analyses of zygotic and somatic embryos. *Plant Sci* 164: 955-969. [https://doi.org/10.1016/S0168-9452\(03\)00079-7](https://doi.org/10.1016/S0168-9452(03)00079-7)
- Roberts DR, Sutton BC, Flinn BS (1990) Synchronous and high-frequency germination of interior Spruce Somatic embryos following partial drying at high relative humidity. *Can J Bot* 68: 1086-1090. <https://doi.org/10.1139/b90-136>
- Rose R, Carlson WC, Morgan P (1990) The target seedling concept. In *Target Seedling Symposium, Proceedings of the Combined Meeting of the Western Forest Nursery Associations*, Roseburg, OR, U.S.A., August 13–17, 1990; Rose R, Campbell SJ, Landis TD (eds); U.S. Department of Agriculture, Forest Service, Fort Collins, CO, U.S.A., RM-GTR-200, pp. 1-8.
- Sword-Sayer MAS, Brissette JC, Barnett JP (2005) Root growth and hydraulic conductivity of southern pine seedlings in response to soil temperature and water availability after planting. *New Forest* 30: 253-272. <https://doi.org/10.1007/s11056-005-7481-x>
- Stebbing L (1993) *Quality assurance: The route to efficiency and competitiveness*, 3<sup>rd</sup> ed.; Prentice Hall, Ellis Horwood series in applied science and industrial technology, New York, NY, U.S.A., 336 p.
- Styer RC, Koranski DS (1998) *Plug & Transplant Production*. Ball Publishing, Batavia IL.
- Sutton BC, Attree SM, El-Kassabi YA, Grossnickle SC, Polonenko DR (2004) Commercialization of somatic embryogenesis for plantation forestry. In *Walter C, Carson M (eds), Plantation forest biotechnology for the 21st century* (pp. 275-301). Research Signpost.
- Teskey RO, Will RE (1999) Acclimation of loblolly pine (*Pinus taeda*) seedlings to high temperatures. *Tree Physiol* 19: 519-525. <https://doi.org/10.1093/treephys/19.8.519>
- Teskey RO, Bongarten B, Cregg, BM, Dougherty PM, Hennessey TC (1987) Physiology and genetics of tree growth response to moisture and temperature stress: an examination of the characteristics of loblolly pine (*Pinus taeda* L.). *Tree Physiol* 3: 41-61. <https://doi.org/10.1093/treephys/3.1.41>
- Tikkinen M, Varis S, Aronen T (2018) Development of Somatic Embryo Maturation and Growing Techniques of Norway Spruce Embryos towards Large-Scale Field Testing. *Forests* 9: 325. <https://doi.org/10.3390/f9060325>
- Teasdale RD, Dawson PA, Woolhouse HW (1986) Mineral Nutrient Requirements of a Loblolly Pine (*Pinus taeda*) Cell Suspension Culture. *Plant Physiol* 82: 942-945. <https://doi.org/10.1104/pp.82.4.942>
- Tinus RW, McDonald SE (1979) *How to grow tree seedlings in containers in greenhouses*. U.S. Department of Agriculture, Forest Service, Fort Collins, CO, U.S.A, GTR-RM-60, 1979.
- Wakeley PC (1954) *Planting the southern pines*. U.S. Department of Agriculture, Forest Service, Washington, DC, U.S.A, Agriculture Monograph No. 18.
- Wang JY (1960) A critique of the heat unit approach to plant response studies. *Ecology* 41:785-790. <https://doi.org/10.2307/1931815>