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EMBRIOGENESIS SOMATICA DE CONIFERAS

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CONIFER SOMATIC EMBRYOGENESIS

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Summary

Somatic embryogenesis in conifers allows the establishment of stable cultures which exhibit polyembrony (or repeated embryo cleavage). These cultures may be cryopreserved or used for production of somatic embryos capable of germination and growth into normal plants. This process has been developed for several commercial species, including spruce and pine species, and Douglas fir, with some successful research on other species. The current challenges involve the development of clonal testing programs and production processes required for commercial application. BC Research Inc. and Silvagen Inc. have established field tests of 1,400 clones of interior spruce (*Picea glauca/engelmannii* complex) in British Columbia, Canada. In order accomplish this, the establishment and freezing of cultures, the maturation of embryos from diverse genotypes and the production of uniform plants had to be completed successfully. Silvagen Inc. is involved in similar clonal selection programs with Sitka spruce, Douglas fir and pine species for various geographic regions. Initial assessments of phenotypic variation, and hence the basis for selection, among clones of spruce have been completed. In parallel, commercial production systems have been developed, with annual production currently exceeding 500,000 seedlings.

Introduction

Somatic embryogenesis (SE) is a tissue culture method of asexual propagation that involves recapitulating the normal process of seed embryo development (Tautorus et al. 1991). The term somatic refers to embryos developing asexually from vegetative (or somatic) tissue. This method has the means of rapidly multiplying elite varieties or clones. Through the application of bulk-handling techniques, SE is rapidly developing into a cost-effective propagation approach for conifers (Roberts et al. 1995). Distinct from conventional cuttings and organogenesis tissue culture, SE offers the advantage of long-term storage of germplasm through cryopreservation (Kartha et al. 1988, Cyr et al. 1994, Cyr 1998). SE is a method that is just starting to be used in forestry as a means of rapidly multiplying elite varieties or clones .

SE has great potential as an effective vegetative propagation method to fulfill the needs of both bulking-up valuable seed, and perhaps more importantly, to capture and select elite clones for subsequent mass propagation. In many conifer species and tree species more generally, rooted cuttings are an effective means of vegetative propagation. However, rooting success and consequently, production costs vary considerably between species. A large-scale SE system may be competitive with rooted cuttings in many situations provided it is cost effective. However, the same is not true of the selection and propagation of elite clones. In most conifer species and also some broadleaf species, once trees are more than a few years old, rooted cutting production becomes less successful and often leads to other problems associated with the maturation such as slow growth and plagiotrophism. Ultimately, the only way in which clonal forestry is feasible in such species is through a tissue culture system which can be applied to create plant cell cultures which can be preserved and stored for extended long periods of time through cryopreservation, and that are capable of sustained high rates of multiplication. Presently, only SE satisfies these criteria. For this reason, a clonal testing and selection program has been designed and implemented in British Columbia for interior spruce through the joint efforts of BC Research Inc. (BCRI) and the BC Ministry of Forests (Sutton et al. 1993). In some species, other *in vitro* propagation methods have been developed (e.g., organogenesis). These methods generally rely on the multiplication of shoots in tissue culture and is often termed as micropropagation. Most attempts to develop cost-effective micropropagation in conifers have been unsuccessful, although micropropagation of *Pinus radiata* has been implemented on a large-scale in New Zealand by Fletcher Challenge Forests Ltd. and in Chile by Bioforest. Nonetheless, micropropagation has some inherent disadvantages, including the fact that shoots have to be divided and transferred individually and by hand, a process which has proven difficult to automate cost-effectively. Also complex plant structures such as shoots are hard to cryopreserve and the duration for storage of this type of culture is dependent on conventional refrigeration of metabolically active shoots.

To date, the development of conifer SE has been focused on several commercial species including interior and Sitka spruce (Roberts et al. 1991, Sutton et al. 1993), white spruce (Park et al. 1993), loblolly pine (Handley et al. 1994), Douglas-fir (Gupta et al. 1994) and radiata pine (Smith et al. 1994). The Forest Biotechnology Centre at BCRI has placed considerable emphasis on the development of SE for spruce since 1988 (Roberts et al. 1993). This program has evolved from basic research to the implementation of clonal selection programs and the large-scale production of plants for operational testing, through a commercial affiliate, Silvagen Inc. In the last 3 years, the basic know-how developed for spruce has been successfully extended to a variety of commercially important pine species, including radiata pine, loblolly pine, *Pinus patula* and Western white pine (*Pinus monitcola*).

Somatic embryogenesis process

In conifers, embryogenic cultures are initiated (induction) from embryos of mature (spruce spp.) or developing seeds (pine spp., Douglas-fir). Through a series of steps performed under sterile conditions, these cultures can be multiplied (proliferation) and induced to form somatic embryos that are genetically identical to the original seed embryo (maturation). The embryos are subsequently harvested, dried for storage, and then germinated to produce somatic seedlings (Grossnickle et al. 1997).

Within each of these process steps there are specific manipulations which are important to the quality of the embryogenic culture and the proportion of embryogenic clones which can be captured from original seeds placed in culture. During induction, after a period of weeks, proliferating embryos emerge from the isolated embryo, in spruce and Douglas fir, or originate from the embryo inside the megagmetophyte and are extruded from it, in pine species. Once the initial culture has appeared, a period of several weeks to several months is required to obtain a stable culture. Thus the actual yield of cultures as a percentage of seed placed in culture is considerably lower than the initial induction rates often reported in the literature. For example, in radiata pine, initial extrusions may occur on 50% or more of the explants but final yields of stable embryogenic cultures (net induction rate) are likely to be 10% or less. Furthermore, the induction rate is strongly influenced by the source tree and consequently different families exhibit quite widely varying induction rates. Nonetheless, since induction in pines is very dependent on the exact developmental stage of the immature seed, the induction rate as a proportion of the seeds at the correct developmental stage may be substantially greater. Based on the literature, successful induction of embryogenesis has only been obtained routinely from seed material or very young germinants of conifer species. Thus it has not yet been demonstrated as a method for propagation of mature trees.

Once stable embryogenic cultures are obtained, they are typically proliferated and maintained on semi-solid medium. For storage, a portion of the culture is treated with cryoprotectants and frozen at a controlled rate and placed in liquid nitrogen storage (temperature -196°C). For long term stability, storage temperatures of less than -140°C are used; this corresponds to the glass transition phase of water, below which no chemical activity takes place (see Cyr 1998).

Embryogenic cultures contain immature somatic embryos (typically about 5,000-10,000 per gram fresh weight). In order to obtain mature embryos, which can be used for germination and plant production, the cultures are transferred to a different medium which usually contains the hormone abscisic acid and often a water stress agent. Although many different media have been developed, all protocols for conifer embryo maturation involve completing maturation on a solid or semi-solid medium, often following a period of liquid culture. Maturation in liquid culture has been largely unsuccessful. Following the maturation step, or as an integral part of late embryo maturation, removal of water from the embryos is critical to achieving subsequent germination and plant conversion. In addition, drying to moisture contents in the range of zygotic seed is required for storage of the embryos. The latter is important for coordinating plant production as well as for shipping embryos to nursery environments. BC Research Inc. was the first to develop drying protocols for conifer embryos, based on the general approaches for angiosperm embryos, and Silvagen routinely uses these methods as part of its proprietary production process (McKersie at al. US Patent # 5,238,835; Roberts US Patent # 5,183,757).

Once somatic embryos which are capable of vigorous germination have been obtained, they are usually germinated *in vitro* on agar-based medium. This standard approach provides good germination provided the embryos are of good quality. It is generally a convenient approach for the production of hundreds or thousands of plants which can be transferred by hand to soil in nursery containers. However, for large scale production, this approach is expensive and relatively cumbersome. Recently, Silvagen Inc. was successful in developing methods for direct seeding of somatic embryos into soil using conventional seeding machinery. This approach promises to provide a production system suitable for large scale commercial production in the millions of plants per annum.

Commercial application of conifer SE

Clone establishment and clonal testing

In order to apply the technology to forest tree improvement, an array of embryogenic clones must be established from valuable seed from selected trees identified in a tree breeding program. Typically the goal will be to select a number

of excellent clones following field testing. To design an appropriate strategy one needs to consider the following:

- The number of selected clones required
- The required genetic diversity of the selected clones
- The intensity of selection in order to achieve the desired genetic gain
- The pedigrees, or families, from which to establish the clones, based on their economic traits.

Quantitative genetics data can be used to estimate these variables for a given species and breeding program. BC Research Inc. and the BC Ministry of Forests developed a program for interior spruce (*Picea glauca/engelamanni* complex) with the following initial goals: 50 clones required following selection at an intensity of 5% from a total of 20 control pollinated families. Genetic diversity among these families must be satisfied by having at least 5 unique parents (8 were chosen initially). This general approach, reviewed in Sutton et al. 1993, resulted in the goal of testing 1,000 clones via SE with approximately 50 clones from each of the 20 families. In practice, this program evolved to include additional parent trees. By 1998 an enlarged comprehensive program of initiation and cryopreservation of embryogenic clones, production of plants and establishment of field trials for clonal selection had been completed as outlined in Table 1.

Table 1. Summary of the Interior Spruce Clonal Selection Field Trials.

Trial Year	Families	Pedigree[1]	Lines
1994-95	12	CP	181
1996-97	21	CP	571
1998	15	OP	700
Total:	**48**		**1,452**

1. CP= control pollinated; OP= open pollinated.

As outlined above, the induction of stable clones, their successful storage in cryopreservation and effective plant production from a substantial proportion of the stored clones, are all critical to the success of this program. In this case, induction of stable clones from mature seed occurred on average at a frequency of 30% and about 70% of the cultures were readily used for production of plants for clonal tests. While some families had lower induction rates (5% or less) and others very high induction rates (up to 60%), no families were lost from the trials due to an

insufficient number of clones. In addition, any such limitations could have been readily remedied by using the reciprocal of specific control pollinated families, since there is a significant maternal effect on induction. Also, higher induction rates may be achieved with immature seed (average over 50%).

In the case of pine species, overall induction rates are lower. In the case of radiata pine, for example, net induction rates of 5% may be obtained (Aitkin-Christie et al. 1994). Significant variation is observed among families with about 20% of families exhibiting low success (less than 1% induction).

Silvagen and BC Research are in the process of clone establishment and field testing from a range of species of pine and other conifers resulting in a cryopreservation clone bank (totaling over 5,000 clones) which is considerably larger than any other such bank in the world (Table 2).

Table 2. Summary of Silvagen/ BC Research clone bank October 1998.

Species (source)	Principal Region	Families	Clones
Interior Spruce	Canada	48	2,000
Sitka Spruce	Pacific NW	23	600
Douglas-fir	Pacific NW	12	200
Pine spp.	South Temperate	61	2,300
Total		144	5,100

Characteristics and Performance of somatic seedlings

Nursery production

The establishment of germinated embryos in soil requires conditions similar to germinating seed but with some important differences. Because somatic embryos lack a megagametophyte and seed coat, some adjustments are required for early seedling development to compensate for differences in water relations and nutrients which are provided by the megagametophyte. Studies using conventional *in vitro* germination show that the initial response of germinants to the nursery environment has a profound influence on subsequent morphological development. Past studies have found variable success in obtaining normal morphological development of interior spruce somatic, compared to zygotic, seedlings during the first stages of

development in the nursery (Webster et al. 1990, Grossnickle et al. 1994). As a result of this variable performance, initial production runs of somatic seedlings resulted in a higher than desired culling rate (up to 20%) based on BC Ministry of Forests shoot-height culling standards (Grossnickle and Major 1994a). Current cultural practices show that when germinants are initially treated more like rooted cuttings (e.g., shade with misting and bottom heat), they quickly acclimate to the nursery environment. Recent nursery performance has shown that a proper environment during the initial establishment stage allows somatic seedlings to achieve morphological development typical of young zygotic seedlings. This improved morphological development has reduced culling rates for 1996 to 5-8% (based on BC Ministry of Forests shoot-height culling standards).

Clonal variation

Somatic seedlings that are produced from various clones can have a wide range in morphological development. An example of this clonal variation in interior spruce is shown in Table 3. For height growth T703 was the tallest and N366 the shortest, with clones G351, T703 and W460 exceeding, and clone N366 meeting the target height standards for 1+0 interior spruce seedlings. These clones also showed a wide range in diameter development, with W460 having the largest and G351 the smallest diameter. Clones N366, T703 and W460 exceeded the target diameter standards for 1+0 interior spruce seedlings. Measurements of shoot architecture indicated that these seedlings had variation in the number of branches, mean branch length and branch angle. Seedlings from clones G351 and N366 had 35 and 20%, respectively, greater number of lateral branches than clones T703 and W460. Clones T703 and W460 tended toward shorter branches with a steeper branch angle compared to clones G351 and N365. Seedlings from clone W460 had ~20 buds on their terminal shoots while clones G351 and N366 had ~75%, and clone T703 had ~50% of this value. Terminal buds from clone N366 contained 173 needle primordia, while clones G351 had 92%, and clones T703 and W460 had ~74% of this value. Seedlings from clone T703 had the greatest shoot dry weight, which is reflective of larger shoot systems. Seedlings of clone G351 had a low level of root development, while clones N366, T703 and W460 exceeded the interior spruce stock type standards for root development.

For the most part, variability within any single morphological parameter measured on seedlings from any single clone was very low (Table 3). This is indicative of the genetic similarity of seedlings within a single clone. All somatic seedlings had general morphological traits comparable to zygotic seedlings and did not display any traits representative of early maturation. Somatic seedlings produced from these clones met the morphological standards that define a plantable seedling. However, there were large differences in morphological development among these interior spruce clones. As a result, seedlings from certain clones did not always meet specific stock type morphological specifications designed for general

containerized interior spruce zygotic seedling populations. It is important to recognize this variability in morphological development of clonal spruce seedlings when developing stock type morphological standards for clonal material. Modifications to these morphological standards may need to be considered to allow the inclusion of clonal material that has other desirable attributes, but does not always meet these generalized standards. On the other hand, if uniform standards are going to be required, then clones need to be grouped based on morphological development to allow for the application of nursery cultural practices that will produce clonal-stock with uniform morphological characteristics.

Table 3. Morphological development (Mean ± SE; n=20) of interior spruce clones G351, N366, T703 and W460. Somatic seedlings were produced in a containerized nursery culture program as a 1+0 frozen stored stock type in 415B containers (Grossnickle 1998).

Measured Parameter	G351	N366	T703	W460	Comments
Height (cm)	25.6 ± 0.4	22.1 ± 0.5	32.1 ± 1.0	25.2 ± 0.4	BC MoF target @ 22.0 [2]
Diameter (mm)	3.0 ± 0.1	3.8 ± 0.2	3.9 ± 0.1	3.1 ± 0.1	BC MoF target @ 3.5
Number of Branches	18.7 ± 0.8	17.2 ± 1.2	13.1 ± 1.3	13.1 ± 1.0	1+0 Z-S @ 16 [3]
Mean Branch Length (cm)	3.4 ± 0.1	3.4 ± 0.3	3.1 ± 0.2	2.7 ± 0.1	
Branch Angle	41.3 ± 0.7	44.2 ± 1.0	78.1 ± 2.5	52.4 ± 1.3	
Number of Buds on Terminal Shoot	14.5 ± 2.0	14.0 ± 1.1	10.6 ± 0.7	20.2 ± 1.1	1+0 Z-S @ 12
Terminal Bud Needle Primordia [1]	159.5 ± 8.4	173.3 ± 12.4	123.6 ± 38.4	132.1 ± 26.9	1+0 Z-S@ 150 to 230
Shoot Dry Weight (g)	3.1 ± 0.1	3.1 ± 0.1	4.0 ± 0.2	3.5 ± 0.1	1+0 Z-S @ 2.7
Root Dry Weight (g)	0.5 ± 0.0	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	BC MoF Target of 0.7

1) Needle primordia number was determined on a sample size of eight seedlings.

2) BC Ministry of Forests target standards for containerized zygotic interior spruce seedlings (Scagel et al. 1993)

3) Abbreviation represents standards of containerized 1+0 zygotic seedlings (Z-S). These standards were determined from previous morphological characterization of this stock type (Grossnickle, unpublished data).

In addition to morphology and growth characteristics, BC Research Inc. has carried out intensive analysis of variation in physiological characteristics. Measurement of gas exchange in response to summer atmospheric conditions (Grossnickle and Fan 1998a), in response to summer drought conditions (Grossnickle and Fan 1998a), and to fall acclimation (Fan and Grossnickle 1998b) together show that substantial clonal variation occurs at all levels of plant function. Such variations suggest significant opportunities for selection of clones which have superior water use efficiency, are able to tolerate severe drought, or withstand late summer or fall frost events.

Field performance

In a series of studies conducted with interior spruce by Grossnickle and co-workers of BC Research Inc. in 1991-1992, somatic and zygotic seedling incremental growth rates were examined over two seasons in the field on a boreal reforestation site. Both stock types, derived from the same families, had comparable height and diameter growth over the first two growing seasons. Initially zygotic seedlings had greater new root growth during the first year in the field (due to delayed nursery development of the somatic seedlings), while somatic seedlings had greater root growth during the second year in the field. Thus, by the end of the second growing season, both somatic and zygotic seedlings had comparable new root development in the field (Grossnickle and Major 1994b).

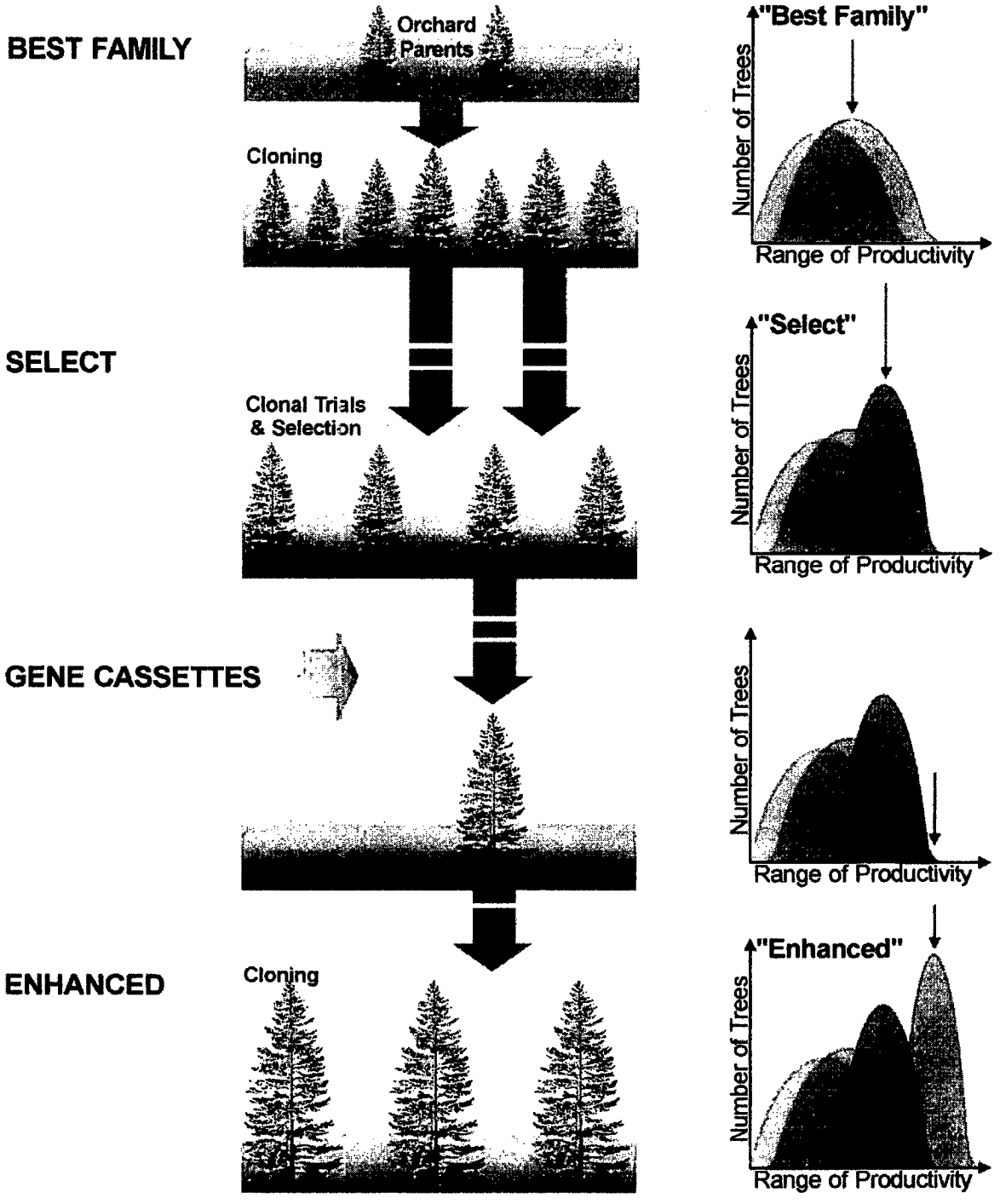
Long-term growth patterns indicate that somatic seedlings are capable of sustaining good incremental shoot development if seedlings are grown under plantation silviculture. Field grown somatic seedlings had morphological traits comparable to zygotic seedlings during the initial years of plantation development and did not display any traits which were representative of early maturation. Somatic seedlings also had very uniform shoot growth patterns, within measured clones, over seven years in the field. This has also been reported in genetically similar material produced through other vegetative propagation systems. Thus, interior spruce somatic seedlings have the inherent capability for long-term rapid shoot development that is desirable in the development of forest plantations.

Current and future commercial products

Currently, Silvagen Inc. is carrying out commercial production for the North American market and is engaged in a number of clonal selection programs with partners in various regions. Commercial products include various levels of genetic improvement. For example, in British Columbia, Canada the production of the best families of interior spruce and in the breeding program results in a so-called BEST product. This provides substantial increases in insect (weevil) resistance (60% improvement) and growth (15% increase in volume increment) over the conventional seed orchard material, since selected full sib families are delivered in operational quantities. Currently data is being collected which will allow further improvement through identification of selected clones (so-called SELECT product). In other regions, where cuttings production is routine, clearly the purpose of SE is to deliver selected clones following testing, either for direct operational planting of somatic seedlings (now being initiated for loblolly pine) or for subsequent cuttings propagation. Silvagen has produced and sold approximately 500,000 somatic seedlings in the last year and expects demand to increase substantially over the next few years. It is expected that future products will incorporate new traits introduced by genetic engineering (so-called ENHANCED product). This product

development strategy is outlined in Figure 1 (reproduced from Sutton and Polonenko 1998). The availability of embryogenic clones provides a necessary prerequisite for genetic engineering since a tissue culture system is normally required in order to deliver recombinant DNA to individual cells which are capable of regeneration into non-chimeric plants expressing the introduced gene. The initial transformants are, by definition, clonal and it is therefore likely that selected clones will be the chosen targets for genetic engineering (Ellis et al. 1993). Because of the need for a reliable production system and the need for preservation of selected transformed clones, SE is the system of choice in species such as conifers where, with some exceptions, conventional micropropagation has been relatively unsuccessful. Traits currently under development for genetic engineering include, increased productivity and cellulose deposition, altered lignification, insect resistance, herbicide resistance, and reproductive sterility. The sustained propagation of individual clones carrying these desirable traits through SE clearly represents a significant future market opportunity.

Figure 1. Illustration of SE products resulting from multiplication of seed families derived from elite parents families ("Best" product), followed by selection of the best individuals within these families ("Select" product). Finally, selected clones may be improved further through genetic engineering with gene" cassettes carrying valuable traits to create "Enhanced" products. Curves on the right refer to the increasing productivity of the stands planted with these products relative to unimproved seed.



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