Research and development of commercial tissue culture systems in loblolly pine

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ABSTRACT: Tissue culture systems offer many opportunities for the mass propagation and deployment of superior clones of forest trees. Somatic embryogenesis, micropropagation, and rejuvenation may all play a part. Micropropagation and the maintenance of juvenility, which were once greatly limited by genotype-specific responses in culture, are showing promise for clonal deployment via hedge production and rooted-stem cuttings. Rejuvenation systems using micropropagation techniques may also be possible. Somatic embryogenesis can provide a large-scale method for the mass production of thousands of somatic seedlings from superior clones, and it offers the potential for automated systems to produce large numbers of synthetic seeds. Limitations due to low initiation frequencies and the genetic specificity of explants are important problems. Establishment of field tests of somatic seedlings via somatic embryogenesis is now considered routine, and comparisons between somatic plants and zygotic seedlings are under way.

KEYWORDS: Breeding, clones, forest genetics, Pinus taeda, *propagation, R and D, somatic embryogenesis, tissue culture.*

A major commercial advantage of tissue culture systems for forest trees is their potential for producing thousands to millions of copies of superior individuals. The mass propagation of high-value genotypes using micropropagation, rejuvenation, or somatic embryogenesis systems may offer a significant advantage in some woody plants. These techniques can be applied to forest plantation species by clonally propagating superior individuals identified in a genetic improvement program. Clonal propagation through tissue culture systems must be integrated within a conventional tree improvement program to identify, test, and select those genetically superior individuals. Micropropagation and somatic embryogenesis systems are currently under development for loblolly pine (*Pinus taeda*) at Westvaco. Progress made in the development of these systems and some of our current research problems will be reviewed.

Different tissue culture systems offer advantages or disadvantages depending upon the commercial application. Micropropagation systems offer the ability to propagate and maintain clonal material in a juvenile state in small amounts of space. Rejuvenation through an organogenic or micropropagation system may also be possible. Micropropagation systems for loblolly pine are becoming routine and appear to work across a wide range of genotypes. These systems integrate well with rooted cutting technology; however, for large-scale deployment the high labor costs involved are a disadvantage. Low multiplication rates for some genotypes can also be limiting, especially if micropropagation is used as the sole means of mass propagation.

Somatic embryogenesis will be useful for clonal propagation since it has the potential to rapidly produce millions of propagules. This method, while presently quite expensive, offers the possibility of automation to reduce production costs. Somatic

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embryogenesis also offers the possibility of long-term cryopreservation of superior clones for later deployment. Major breakthroughs have been made in the development of a somatic embryogenesis system for loblolly pine. Embryogenic tissues can be derived from immature seed and somatic embryos can then be developed from these tissues, germinated, and planted in the field. The current major limitation in loblolly pine is the widely variable culture-initiation frequencies experienced from different genetic families; a major portion of Westvaco's current research effort is addressing this problem.

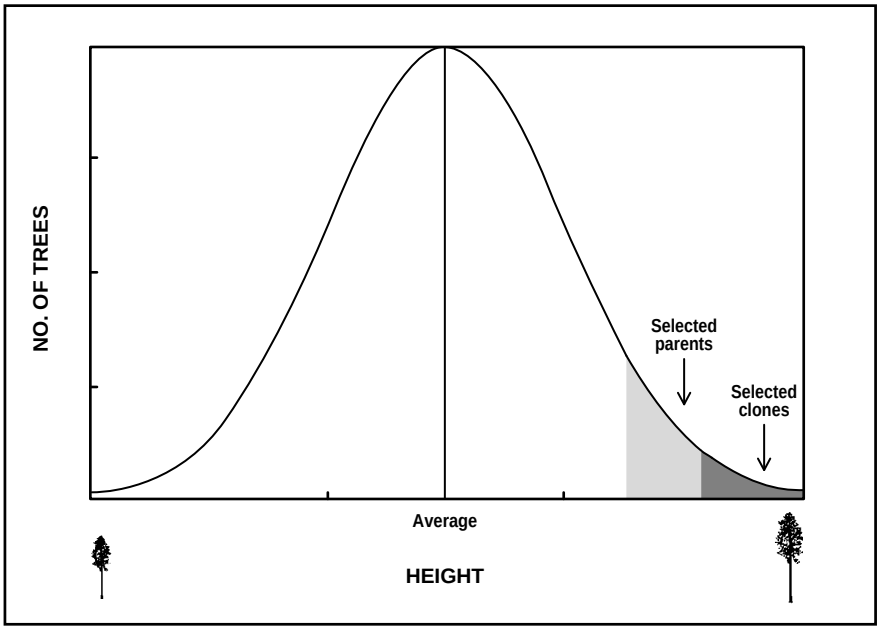
Integration with genetic improvement

Response of explants in a tissue culture system is inherently dependent upon the genotype of the material placed in culture. Numerous studies have shown that response of a tissue to a given cell culture system is dependent upon the genotype. In alfalfa, corn, and wheat embryogenic systems genetic backgrounds influence culture responses, and these responses are often controlled by just a few genes *(1–3)*. In such systems, efforts are being made to integrate these "regeneration genes" into mainline breeding stock and into commercially important cultivars. In a forest plantation species such as loblolly pine, where the goal is to capture and mass produce many superior genotypes, this genotype specificity is important when considering the unique features of tree breeding programs and commercial deployment.

Commercially important traits for forest trees in a breeding population usually occur in a range showing a normal distribution. Traits such as height and volume are good examples (**Fig. 1**). A typical breeding program attempts to capture individuals that occupy the upper end of this distribution. In a recurrent breeding strategy, crosses are made among parents that occupy the upper end of the distribution for the desirable trait, and those parents that produce a high proportion of progeny possessing this trait are placed in seed orchards for seedling production. In a "breeding-cloning" program, individuals from the upper end of this distribution are captured and deployed as clones. Clonal deployment requires testing beyond conventional seedling progeny tests because it is necessary to assess how clones will perform as mass-propagated individuals and not just as parents. In any clonal breeding and testing program, the goal is to capture and mass produce the superior genotypes from the population. The advantage of a clonal deployment program is that it allows one to utilize both the additive and dominance portions of the genetic variation in the population and should therefore increase the genetic gain potential, especially for traits with high dominance variance.

The method for the selection and deployment of clones should not restrict one's ability to mass produce the best genotypes available. Since the response of plants in tissue culture systems is inherently dependent on genotype, care must be taken to

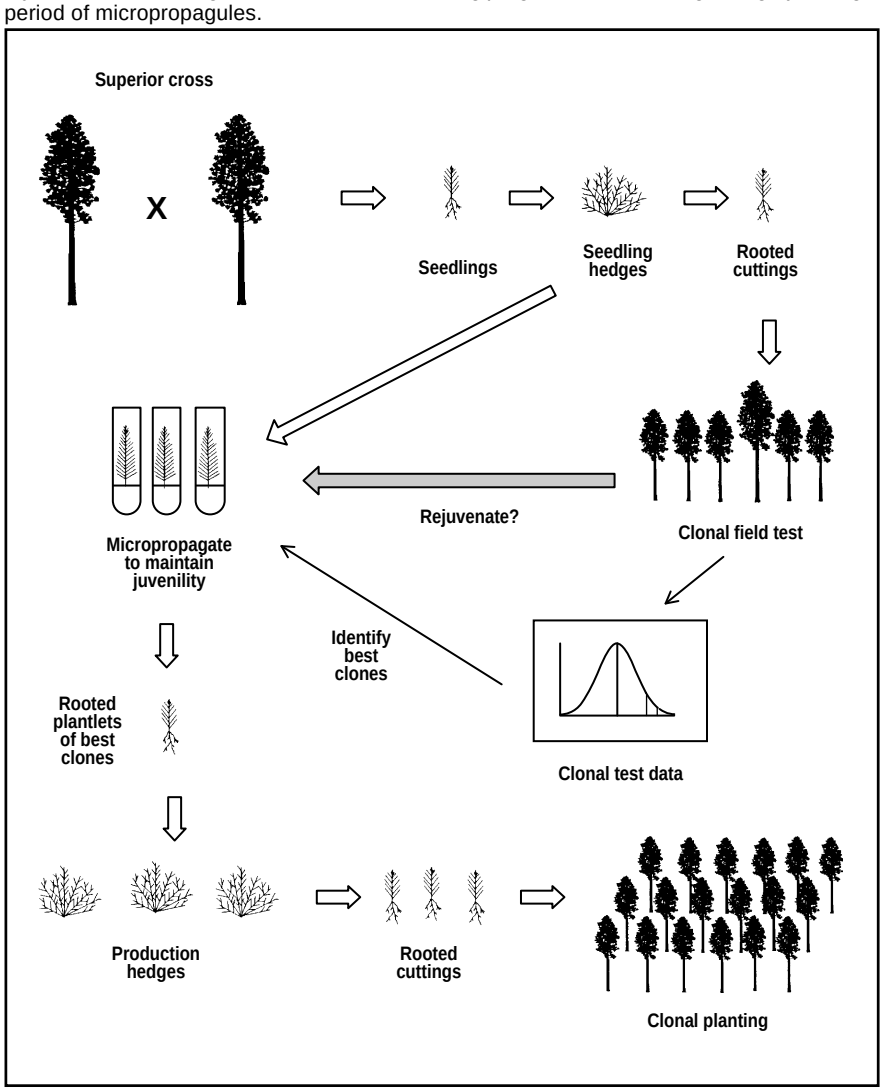
1. The shaded area represents the portion of trees that would be selected and retained for seed orchard establishment or for continued breeding based on progeny test data. The darkest shaded area (far right) represents clones that would be selected for deployment.



I. Performance of five open-pollinated loblolly pine families in a cotyledon regeneration protocol

Family	No. of seeds cultured	No. of seeds regenerated	Percent regenerated	No. of shoots harvested	No. shoots per responsive seed mean + s.e.
A	42	33	79	841	25.5 ± 16.0
B	50	44	88	940	21.4 ± 17.4
C	50	40	80	524	13.1 ± 9.9
D	50	30	60	460	15.3 ± 9.6
E	50	38	76	543	14.4 ± 13.4

2. Using micropropagation to maintain the juvenility of clones during a clonal field test. If rejuvenation becomes possible (shaded arrow), trees from the clonal test itself may be rejuvenated and brought into a rooted stem cutting program, thus avoiding a lengthy storage period of micropropagules.



ensure that valuable genotypes are not eliminated by the culture process. Two concerns must be addressed:

1. Does selection for the ability to grow and propagate in the tissue culture system correlate in a negative or positive way with valuable commercial characteristics?
2. Does selection for those individuals that respond in a tissue culture system eliminate a large portion of the deployment population with superior characteristics? If so, can this be overcome by improving the tissue culture treatments?

These are questions that are just beginning to be addressed in conifer tissue culture systems. With loblolly pine, we have experience in organogenic or micropropagation systems but are still addressing these questions with somatic embryogenesis.

Micropropagation

Micropropagation, or tissue culture systems based on an organogenic process, have been successful in loblolly pine for a number of years. Early studies at North Carolina State University showed that adventitious shoots derived from cotyledons from mature seeds could be successfully micropropagated and rooted *in vitro (4)*. Clonal material produced from cotyledon-derived shoots were planted in a number of field studies *(5)*. In laboratory studies the genetic background of the seed had a large impact on *in vitro* response. Both the ability to produce shoots and the number of shoots produced were dependent upon the genotype of the explant *(6)*. In these studies, from 30% to 96% of the cultured cotyledon explants (each an individual genotype) within a given family produced shoots *(7)*. There was no correlation between family height performance ranking and family response in culture.

In early Westvaco studies, we found that genetically superior families responded well in the cotyledon regeneration procedure developed by Mott and Amerson *(4)*. Responses ranged from 60% to 88% of the genotypes from a given open-pollinated family producing shoots, with the number of shoots produced per seed ranging from 13 to 25 (**Table I**). However, long-term culture of these shoots *in vitro* proved difficult. Only 12–20% of the shoots maintained in culture survived over a six-month period, and only 25% of the surviving shoots could be rooted *in vitro*. We also found that both long-term survival in culture and rooting of shoots *in vitro* or in the greenhouse were dependent upon the culture parameters used. It was evident in these studies that family had an influence on shoot production, although all families tested produced shoots. The lowest response for a given family was 60% of the genotypes responding. We did not examine the extent to which propagation ability was correlated with traits in the field such as height growth or volume.

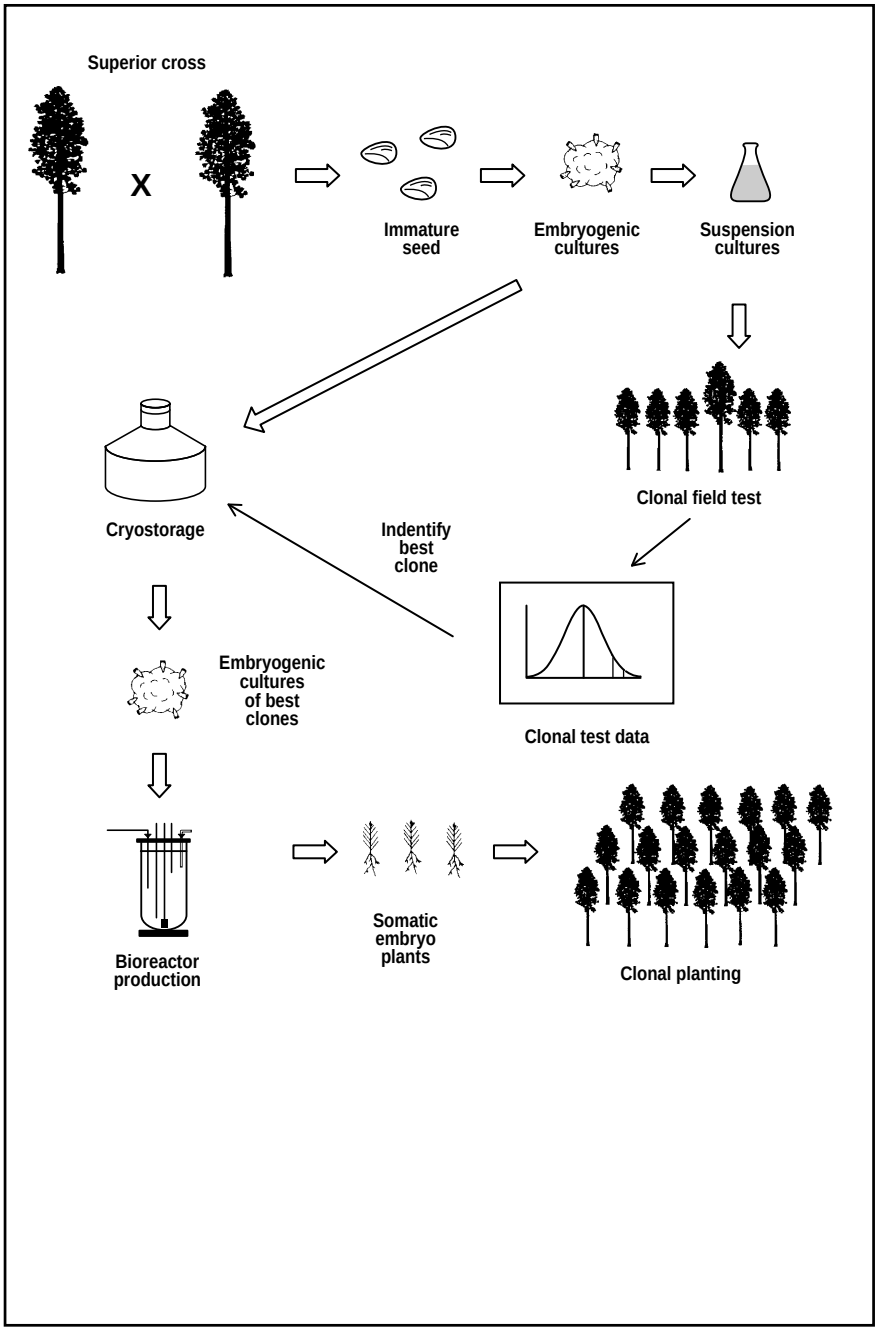
In recent studies, our approach with micropropagation systems in loblolly pine has primarily been toward optimizing the system for the widest range of genotypes. We envision that this system will interface with our rooted cutting project for the maintenance of juvenility during

II. Development of stage three somatic embryos from 13 genetically unique embryogenic suspension culture lines of loblolly pine

Line code	*Embryos developed**
A	11
B	198
C	202
D	422
E	475
F	754
G	791
H	926
I	2833
J	3471
K	3473
L	3520
M	4537

*Total embryos produced over a nine-week period from 24 mL of suspension cultures plated as 3 mL/plate on a gel-solidified development medium in petri dishes.

3. Using somatic embryogenesis and cryopreservation to produce and maintain clones during a clonal field test. Embryogenic tissues are derived from immature seeds from a cross of genetically superior trees. These cultures can then be multiplied in suspension culture and placed in cryostorage. After the clonal test, superior clones are retrieved from cryostorage, proliferated in bioreactors, and deployed in clonal plantings.

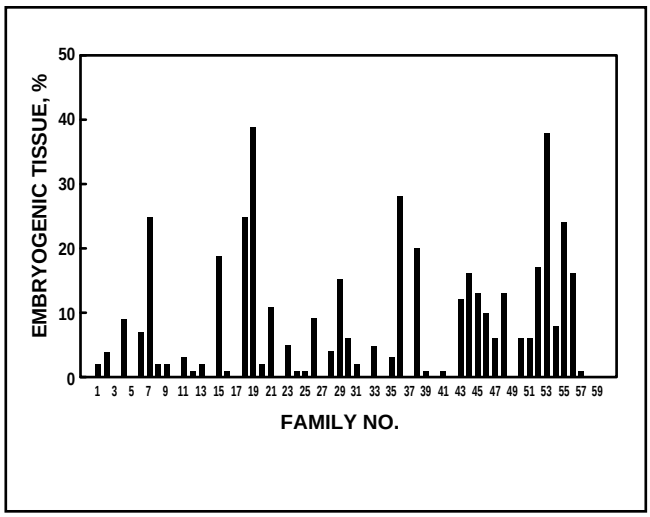


clonal testing or will be used as a component of a rejuvenation process (**Fig. 2**). Our goal is to keep as many genotypes as possible in a juvenile state and to recover those genotypes after a clonal test.

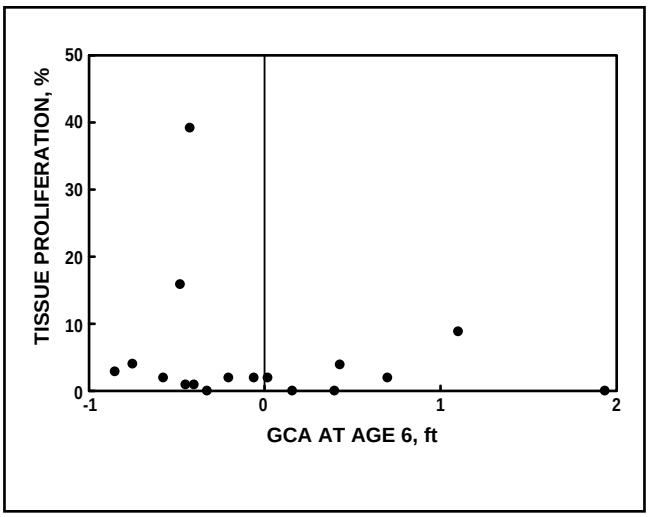
We are currently testing the *in vitro* maintenance of juvenility. During the research phase, we are using a seedling-based model system. Shoots are removed from young seedlings and placed in the tissue culture system. The goal is to develop a system that will maintain shoots in a juvenile state during the span of the clonal test. These shoots would then be rooted and sheared to produce hedges for rooted stem cutting production. Our efforts have been successful; we can now achieve 100% survival for all families placed in our micropropagation system while maintaining a juvenile morphology during culture. Most of our effort is now focused upon rooting shoots derived from the micropropagation system in the greenhouse.

Our research has demonstrated that the micropropagation system for loblolly pine can be optimized so that our best genetic material will not be eliminated by the process. We can routinely propagate around 80% of the genotypes within a family when placed into culture and mass produce those for subsequent greenhouse rooting for the production of hedges. We do not yet know to what extent our ability to root micropropagules of loblolly pine may be limiting, however. Results from our research, and work conducted by others, indicates that rooting percentages of micropropagules should not significantly reduce the number of genotypes that can be propagated *(8)*. For example, in our rooted cut-

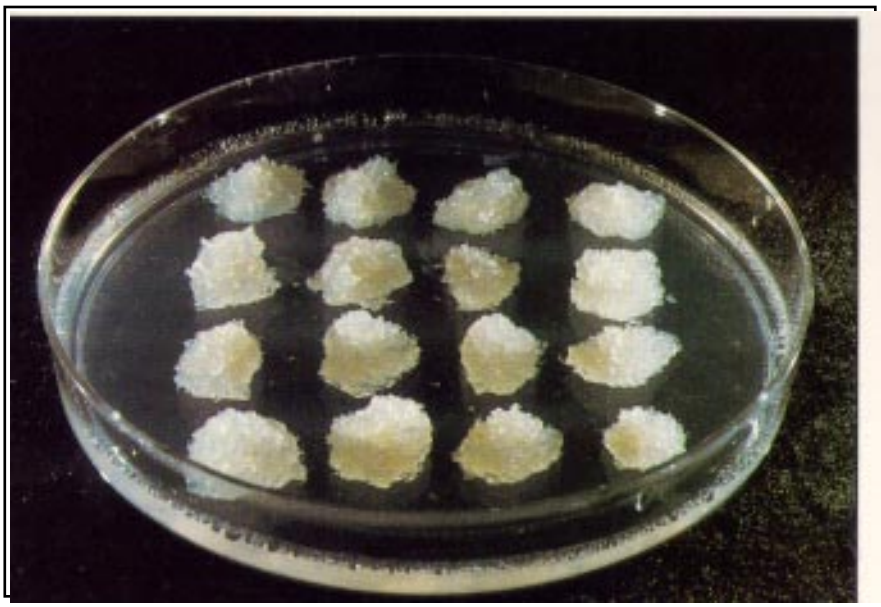
4. Embryogenic tissue proliferation from immature seeds of 59 families of loblolly pines.Tests were conducted over a number of years. Data are expressed as a percentage of immature seeds producing embryogenic tissues and are based on experiments containing at least 48 seeds per family.



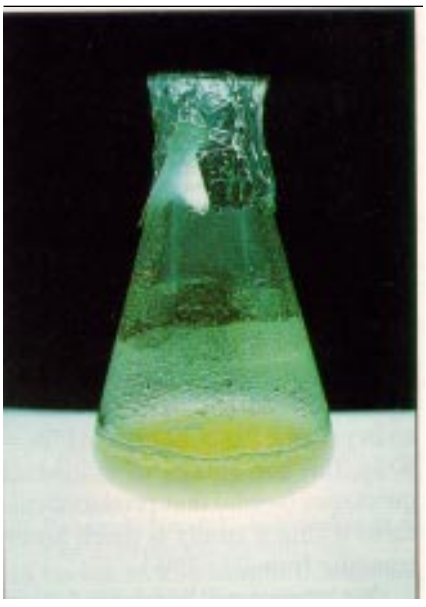
5. Embryogenic tissue proliferation from immature seeds of 17 open-pollinated loblolly pine families of various general combining abilities (GCA) for height. GCA values were calculated as the deviation from the mean (in feet) of the tested population at age six. Data are expressed as in Fig. 4.



6. Embryogenic tissue culture of loblolly pine growing on a gelled medium. Cultures composed of masses of tissue derived from immature seeds of loblolly pine are proliferated in petri dishes.



7. Liquid suspension culture of loblolly pine derived from embryogenic tissue cultures. The suspension cultures are subdivided weekly and, due to rapid growth, the embryogenic cells can be multiplied very quickly.



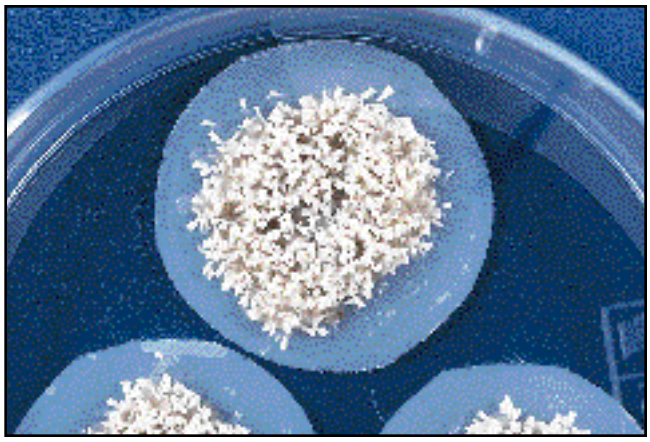
ting program we can now achieve 70–90% rooting using juvenile shoots from container-grown seedling hedges. Therefore, micropropagation for maintaining clones in a juvenile state should not severely narrow the genetic base of the material that is to be deployed in loblolly pine.

Somatic embryogenesis

Somatic embryogenesis research is also aimed toward the deployment of superior clones of loblolly pine (**Fig. 3**). Like micropropagation, we envision that this system will integrate with a genetic improvement program. Our goal is to identify and deploy clones from our best genetic families using this process.

As in organogenic systems, genotype has had a profound influence on the ability of material to be produced by somatic embryogenesis. In conifers, the genotypic specificity of plant regeneration via somatic embryogenesis has been well docu-

8. Development of hundreds of somatic embryos from a liquid culture medium after plating on a solid development medium. These somatic embryos were produced from 1mL of liquid culture.



9. Field planting of loblolly pine somatic seedlings. Somatic embryo plants are on the left and conventional seedlings are on the right. THis field planting was established in 1992 and the photo taken in 1994.

mented. Tremblay *(9)* has reported that somatic embryogenesis of white spruce was significantly affected by the seed provenance. Cheliak and Klimaszewska *(10)* found significant differences among open-pollinated families in black spruce. They found that up to 85% of the families used could be successfully cultured through somatic embryogenesis. However, the proportion of explants (genotypes) that produced embryogenic tissue within a family was low (0–18%). We have found this same type of response in loblolly pine. **Figure 4** shows the percentage of embryogenic tissues that were produced from seeds of 59 different families of loblolly pine in our embryogenic system. While the overall number of families that produced embryogenic tissue is high (44/59 = 80%), the percentage of individual genotypes (seeds) that produced cultures within a family is much lower (ranging from 0 to 39%).

Our current method for inducing embryogenic tissue cultures in loblolly pine uses immature seeds. Seeds are collected from cones in mid-July and are placed on induction medium. Megagametophytes extrude embryogenic tissues which then proliferate and can be subcultured. Proliferating embryogenic tissues can then be induced to form somatic embryos. The embryos are then germinated, planted in the greenhouse, and later established in field plantings. So far, we have established four field plantings of loblolly pine somatic embryo plants. Two of these are comparing growth of somatic embryo plants with growth of seedlings from the same families.

As Fig. 4 shows, a major obstacle is achieving adequate extrusion and proliferation rates from a wide range of genotypes. While up to 40% of the explants in some families will produce embryogenic tissues, this level of response is rare. The average response for the families shown here is about 7%. The median value for response is 3%. Also, in this group, 20% of the families that were used failed to produce any tissue at all.

Figure 5 shows tissue proliferation data plotted against general combining ability (GCA) predictions of age-six height for 17 different open-pollinated families. GCA is a measure of the mean performance of progeny and indicates the relative value of parents for future breeding or deployment. The same families were also involved in embryogenic tissue initiation and proliferation tests. The figure suggests that there is not an association between the GCA of a family and its ability to produce embryogenic tissues. There were three families where 10–40% of the seeds cultured gave rise to proliferating cultures, but overall tissue induction and proliferation percentages were very low. Some of the best tissue-producing families had low GCA values. However, Fig. 5 shows that there are families with high GCA prediction values that produced 3–15% proliferation. Our goal is to increase these percentages across all families.

If somatic embryogenesis is to be used for clonal deployment, there is probably a certain threshold value for successful extrusion, proliferation, and development that should be obtained across most of the families in a genetic improvement program. This may be especially important for genetically superior families from which one is most likely to find superior clones. If somatic embryogenesis provides only a few genotypes, a serious loss of genetic diversity could occur in an operational breeding-cloning program *(11)*. Ekberg *et al. (12)* have estimated, in *Picea abies*, that there should be minimal risk of directional selection for growth rhythm traits when only progeny that form embryogenic cultures are used. We do not know to what extent this is true in loblolly pine.

For loblolly pine, we know that we must increase the rate of embryogenic tissue establishment and

maintenance across a range of our best genetic families. We are therefore continuing to work toward increasing the percentage of genotypes that perform in the system from all of our families. Just as our strategy has been to achieve a higher percentage of genotypes performing in the micropropagation system, so it is our strategy in the embryogenesis system. Park *et al. (11)* have shown that for some families the percentage of explants becoming embryogenic can be increased by using improved treatment combinations. We have encouraging evidence that this can be done in loblolly pine as well.

In the families that do produce embryogenic cultures a great deal of success has been achieved. Recently we have begun producing thousands of embryos from liquid suspension cultures. Again, just as in tissue proliferation, the development of embryos from liquid suspension cultures is dependent on the genotype. **Table II** shows the production of somatic embryos from 13 different suspension culture lines of loblolly pine. We have found that nearly all embryogenic tissue cultures that grow on a gelled medium **(Fig. 6)** will grow in a liquid suspension culture system **(Fig. 7)** and retain their ability or inability to produce fully developed somatic embryos. Generally, embryo quality from suspension culture is quite good; around 40% readily germinate.

We see variation in the genotypes from which embryogenic cultures can be established as well as in the numbers of embryos that can be developed among the lines that are established. This has been documented in embryogenic callus cultures of conifers *(13)*, and we have found the same phenomenon in liquid cultures. If deployment of plants through the embryogenic process is to become routine, we must find ways to deal with this variation or devise methods for narrowing this variation. Our major objective now is to get a higher proportion of the lines that can be successfully maintained in liquid to produce a minimum number of embryos.

As in other conifer systems, we have seen different embryo morphologies in our liquid culture systems. We see the polar, solar, and undeveloped patterns of cell proliferation as shown by Jalonen and von Arnold *(14)* for spruce. Generally, as in spruce cultures, we find that those cultures having distinct, highly developed suspensor-type cells with prominent head cells produce advanced-stage embryos when plated on a gel-solidified development medium **(Fig. 8)**. Cultures that have no early embryos present or that have a solar pattern with no evident suspensors give rise to few if any somatic embryos, and most of the embryos developed are malformed.

Our first embryos from liquid suspension cultures are now going into field tests, and we are confident that the production of somatic embryo plants from liquid suspension can be routine (at least for the families that respond to the system). Field test establishment of somatic plants from both embryogenic tissues grown on a gelled medium and from liquid cultures is also considered nearly routine **(Fig. 9)**.

Conclusion

Tissue culture systems in loblolly pine have developed to a point where they are becoming useful tools that can interface with traditional breeding programs and clonal forestry. Applications such as micropropagation and the maintenance of juvenility, which were once greatly limited by genotype-specific responses in culture, are showing promise for interfacing with clonal deployment via hedge production and rooted stem cuttings. Somatic embryogenesis from immature seeds of loblolly pine is routine from a range of families, and large numbers of somatic embryo plants can be produced for routine field planting. Limitations due to low initiation frequencies and the genetic specificity of genotype of starting material are important problems. However, we can use responsive families and clones to optimize portions of the embryogenesis system while we solve these problems. We can also assess the quality of plants in the field using both micropropagation and somatic embryogenesis tissue culture systems that are under development.

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