

Genetics of Somatic Embryogenesis in Loblolly Pine

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ABSTRACT

Clonal propagation methods must be adaptable to trees from a wide range of genetic backgrounds to meet objectives of tree improvement programs. Recently, major advances have been made in somatic embryogenesis (SE) of loblolly pine, making it a promising method for the implementation of clonal forestry for the Southeastern United States. However, initiation of SE cultures is highly variable among diverse loblolly pine families and thus it remains a bottleneck to the implementation of this technology. We investigated genetic control of SE culture initiation and naturally occurring polyembryony, using a factorial mating design. The two stages of the SE culture initiation process, i.e., extrusion of zygotic embryos and formation of somatic embryos, are both under genetic control. While initiation is primarily under additive control, extrusion is controlled both by additive and non-additive gene action (maternal and reciprocal effects). We also found that individual trees can affect initiation differently when used as mother-trees vs. as pollen parents.

Our study also included an analysis of polyembryony, a naturally occurring process by which multiple embryos are formed early during development of pine seeds. Only one of the embryos reaches maturity in each seed. The number of immature embryos formed in a seed was highly variable among full-sib families and was under strong genetic control, primarily but not exclusively of the mother-tree. Our analysis suggested that variation of the number of immature embryos per seed is linked to cleavage polyembryony rather than variation in the number of fertilized archaegonia (simple polyembryony). We also studied the relationship between the number of zygotic embryos per seed and initiation of SE cultures. Our data indicated that the number of zygotic embryos per seed may be a driver of initiation and thus could be a useful indicator of initiation potential.

INTRODUCTION

The implementation of clonal forestry is an opportunity to achieve major benefits for the forest products industry in coming years. Clonal forestry is expected to increase productivity by some 20 to 30% over current practices, which deploy seedlings obtained by open-pollination of selected mother-trees. Such an increase in productivity will increase competitiveness due to reduced fiber cost from the shorter time to harvest (quicker return on investment) and reduced land-base needs to produce the fiber. Another advantage of clonal forestry is increased uniformity, which will lead to higher conversion to product, higher mill performance, and reduced waste (Libby and Rauter, 1984). Unfortunately, significant barriers still exist to large-scale clonal multiplication and plantation of forest trees important to U.S. industry, namely

economic barriers (high cost relative to expected returns) and biological barriers (robust propagation methods).

Loblolly pine is the most widely planted tree by the US Forest Products Industry. It is also the object of several genetic improvement programs, which aim to develop high performing trees adapted to diverse plantation needs. Clonal propagation methods must therefore be adaptable to trees from a wide diversity of genetic backgrounds. Somatic embryogenesis is becoming the method of choice for clonal propagation of spruce, but development of somatic embryogenesis methods for southern pines has lagged behind. In particular, initiation of somatic embryogenic cultures from diverse loblolly pine genotypes continues to be a bottleneck, even with recent breakthroughs in method development (Becwar and Pullman, 1995). The objective of our research was to develop new information that will directly overcome this limitation and help to establish somatic embryogenic cultures of numerous and diverse loblolly pines.

MATERIALS AND METHODS

The goal of this study is to investigate the genetic control initiation of somatic embryogenesis from immature loblolly pine seed. We analyzed the genetic variability of extrusion of zygotic embryos from the megagametophyte, initiation of somatic embryos proliferation, and naturally occurring variation in polyembryony within pine seed (see concept definitions below). A diallel mating design was selected, and crosses were carried in Winter 1998 by Westvaco, resulting in 33 full-sib families (Table 1). The parent-trees were previously characterized as producing seed with high, intermediate, and low potential for initiation (Becwar and Chesik, 1994). Open-pollinated seed from the mother-trees were also analyzed to control for artificial vs. wind pollination.

- **Initiation Media.** A single medium was used and was composed of ½ P6 salts, activated carbon 100 mg/L, 1.5% maltose, 2% myo-inositol, 500 mg/L case amino acids, 450 mg/l glutamine, 2 mg/l NAA, 0.45 mg/l BAP, 0.43/l kinetin, and 2 g/l gelrite.
- **Extrusion.** Extrusion is defined as one or more zygotic immature embryos moving from within the megagametophyte out onto the initiation medium.
- **Initiation.** Initiation occurs when one or several extruded zygotic embryos begin to form new proliferating embryogenic tissue. In this study, initiation was considered successful if at least three somatic embryos were present or if a mass of proliferating cells typical of somatic embryogenic cultures was present after 9 to 10 weeks on the initiation medium.
- **Polyembryony.** Multiple embryos (polyembryony) are formed early during development of pine seeds, although only one embryo reaches maturity (Ferguson, 1904). Each seed forms one to several archaegonia (usually 1 to 4), each containing an egg cell. Each archaegonium may host a separate fertilization event (simple polyembryony). Each of the resulting 1 to 4 embryos within a seed divide into four embryos soon after fertilization (cleavage polyembryony), thus generating up to 16 embryos per seed (Skinner, 1992). A dominant embryo emerges from this cohort of embryos, while the other (subordinate) embryos soon stop developing.
- **Number of Zygotic Embryos per Seed.** In this study the number of embryos per seed, determined by aceto carmine staining followed by microscope examination,

is the total number of post-cleavage embryos (dominant and subordinate) present in the seed at the beginning of an initiation experiment.

- **Statistics.** Analyses of variance were carried out with the statistical analysis software *Statgraphics* (Manugistics). The percent extrusion and initiation data were transformed prior to analysis using a $\sqrt{\arcsin}$ transformation. Quantitative genetic analysis was carried out with the DIALL statistical analysis program (Park and Bonga, 1993) to identify genetic variance components for initiation and extrusion.

RESULTS

Variation Among Full-Sib and Half-Sib Families

The initiation rates showed extensive variation among full-sib and half-sib families, ranging from 0% to 32% and averaging at 11% (Table 1). Analysis of this variation indicated that both the mother-tree and the pollen parent had highly significant effects, although the effect of the mother-tree is more pronounced (Table 2). Beneficial pollen-parent effects were observed with two trees, C5 and E5 (Table 1). Seed from crosses with C5 or E5 pollen had significantly higher initiation rates than average, and higher than the open-pollinated seed. Interestingly, Tree E5 gave low initiation as a mother-tree but promoted initiation as pollen-parent, suggesting differences between maternally acting genes and paternally inherited genes. Pollination with three of the trees (B5, D5, and G5) suppressed initiation. This was most apparent when crossed with the “good” mother-trees C5 and F5.

Significant differences were observed among reciprocal crosses. For each pair of trees that were mated, two different crosses may be considered, e.g., A X B or B X A, where the first tree is the mother and the second is the father. On average there was a 95% difference between the two crosses, i.e., the lowest of each pair gave an average of 8.3% and the highest of each gave 16.2% initiation. The average for all families was around 11%; therefore, the ability to select the most favorable crosses could give an increase of 46% efficiency over no selection.

TABLE 1. Average initiation rates (%) of full-sib and open-pollinated families.

	Father (Pollen Source)								Mean (s.e.)
Mother	A5	B5	C5	D5	E5	F5	G5	O-P	Mother
A5		4.1	11.3	3.0		7.1	5.1	6.1	7.3 (1.39)
B5	5.0		24.9	6.3	8.4	0	1.0	6.1	6.9 (1.27)
C5	23.1	15.5		14.6	30.1	30.2	15.5	26.1	23.7 (1.26)
E5		3.0	8.3			8.3	8.3	4.0	6.4 (1.54)
F5	16.6	cont.*	32.4	10.4	27.4		10.4	23	19.4 (1.39)
G5	3.0	1.0	5.3	7.4	10.25	2.0		2.7	3.9 (1.29)
Mean (s.e.)	9.8 (1.70)	7.6 (1.70)	18.8 (1.50)	7.4 (1.50)	16.8 (1.71)	11.1 (1.50)	6.6 (1.49)	11.4 (1.37)	<i>Grand Mean 11.1</i>

*cont. Contamination prevented evaluation of this family

TABLE 2. Analysis of variance of initiation rates (type III sum of squares)

Source	Sum of Squares	Df	Mean Squares	F-Ratio	P-Value
MAIN EFFECTS					
Mother-Tree	16134.1	5	3226.8	37.02	0.0000
Father-Tree	5092.3	7	727.5	8.34	0.0001
RESIDUAL	25191.6	289	87.17		
TOTAL (Corrected)	45860.2	301			

All F-Ratios are based on the residual mean square error.

Genetic Effects – Extrusion and Initiation

Variance components were estimated for extrusion and for initiation (Table 3). In both cases the variance component due to general combining ability (σ^2_{GCA}) was significant, explaining 15.8% of the total variance for extrusion and 26.0% for initiation. In contrast, extrusion and initiation had striking differences for other variance components. The variance component for specific combining ability (σ^2_{SCA}) accounted for 10.4% of the variance in initiation, but was negligible for extrusion. In addition, variance components for maternal (σ^2_{MAT}) and reciprocal (σ^2_{REC}) effects were large for extrusion (19.8% and 17.1%, respectively), but non significant for initiation. Therefore, initiation was primarily under additive genetic control, while extrusion was under additive as well as non-additive genetic control (maternal and reciprocal effects).

TABLE 3. Estimated variance components and the percentage of total variance for the rate of extrusion of zygotic embryos and for initiation rate of embryogenic tissue.

Variance component	EXTRUSION		INITIATION	
	Estimate	Percentage	Estimate	Percentage
σ^2_{GCA}	91.0*	15.8	124.7*	26.0
σ^2_{SCA}	12.8	2.2	50.1*	10.4
σ^2_{MAT}	114.1*	19.8	25.5*	5.3
σ^2_{REC}	98.4*	17.1	7.3	1.5
σ^2_e	261.0	45.2	272.6	56.8
Total	577.2	100.0	480.2	100.0

* Significant at $P < 0.01$

The breakdown of variance components indicating different types of genetic control suggested further investigation of the relationship between extrusion and initiation. Extrusion is required for initiation because somatic embryogenic masses are initiated from zygotic embryos that are extruded from the megagametophyte. Initiation increased significantly only above a relatively high threshold of extrusion, around 40% to 45%. In practice, this could mean that the improvement of initiation should focus on conditions that will cause more extruded zygotic

embryos to give rise to somatic embryos. Alternatively, extrusion may be considered as limiting initiation.

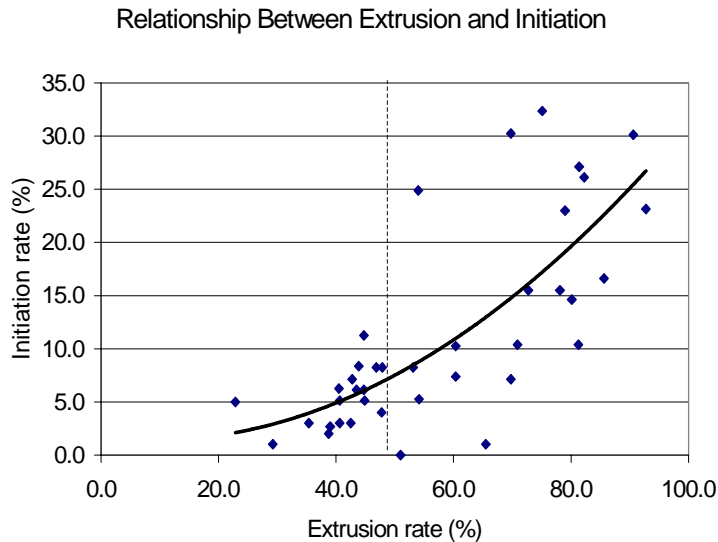


Figure 1. Relationship between extrusion and initiation.

Each data point represents a full-sib family (33 families) or an open-pollinated family (6 families). The trend line and a "threshold" line at ~50% extrusion were added for illustrative purposes only.

Variation in the Number of Zygotic Embryos per Seed

Previous studies in our laboratory showing that polyembryony is variable in loblolly pine suggested that the average number of embryos in immature seed was under genetic control of the mother-tree. We examined the number of archaegonia (post-fertilization) in open-pollinated seed from each of the mother-trees to determine if it is linked to the number of embryos per seed. We also examined the number of post-cleavage embryos in immature seed from open-pollinated seed and each of the full-sib families included in this study.



Figure 2. Polyembryony in loblolly pine. Six early-stage embryos, shortly after cleavage.

The number of fertilized archaegonia per seed showed very little variation among mother-trees; means ranged from 2.30 to 2.45. The observed number of post-cleavage embryos in immature seed from full-sib families varied much more significantly, ranging from 5.0 to 10.9 and averaging at 7.4 (Figure 3). Both the mother-tree and the pollen-parent effects for this trait

were highly significant, although the mother-tree effect was more pronounced (Table 4), supporting the hypothesis of maternal control over this trait. In exception to this finding, Tree E5 significantly increased the number of embryos per seed when used as pollen parent. Pollination with the six other trees gave an average number of embryos per seed ranging from 6.7 to 7.6, whereas, pollination with Tree E5 gave an average of 9.2 embryos per seed. This phenomenon is striking because E5 was the mother-tree with the lowest average number of embryos per seed.

TABLE 4. Analysis of variance of number of post-cleavage embryos per seed (type III sum of squares).

Source	Sum of Squares	Df	Mean Squares	F-Ratio	P-Value
MAIN EFFECTS					
Mother Tree	448.0	5	89.6	7.29	0.0000
Father Tree	320.9	7	45.8	3.73	0.0006
RESIDUAL	9406.6	765	12.3		
TOTAL (Corrected)	10392.4	777			

All F-Ratios are based on the residual mean square error.

The observed number of post-cleavage embryos per seed was lower than the expected number based upon the number of archaegonia, except for crosses with Tree E5 as a pollen parent. Our observations indicate that the variability in the number of embryos per seed among mother-trees was due either to (1) more rapid degeneration of subordinate embryos in some trees or; (2) a deficiency in cleavage poly-embryony, i.e. fewer embryos undergoing cleavage or cleavage resulting in fewer than 4 viable embryos.

Relationship Between the Number of Zygotic Embryos per Seed and Initiation

We investigated the relationship between the number of embryos per seed and the initiation rate for all the families in this study. We aimed to test the hypothesis that genetic factors that control initiation may act by influencing the number of embryos formed in each seed. Therefore we searched for correlation between the number of embryos per seed and the rate of initiation. The relationship between the number of embryos and initiation rate appeared to be complex and did not follow a highly consistent pattern. Higher initiation rates were associated with greater number of embryos per seed; however, a greater number of embryos per seed was not sufficient to obtain higher initiation rates. More specifically, seed lots giving high initiation rates had more embryos per seed, but not all seed lots with many embryos per seed gave high initiation rates. The effect of pollination with Tree E5 was apparent, significantly increasing both initiation rate (Figure 4) and the number of embryos per seed (Figure 3). A better understanding of this relationship may require reducing the genetic variation in order to decrease the experimental complexity.

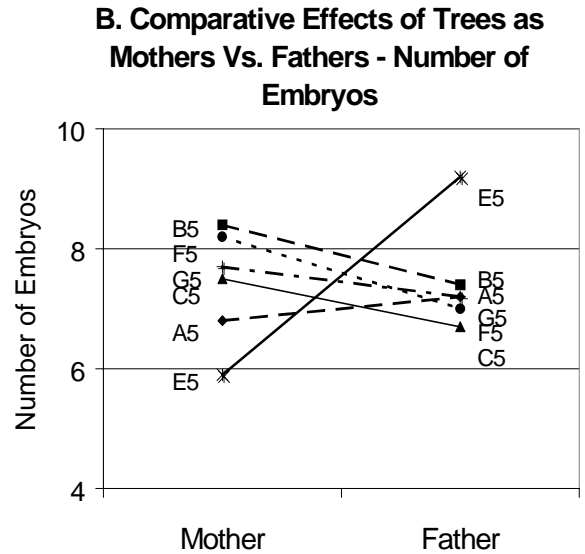
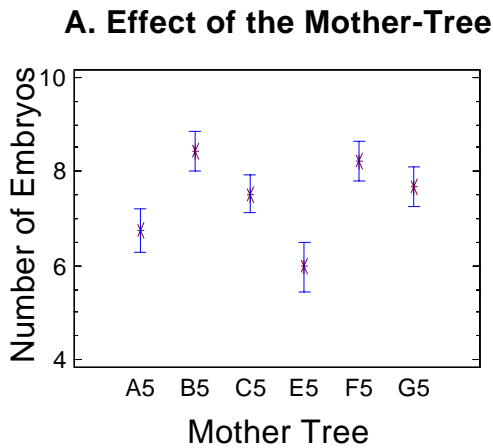


FIGURE 3. Variation in the number of post-cleavage embryos in immature seed.

A. Mean and 95% LSD intervals for each mother-tree calculated from all control-pollinated families and one open-pollinated family **B.** Compared means of each tree when used as a mother-tree or father (pollen parent).

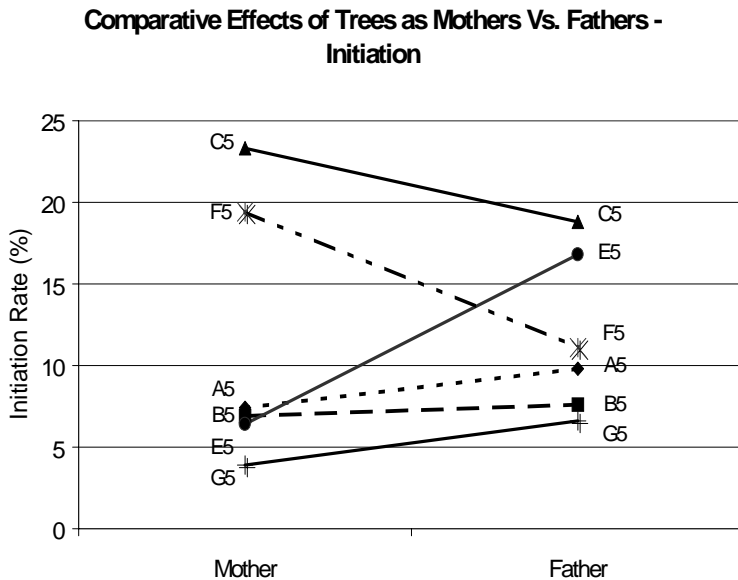


FIGURE 4. Comparative effects of trees as mothers vs. fathers for initiation.

Each point represents a mean for a mother or father-tree. Lines connect the two means obtained for a given tree as mother and father.

DISCUSSION AND CONCLUSIONS

Potential Methods for Improving the Initiation of Somatic Embryogenic Cultures

The findings from these studies lead to recommendations that will help maximize initiation rate, establishment of SE cultures, and genotype capture. Recommendations include:

1. General combining ability was the largest variance component for initiation. Therefore controlled pollination with mother-trees that produce seed with good initiation potential should produce high rates of somatic embryogenic culture establishment. In our study, selection of the most favorable crosses would give a 46% increase in efficiency over no selection.
2. The pollen parent can occasionally be used to enhance the initiation potential of mother-trees that are recalcitrant to initiation of somatic embryogenic cultures.

Savings/Improvements

We provide an example where initiation rate would be improved by 46% if our recommendations are followed. These improvements in efficiency would directly translate to cost reductions in the establishment of clonal propagation methods. Such increases also move somatic embryogenesis significantly closer toward commercial application. For clonal selection and deployment to be effective, somatic embryogenic cultures of diverse genotypes must be established. Our recommendations will greatly improve the ability to meet this goal. Specifically, many trees produce seed that are recalcitrant to somatic embryogenesis, and we showed that cultures could quite readily be established with “recalcitrant trees” if used as a pollen-parent.

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