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Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding

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Abstract Major advances have been achieved in somatic embryogenesis (SE) of loblolly pine, making it a promising method for the implementation of clonal forestry. However, the frequency of initiation of SE cultures, which is highly variable among loblolly pine families, needs improvement to further advance the implementation of this technology in conjunction with tree breeding. Genetic control of SE initiation was investigated using a diallel mating design with six parent trees. The results showed that SE initiation is under the control of strong genetic additive effects, as 42% of the total variance was explained by the variation due to general combining ability effects. The variation due to maternal effects explained a moderate proportion of the total variance, whereas other components of variance had small but significant effects. The conclusions regarding the strong genetic control of SE initiation were drawn from two independent experiments in which consistent results were obtained with seed from the same controlled pollinations but using entirely different procedures. Practical implications for breeding and clonal propagation were tested in independent experiments with targeted matings. Our results indicated that large improvement in SE culture initiation could be achieved in a predictable manner by selecting the most favorable female parent, or in some cases, a favorable male parent.

Abbreviations op: open-pollinated · cp: control pollinated · SE: somatic embryogenesis · GCA: general combining ability · SCA: specific combining ability

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Introduction

Clonal forestry provides opportunities for the forest products industry to achieve greatly improved forest productivity and greater uniformity of raw material [12]. Furthermore, clonal forestry offers flexibility to adapt to changing breeding goals or program [14]. However, for many commercial softwood trees, large-scale clonal multiplication of planting stock is not in widespread use, partly because of the high costs relative to expected returns and the lack of robust propagation methods. Somatic embryogenesis (SE) is a clonal propagation method that holds great promise for overcoming these hurdles for softwood trees [1]. SE is amenable to high-throughput production, necessary to reduce production costs. The most important advantage of SE is that embryogenic cultures may be maintained practically indefinitely through cryopreservation [17]. Long-term maintenance of propagation potential is essential because several years of field-testing are required to select the best clones before their deployment in commercial plantations. Finally, in order for SE to be an integral part of tree improvement programs, it must be sufficiently robust to include a wide range of genotypes [14] to maintain a high level of genetic diversity in the breeding population. Consequently, variability in SE initiation should be examined.

Loblolly pine (*Pinus taeda*) is the tree most widely planted by the US forest product industry with approximately 1.4 billion trees planted annually [23]. The development and use of SE in loblolly has progressed significantly in recent years. However, there still exists large variation in SE initiation among different families in loblolly pine [13, 18]. Genetic variation has been shown to represent a major source of variability in SE of crop plants such as maize (*Zea mays*; [24]) and *Brassica* species [25] and in spruce species [5, 15, 16]. In white spruce (*Picea glauca*), culture initiation was clearly shown as the step of the SE process under the strongest genetic control [16]. In most species, genetic control has been assigned primarily, but not exclusively, to additive genetic effects. Dominance effects were also reported in *Brassica* [25], whereas maternal and heterosis effects were found in maize [24]. Significant reciprocal effects, which are related to the direction of crosses whether the parents are used as the male or the female, were observed in spruce [15] and loblolly pine [3]. As a whole, the factors controlling SE are multiple, and the nature and extent of genetic control appear to vary depending on the type of explants and the experimental conditions, among other things.

The objective of our research was to conduct a quantitative assessment of the genetic control of SE culture initiation in loblolly pine using progenies from diallel crosses and factorial matings and compare the results with those obtained in spruce [15]. As opposed to spruce, where mature seeds are suitable for SE initiation even after years in storage, only a narrow window during early zygotic embryo development is optimum for loblolly pine. Also, unlike spruce, pine seed development involves both simple and cleavage polyembryony, resulting in the formation of multiple zygotic embryos during early development, followed by the degeneration of all but one embryo during mid to late seed development. In light of these differences, our hypothesis was that significant differences are likely to exist in genetic control of SE initiation in pine compared with spruces, although some similarities appear to be present. The second objective was to evaluate whether a better understanding of the genetic control in SE initiation could be applied through breeding to improve SE initiation and thus increase the capture of a wide diversity of genotypes from a breeding program.

Materials and methods

Plant material

Three distinct experiments were conducted to investigate the genetic control of SE initiation. These experiments used three separate sets of plant material, as well as experimental procedures and designs that differed from one experiment to the next. The experiments were designated one (1) through three (3) for easy reference; plant materials and procedures are described separately for each experiment.

Experiment 1: six-tree diallel mating This experiment analyzed SE initiation in seed from a complete diallel mating using six parent trees (trees 1 to 6) including reciprocals, but excluding selfs, for a total of 30 control-pollinated crosses. Westvaco made crosses with trees in their breeding program and collected control-pollinated (cp) and open-pollinated (op) cones. Seeds from the op mother trees were also tested for SE initiation. Two parallel SE initiation experiments were carried out, one at the Institute of Paper Science and Technology (IPST; Experiment 1A) and one at Westvaco (Experiment 1B), both using seed from the same controlled pollinations, but different laboratory procedures (see below).

Experiment 2: 3×3 factorial mating This experiment aimed to verify the effect of tree 5 as pollen-parent (male) with females having different SE initiation potentials. The mating design involved three maternal parents and three different pollen parents with previously assessed initiation potentials, to produce nine different cp families. Westvaco made the crosses and collected the cones.

Experiment 3: three-tree diallel mating The mating design of this experiment was a diallel with three parent trees that had previously shown high, intermediate, and low (trees 9, 10, and 11, respectively) SE initiation potential as op mothers. Seeds were tested from the resulting six cp crosses, along with op seed from each of the three mother trees. Mead Corp. and the Timber Co. made crosses from trees in their breeding programs and collected cp and op cones.

Seed explants Megagametophytes with the immature zygotic embryos intact were used as explants in all of the experiments. Immature seed cones were collected when dominant zygotic embryos were at the precotyledonary stage, which correlated with high initiation of SE in *P. taeda* [2]. The optimum time for the collection of seed was assessed separately for each mother tree to target the most suitable developmental stage. For any given experiment, all of the seed cones from a cp or op family were collected on the same date, but the different families were collected over a period of approximately 10 days. Seed cones were held at 4°C for 2–3 weeks before extracting seed for culture.

Culture initiation medium and experimental design

Experiment 1 Seed explants from the six-tree diallel mating were tested in two parallel SE initiation studies conducted in two laboratories—one at IPST (Experiment 1A) and one at Westvaco (Experiment 1B). The experiments each used a subset of the cones obtained from the same control pollinations, but each lab used different seed sterilization procedures, culture medium, and vessels for SE initiation, representing standard protocols in each laboratory, as well as different experimental designs.

Experiment 1A (IPST) The culture medium 505 formulation for Experiment 1A contained inorganic salts and vitamins of Pullman and Johnson [18], *myo*-inositol (20 g/L), activated carbon (50 mg/L), case amino acids (500 mg/L), naphthalene acetic acid (NAA; 2.0 mg/L), N^6-benzyladenine (BA; 0.45 mg/L), linetin (0.43 mg/L), maltose (15 g/L), and Gelrite (2.0 g/L). The pH of each medium was adjusted to 5.7 before autoclaving at 121°C for 20 min. Finally, 2 mL of medium was poured into the wells of 24-well cell cluster plates (Costar no. 3526).

Seeds extracted from cones were rinsed for 10 min in cold running tap water, surface cleansed 10 min in 10% Liquinox detergent with 2 ml/L Tween 20, and rinsed for 30 min under running tap water. Seed surface sterilization was done by immersion in a solution of 20% H_2O_2 for 10 min, followed by five rinses (5 min each) in distilled autoclaved water. Seeds were stored in sterile Petri plates on moist filter paper for less than 10 h before culturing.

An average of 80 randomly selected megagametophyte explants for each cross were placed in cell cluster plates and incubated in the dark. Each plate contained ten explants, each in an individual well, thus using a total of eight different cell cluster plates per family. The plates were randomly mixed and placed in the same area of the culture room. As eight plates were used for each family, they are considered to be replicates (each comprised of ten seeds). After placement on the plates, the cultures were incubated in the dark at 25°C.

The assessment of extrusion rate was carried out after 4 weeks in culture. Extrusion was defined as one or more immature zygotic embryos having moved from within the megagametophyte onto the culture medium: presence or absence was scored. Initiation of SE was scored as positive when at least three somatic embryos were present or a mass of translucent, proliferating cells typical of conifer somatic embryogenic cultures was present after no more than 9 weeks in culture. The extrusion and initiation were assessed for each explant in each plate and computed as percentages per plate (out of ten explants contained in each plate).

Experiment 1B (Westvaco) The culture medium formulation used in Experiment 1B was WV5 inorganic salts [6], DCR vitamins [8], *myo*-inositol (0.5 g/L), casein (0.5 g/L), 2,4-dichlorophenoxy acetic acid (3 mg/L), BA (0.5 mg/L), abscisic acid (10 mg/L; [9]), maltose (30 g/L), and Gelrite (1.5 g/L; [4]). The pH was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Each sterile plastic culture plate (Falcon 100×15 mm, no. 1029) contained 25 mL of gelled medium.

A total of eight randomly selected megagametophyte explants for each family were placed in 24 to 29 (mode 25) Petri dishes, which were considered as replicates. The plates were randomly mixed and placed in the same area of the culture room. After 8 weeks in culture, initiation rate was assessed. The number of responsive explants on each noncontaminated plate was counted by viewing each explant under a dissecting microscope at 8× magnification. An explant was scored as responsive if zygotic embryos, extruded from the megagametophyte, had vigorously proliferated embryogenic tissue with somatic embryos. The initiation rate was computed as a percentage of responsive explants per plate (out of eight explants contained in each plate).

Experiments 2 and 3 Experiments 2 and 3 used culture medium formulation 1,253 [20], a modification of medium 505 that contained *myo*-inositol (20 g/L), activated carbon (50 mg/L), case amino acids (500 mg/L), MES buffer (2(*n*-morpholino)ethanesulfonic acid) (250 mg/L), NAA (2.0 mg/L), BA (0.63 mg/L), kinetin (0.61 mg/L), silver nitrate (3.4 mg/L), guanosine 3′,5′-cyclic monophosphate, 8-Bromo-, sodium salt (4.5 mg/L), brassinolide (0.5 mg/L), biotin (0.05 mg/L), folic acid (0.5 mg/L), maltose (15 g/L), and Gelrite (2.0 g/L). The pH of each medium was adjusted to 5.7 before autoclaving at 121°C for 20 min.

The megagametophyte explants were cultured in plastic culture plates (Falcon 100×15 mm, no. 1029) containing 25 mL of medium, with ten explants per each culture plate as one experimental replicate. A total of 20 replicates were used in experiment 2, and between 40 and 60 replicates were used in experiment 3 (ten seeds per replicate in each case). Seed surface cleansing and sterilization and assessment of initiation rate were described in Experiment 1A.

Table 1 Combined analysis of variance of SE initiation data from Experiments 1A and 1B using the diallel mating

Source	*df*	Sum of squares	Mean squares	*F* ratio	*p* value	Estimated	Percent
Procedure (P)	1	329,350	329,350	71.9	0.0000	1,001.7	41.5
Families (F)	27	626,646	23,209	3.4	0.0019	529.2	21.9
GCA	5	367,268	73,453	26.9	0.0001		
SCA	8	21,726	2,715	0.4	0.9160		
MAT	5	210,998	42,199	14.3	0.0005		
RECP	9	26,654	2,961	0.4	0.9022		
P × F	26	114,338	4,398	7.9	0.0000	323.3	13.4
Error	811	453,653	559			559.4	23.2
Total	865	1,523,987		2,413.6			100.0

The significance test is based on Satterthwaite's [21] procedure
GCA general combining ability, *SCA* specific combining ability, *MAT* maternal *RECP* reciprocal

Table 2 Estimated variance components (standard deviation) and percentages of total variance for SE initiation in a six-tree diallel experiment

Variance component	Experiment 1A		Experiment 1B	
	Estimate (SD)	%	Estimate (SD)	%
$2\sigma_{GCA}^2$	249.4 (154.4)*	41.5	865.4 (478.5)*	48.6
σ_{SCA}^2	50.4 (31.7)*	8.4	36.6 (24.4)*	2.1
σ_{MAT}^2	25.5 (16.3)*	4.2	175.6 (100.5)*	9.9
σ_{REC}^2	7.6 (11.0)*	1.3	54.1 (29.1)*	3.0
σ_{ERR}^2	267.5 (27.4)	44.6	648.1 (36.7)	36.4
σ_{TOTAL}^2	600.4	100.0	1,779.8	100.0

Variance estimates are for general combining ability (GCA), specific combining ability (SCA), maternal (MAT), reciprocal (REC) and error (ERR) effects
*Significant at p=0.05

Data analysis

Statistical analyses were applied to SE extrusion and initiation frequency, which are respectively the percentage of positive extrusions and initiations per replicate plate. The SE extrusion or initiation rate was transformed by taking the arcsine value of the square root of the extrusion or initiation percentage ($sin^{-1}\sqrt{x}$) multiplied by 100.

The initiation data from Experiments 1A and 1B were analyzed together to determine the effects of different procedures and their interactions with genotypes (families), using the model:

$$Y_{hijk} = \mu + P_l + F_{ij} + PF_{lij} + e_{lijk}, \quad (1)$$

where Y_{lijk} is the kth observation of SE initiation of the cross between ith female and jth male using lth procedure; μ is mean, P_l is the lth procedure; F_{ij} is the ijth family; PF_{lij} is the interaction effects of lth procedure and ijth cross; and e_{lijk} is the random error component. The effect of crosses was further partitioned using the diallel model described below. After examining the effects of the procedure and their interactions with crosses, data were analyzed separately for each procedure using the model:

$$F_{ijk} = \mu + g_i + g_j + s_{ij} + m_i + m_j^{'} + r_{ij} + e_{ijk}, \quad (2)$$

where F_{ijk} is the kth observation of SE initiation of the crosses between the ith female and jth male parents; μ is the experimental mean; g_i (g_j) is the general combining ability (GCA) effect of the ith (jth) parent; s_{ij} is the specific combining ability (SCA) effects for the cross between the ith and jth parents; m_i is the maternal effect of the ith parent in contrast with jth parent, where $m_j'=-m_j$; r_{ij} is the reciprocal effect involving the ith and jth parents, where $r_{ij}=-r_{ji}$; and e_{ijk} is the random error component. Differences between reciprocal crosses are fit by the maternal and reciprocal effects in a manner analogous to the fitting of the sums of the reciprocal crosses by the general and SCA effects [22]. All the terms in the model, except the experimental mean, were considered to be random effects, and variance components were estimated. The significance of variance components was determined approximately using Satterthwaite's [21] procedure. The analyses of variance were carried out using a software program, DIALL [22], and the variance components were estimated. The analysis was applied to the percentage of extrusion assessed in the IPST procedure. Narrow sense heritability estimates were computed as previously described [15].

For Experiments 2 and 3, multi-factor analyses of variance were conducted assuming fixed genotypic effects, and least significant difference (LSD) multiple range tests for separation of means were carried out using the software STATGRAPHICS Plus (Manugistics, Rockville, MD, USA).

Results

Experiment 1

Genetic control of SE initiation

Combined analysis of variance for the 30 families derived from diallel crosses indicated that the initiation frequency of SE cultures varied significantly between procedures (Experiments 1A and 1B), among families (Table 1). The variance due to procedures accounted for 41.5% of total phenotypic variance, whereas that due to families accounted for 21.9%. Significant variance due to interactions between families and procedures was also found, accounting for 13.4% of the phenotypic variance. The mean

Table 3 Mean initiation percentages in Experiment 1 (six-tree diallel) of loblolly pine parents as female, male, mid-parent, and open pollinated (op) with the rankings in parenthesis

Parent	Experiment 1A				Experiment 1B			
	Female	Male	Mid-parent	op	Female	Male	Mid-parent	op
1	6.9 (5)	11.9 (3)	9.4 (4)	6.1 (3)	15.9 (6)	47.8 (3)	31.9 (5)	16.5 (6)
2	7.9 (3)	5.9 (6)	6.9 (5)	6.1 (3)	59.8 (2)	42.4 (5)	51.1 (3)	23.7 (4)
3	22.9 (1)	16.4 (2)	19.7 (1)	26.1 (1)	73.4 (1)	43.6 (4)	58.5 (1)	67.4 (1)
4	4.3 (6)	8.1 (5)	6.2 (6)	2.7 (6)	37.0 (4)	21.7 (6)	29.4 (6)	28.5 (3)
5	7.0 (4)	19.1 (1)	13.1 (3)	4.0 (5)	23.4 (5)	66.9 (1)	45.1 (4)	18.5 (5)
6	21.7 (2)	9.5 (4)	15.6 (2)	23.0 (2)	54.8 (3)	56.4 (2)	55.6 (2)	63.6 (2)

Table 4 Spearman's rank correlation coefficients for Experiments 1A and 1B among parental values of SE initiation percentages (upper value) with probability of significance (lower value)

	1B fem	1B mal	1B mid	1B op	1A fem	1A mal	1A mid	1A op
1B fem	1.00	−0.37	0.77	0.83*	0.77	−0.23	0.37	0.61
		0.47	0.07	0.04	0.07	0.62	0.47	0.20
1B mal		1.00	0.26	−0.26	0.26	0.71	0.60	0.20
			0.62	0.62	0.62	0.11	0.21	0.70
1B mid			1.00	0.66	1.00*	0.20	0.83*	0.90*
				0.16	0.00	0.70	0.04	0.01
1B op				1.00	0.66	−0.09	0.49	0.55
					0.16	0.88	0.33	0.26
1A fem					1.00	0.20	0.83*	0.90*
						0.70	0.04	0.01
1A mal						1.00	0.66	0.17
							0.16	0.75
1A mid							1.00	0.81*
								0.05
1A op								1.00

The 1A or 1B fem, mal, mid, and op represent mean SE initiation percentage as female, male, mid-parent, and open pollinated, for Experiments 1A and 1B, respectively
*Correlation significant ($p \leq 0.05$)

initiation frequencies for Westvaco (1B) and the IPST (1A) procedures were 45 and 11.2%, respectively. The family means ranged from 4 to 73%. Because of this large variation between the two procedures and their interactions with families, the analysis of genetic effects was carried out separately.

The GCA variance was the largest component of genetic variance accounting for 41.5 and 48.6% for Experiments 1A and 1B, respectively (Table 2). The GCA and all the other genetic variance components were statistically significant at a 5% probability level. The relative magnitude of the GCA component reflects a wide range of mid-parent values, i.e., average value of a parent when used as female and male, which ranged from 6.2 to 19.7% and from 29.4 to 58.5% for Experiments 1A and 1B, respectively (Table 3). Although the magnitude of the GCA variance was large and remained relatively similar for both experiments, the magnitude of the remaining genetic variance component estimates was different (Table 2). In Experiment 1B, the variance due to maternal effects accounted for 9.9% of total phenotypic variance, whereas the variance due to SCA and reciprocal effect were small, accounting for 2.1 and 3.0, respectively (Table 2). In Experiment 1A, however, the SCA variance was relatively large, accounting for 8.4% of total phenotypic variance, whereas maternal and reciprocal variances were 4.2 and 1.3%, respectively. Further insight into the genetic control of initiation was revealed by the lack of correlation between female and male values from the same trees, even showing negative, although not significant, correlations (Table 4). Most striking are differences between female and male values for parent tree 5 (Table 3). Tree 5 gave the highest average initiation frequency as a male, but gave low initiation when used as a female. In contrast, tree 4 appeared to have unfavorable effects both as a female and as a male. These results indicate that genes implicated in additive and maternal effects act independently, and their inheritance would be expected to be unlinked.

The validity of genetic interpretations is supported by the consistency between the two separate experiments, in which very different initiation rates were obtained. Although different experimental conditions were used, the significance and relative magnitude of the genetic variance components, the rankings of parents, and the opposite female and male effects of tree 5 were similar in both data sets. Most importantly, the rankings of the mid-parent val-

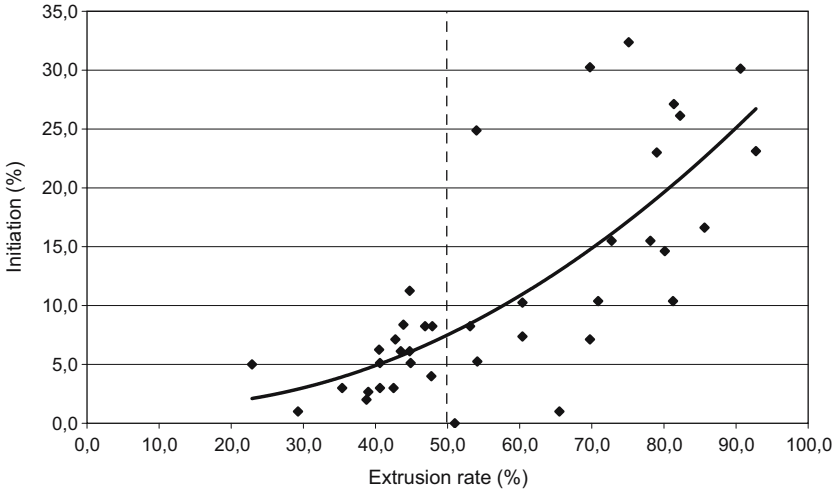

Fig. 1 Relationship between the rate of extrusion and the rate of initiation of SE. The rate of extrusion of zygotic embryos from megagametophytes explants was plotted against the rate of initiation. Each data point represents a full-sib or open-pollinated family mean from the six-tree diallel mating (Experiment 1). The "threshold" line at ~50% extrusion was added to distinguish low vs high extrusion. The trend line is the second-order polynomial regression best-fit (r^2=0.56)

Table 5 Estimated variance components (standard deviation) and their percentages of total variance for extrusion from Experiment 1A only

Variance component	Estimate (SD)	Percent
$2\sigma_{GCA}^2$	182.0 (108.6)*	27.4
σ_{SCA}^2	13.0 (21.7)	2.0
σ_{MAT}^2	114.1 (73.4)*	17.2
σ_{REC}^2	98.6 (51.3)*	14.8
σ_{ERR}^2	257.4 (26.3)	38.6
σ_{TOTAL}^2	665.1	100.0

*Significant at p=0.05

ues between the two sets of data were significantly correlated (r=0.83, Spearman's rank correlation), indicating that parental contribution is consistent despite the differences in procedures (Table 4). Similarly, the rankings of mid-parent values of Experiment 1B were consistent with the rankings of female parent, mid-parent and op values of Experiment 1A (r=1.00, r=0.83, and r=0.90, respectively). Narrow sense heritability for SE culture initiation was very high, with estimates of 0.83 for combined data, and 0.83 and 0.97 for individual experiments 1B and 1A, respectively.

Genetic control of extrusion and its relationship to initiation

The procedure for SE initiation used megagametophyte explants containing early stage, immature, zygotic embryos, requiring the emergence of zygotic embryos from the megagametophyte, or extrusion, in order for culture initiation to take place. The extrusion frequency, was assessed in Experiment 1A only and gave family means ranging from 38.8 to 92.8%, and an overall mean of 57.2%. Approximately one fifth of the extrusions led to SE initiation, clearly showing that extrusion is not sufficient for initiation and indicating that postextrusion events played a large role in controlling SE initiation. The two events showed a moderate positive correlation (r=0.75) at the family level. The distribution of initiation frequency relative to extrusion frequency further illustrates the nature of their relationship (Fig. 1). Initiation was low when extrusion was less than 50%, however, beyond the 50% threshold; increases in extrusion appeared to translate into increased initiations.

All of the genetic variance component estimates computed for extrusion were statistically significant at a 5% probability level, except for the SCA (Table 5). The GCA variance was the greatest, at 27.4% of the phenotypic variance, indicating that extrusion is also under additive genetic control. Maternal (17.2%) and reciprocal (15.4%) variances appear to be relatively important, indicating that extrusion and initiation are partly under different types of genetic control. The significance of maternal effects in extrusion and its relationship to initiation are illustrated by the larger half-sib family correlations between extrusion and initiation for females (r=0.94) than for males (r=0.44).

Experiment 2

We analyzed initiation using a 3×3 factorial mating design, with aim of verifying the effect of the highly responsive male, tree 5, on SE initiation. Both female and male parents were shown to have a significant effect on the initiation rate (Table 6B). The mean initiation frequency obtained with tree 5 as a male parent (26.8%) was statistically greater than those obtained with the two other males (19.0 and 9.3%; Table 6A). Crosses with tree 5 as the male also produced higher initiation frequencies with each mother tree taken individually. These data provide an independent confirmation that tree 5 had a strong positive effect on initiation as a male parent; despite the low initiation, it produced as a female parent (Table 3).

Table 6 (**A**) Mean initiation rate (%) in controlled crosses of loblolly pine in Experiment 2 (3×3 factorial experiment). (**B**) Analysis of variance for initiation for Experiment 2

A

Female parent	Pollen parent			
	3 (High)	4 (Intermediate)	5 (Low)	Mean (female)
1 (Low)	21.0	11.0	31.5	21.7b
7 (Low)	9.0	1.8	19.5	10.5a
8 (High)	27.0	14.0	29.5	23.5b
Mean (pollen parent)	19.0a	9.3b	26.8c	18.5

B

Source	*df*	Sum of squares	Mean square	*F* ratio	*p* value
Female (F)	2	5,725.19	2,862.59	17.19	<0.0001
Male (M)	2	9,344.51	4,672.26	28.06	<0.0001
F × M	4	443.13	110.8	0.67	0.6170
Error	166	27,644.4	166.53		
Total	174	43,127.23			

Means followed by the same letter are not significantly different (p=0.05)
All F ratios are based on the residual mean square error

Table 7 (**A**) Mean SE initiation rate (%) in Experiment 3 (three-tree diallel) of loblolly pine controlled crosses and (**B**) analysis of variance for initiation for Experiment 3

A

Female parent	Pollen parent				
	9 (High)	10 (Intermediate)	11 (Low)	Mean (female)	Open-pollinated
9 (High)	–	54.8	44.3	47.7d	45.2
10 (Intermediate)	18.2	–	12.7	15.8b	16.5
11 (Low)	4.8	8.3	–	5.8a	4.4
Mean (pollen parent)	11.4ab	31.5c	28.5c	23.1	21.6

B

Source	*df*	Sum of squares	Mean square	*F* ratio	*p* value
Female	2	20.36	10.18	241.56	<0.0001
Male	2	4.46	1.49	35.50	<0.001
Error	563	23.72	0.04213		
Total	567	51.16			

Initiation potential for each open-pollinated (op) mother tree is shown in parenthesis
Means followed by the same letter are not significantly different (p=0.05)
All F ratios are based on the error mean square error

Experiment 3

The strong genetic control of initiation suggests that mating plans may be designed to improve initiation frequencies by selecting favorable male and female parents. To obtain data in support of this hypothesis, a three-tree diallel mating design was used with trees known to have low, intermediate, or high SE initiation potential as op mother trees. Initiation family means varied from 4.8 to 54.8%, but female mean values varied more widely than male mean values (Table 7A). Significant effects were determined for female and male parents (Table 7B). The stronger influence of the female parent on initiation (F statistic, p value) was consistent with maternal effects as exhibited in Experiment 1B. Statistically significant differences were observed between pairs of reciprocals (p<0.05), indicating potential differences in maternal and additive effects. For example, a cross of tree 9 (female) with tree 10 (male) gave 54.8% initiation, whereas the reciprocal cross gave 18.2% initiation (Table 7). These results indicate that initiation may be increased simply by choosing a favorable female parent for cross-pollination based on the relative responsiveness of that parent as an op tree.

Discussion

The estimated variance components from diallel analysis (Experiment 1) offer genetic interpretations of the observed variability of SE initiation, under the assumption that the parents are random samples of a breeding population. The op trees produced a range of SE initiation rates that are typical of SE responsiveness in a loblolly pine breeding population. Variance component estimates were computed separately for two experiments (1A and 1B) using the same cp and op seeds, but using different experimental procedures. The variance component due to GCA (σ_{GCA}^2) is the variance of the average performances of parents in cross combinations. The GCA variance represents the covariance of half-sibs that is translated into one quarter of additive genetic variance. The SCA variance (σ_{SCA}^2) is the variance of specific cross combinations after subtracting the GCA effects of both parents. Therefore, the SCA variance represents full-sib covariance after removing two parental half-sib covariances, which is translated into one quarter of dominance genetic variance. The variance component due to maternal effects (σ_{MAT}^2) is the variation due to average maternal effects of each parent. The reciprocal variance (σ_{REC}^2) is the variation due to reciprocal differences not accounted for by σ_{ERR}^2. The error variance (σ_{ERR}^2) includes all genetic–environmental covariances. The total phenotypic variance (σ_{TOTAL}^2) is the sum of all genetic and genetic–environmental variances, which is the covariance of individuals. As σ_{ERR}^2 represents the average variance of parents, the total variance includes $2\sigma_{GCA}^2$ to account for both female and male effects.

Our results clearly establish the importance of genetic control in initiation of SE cultures in *P. taeda* and provide strong indications as to the nature and magnitude of genetic control. Strong additive genetic effects govern SE initiation, as indicated by the large proportion of variance due to the GCA component (σ_{GCA}^2). This major conclusion of our work regarding the genetic control of SE initiation is supported by the consistent results obtained from two independent experiments using different procedures with the same control-crossed immature seed. The computed estimates of narrow sense heritability for loblolly pine SE initiation were very high (0.83 and 0.97) compared with many metric traits in trees, but are similar to those of spruce SE culture initiation [15] and of oilseed rape microspore embryogenic ability [25]. The effects associated with the maternal, reciprocal, and SCA components each explained a smaller proportion of the total variance and were variable in magnitude between experiments. A possible interpretation of our data is that additive effects and maternal effects operate independently, and maternal effects may mask the additive effects. The lowest initiation consistently being associated with certain females provides support for the hypothesis of negative maternal effects in some trees, also

observed in selected genetic backgrounds of maize [24]. The opposite male and female effects were striking in tree 5, which gave the highest SE initiations as a male but low initiations as a female in independent experiments.

Strong additive genetic effects for SE initiation were observed in both loblolly pine (this study) and white spruce [15], both belonging to the *Pinaceae*. In comparing SE initiation of spruce and pine, there appear to be differences in the relative importance of other genetic effects. The variance component due to REC effects was second in importance and represented 21.3% of the total variance in white spruce [15], and only accounted for 3% or less of the variance in loblolly pine. Different variance components were second in importance depending on the loblolly pine experiment: it was either the maternal effects, accounting for 9.9% of the phenotypic variance (Experiment 1B), or the SCA, accounting for 8.4% (Experiment 1A). This indicates that the genetic effect is influenced by in vitro procedure used at the two laboratories. It is possible that overall low initiation rates in Experiment 1B may have masked some genetic effects such as maternal effects, despite the fact that the major genetic effect, the GCA variance, remained similar in magnitude for both experiments. In spruce, maternal effects were not significant and were smaller than both the GCA and REC components. Isolated zygotic embryos are used as explants in spruce, whereas pine SE cultures are initiated with intact megagametophytes containing immature zygotic embryos. The presence of the megagametophyte may extend the influence of the mother tree into culture to a greater degree and thus account for the importance of the maternal effects. The use of the whole megagametophyte usually requires embryo extrusion to take place, and extrusion indeed had a maternal variance component, representing 17.2% of the total variance.

Both pine and spruce SE also share similarity with major crop plants, such as maize and *Brassica*, in which genetic control has been assigned primarily, but not exclusively, to additive genetic effects [24, 25]. With regard to other variance components, Experiment 1B suggested that SE in loblolly pine may be more similar to maize, which shows significant maternal effects [24]. A simple interpretation of our data is that maternal effects account for much of the variation at the level of extrusion, and that additive gene effects may play a larger role in postextrusion events, which appear to be most critical to SE initiation. Therefore, quantitative variation in the differentiation and proliferation of somatic embryos after extrusion would represent the largest proportion of variation between families and different procedures. Several cellular and molecular factors may play a role in regulating these events. For example, in Norway spruce (*Picea abies*), the proliferation of somatic embryos was linked to secreted arabinogalactan proteins [7]; early embryo development and embryogenic potential were related to the expression of the lipid transfer protein Pa18 [10] and the Knox-I gene *PaHB2* [11], respectively. A mechanism for the maternal effect in pine may be linked to the presence of the haploid megagametophyte during culture initiation, the genome of which is solely inherited from the mother tree. The early-stage megagametophyte contains nutritional components and growth regulators important to the growth of the early-stage zygotic embryo. Application of abscisic acid [9] and brassinolide [19] was shown to improve SE initiation in loblolly pine. These compounds could be present in the megagametophytes, and variation in such compounds among seed sources with different genetic backgrounds could be investigated.

Variations in initiation frequencies between Experiments 1A and 1B were likely caused by major differences in culture medium formulations, different basal salts (505 vs. WV5), incorporation of abscisic acid (none in 505 vs. 10 mg/L in WV5; [9]), and a lower concentration of gelling agent (2.0 g/L in 505 vs. 1.5 g/L WV5; [4]). However, other factors that varied between the two laboratories may also have contributed to the difference.

Practical implications for use of SE within a breeding program

Practical implications for breeding and clonal propagation were derived from our study. Previous work showed that many mother trees of *Pinus* species produce seed with low SE initiation frequencies, and practical solutions are needed to overcome this problem. Our hypothesis was that such trees possess unfavorable alleles at loci expressed in the mother tree (noninherited), whereas neutral or favorable alleles act at other loci and may be inherited by zygotic embryos. The large GCA variance component and narrow sense heritability estimates confirmed that breeding could influence SE initiation. Based on findings from Experiment 1, we proposed that trees with favorable alleles at loci expressed in the mother tree could be used as female parents to effectively circumvent putative negative maternal effects in the other trees. This operational approach was tested among a small number of control crosses and their reciprocals with trees that ranged from low to high initiation as op mother trees in Experiment 3. This small data set confirmed that SE initiation may be improved by choosing a favorable female parent for cross-pollination for each pair of parents. The initiation rate was improved from 1.5- to 9.2-fold by switching the mother and pollen parent in each cross (Table 7).

Our findings have several implications for increasing SE initiation in loblolly pine. First, the initiation from a given mother tree in a cp cross can often be predicted based on the response of the op seed, which thus may help plan controlled pollinations to optimize initiation frequency. Second, low initiation encountered with a tree used as female parent can often be overcome by switching to use the same tree as a male parent. Third, some trees appear to have very strong additive effects as male parents (e.g., tree 5), but negative maternal effects. The strong positive effect of tree 5 as a male parent was expressed consistently on three different SE initiation protocols in two experiments. Use of such trees as male parents in cp crosses offers yet another solution to improve initiation in the presence of negative maternal effects (Table 6). Finally, the strong interaction

between families and procedures also clearly indicates that different culture media may be better suited for different genotypes. Our overall understanding of the genetic control of SE initiation shows clear potential to maximize initiation of SE, a key step in the capture of genotypes from a breeding program.

A major application of SE is clonal selection within full-sib families resulting from crosses among parents with high breeding values. Within-family clonal selection requires that a sufficient number of clones per family be propagated and tested in the field to identify the best clones to use in clonal forestry. A simple strategy to ensure efficient capture of many clones is proposed based on our findings. The proposed approach is most practical when targeting a small number of full-sib families, but could be modified for different breeding needs. In year 1, parents are identified based on breeding values. Reciprocal matings are made with each cross. During the same year, SE initiation potential is evaluated using op seed of both maternal and paternal parent tree used in the controlled crosses. In year 2, SE is initiated from controlled crosses using the best initiator as the mother tree based on evaluation of op trees from the previous year. This strategy assumes that improved initiation frequencies will translate into more clonal lines being carried through (captured) for field-testing. This assumption has been validated in our laboratories (data not shown) and is supported by research in white spruce that showed that initiation is the step in the SE process which is the most highly influenced by genetic background [16]. Finally, the strategy is designed for loblolly pine, where seeds are produced in the second growth season after pollination, and immature seed explants need to be used soon after removal from the tree to obtain the optimum SE response. This strategy may also be directly applied to other species, especially other *Pinus* species that share these features.

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