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Somatic embryogenesis in white spruce (*Picea glauca*): genetic control in somatic embryos exposed to storage, maturation treatments, germination, and cryopreservation

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Abstract Genetic controls for growth of embryogenic cultures, storage, maturation treatments, germination and cryopreservation in white spruce somatic embryogenesis (SE) were examined. These SE processes were under genetic control but less strongly so than the initiation phase. For all the SE characters examined, variance due to clones within families was significant and often the largest genetic component of variance. This was further partitioned using an additive-dominance-epistasis model. A relatively-large proportion of the total genetic variance was due to epistatic variance in the maturation and germination of somatic embryos. Embryogenic lines were cryopreserved easily without a distinct genetic influence being noticed.

Key words Somatic embryogenesis · Maturation Germination · Genetic variances · Cryopreservation Tree breeding

Introduction

This is the second part of a study concerning the somatic embryogenesis (SE) of white spruce [*Picea glauca* (Moench) Voss]. In the first part of this study (Park et al. 1993), we analyzed the effects of various experimental treatments on genetic controls and on genotype × environment interactions during the initiation of embryogenic tissue (ET). This experiment was carried out with 30 full-sib families from six-parent diallel crosses. We obtained embryogenic tissue from all 30 families at relatively high initiation percentages.

The initiation of ET was under strong additive genetic control and exhibited genotype × treatment interactions. It was found that freshly excised immature zygotic embryo explants provided a greater embryogenic response than stored immature, and fresh and stored mature ones. When immature explants were used, relatively large variances due to specific combining ability (SCA) and maternal effects were found. When mature explants were used, these variances were no longer present; the variation due to general combining ability (GCA) and reciprocal effects remained. Such changes in variances indicate that, during initiation, we are dealing with rapid shifts in the activity of several genes. While dominance variance expressed by SCA may be transitional, the additive gene effects (GCA) consistently play an important role in initiating SE. We have extended this research to a study of genetic responses during culture maintenance, storage, maturation, germination and cryopreservation of the somatic embryos (SEs).

Embryogenic tissue will grow vigorously as long as the cultures are maintained at approximately 25°C in auxin-containing initiation medium and subcultured every 2 weeks. Unfortunately, frequent subculturing is labor intensive, which leads to severe logistical problems when a large number of clones is involved. Therefore, our first objective was to examine if subculture frequency could be reduced by culture at lower than 25°C and to determine if this process, which we will call storage, is affected by the genetics of the material, both at the clonal and family levels.

Once sufficient stock of ET has been obtained, maturation of SEs is the next critical step. In most research laboratories, this is still the least efficient stage during the regeneration of conifer plants from somatic embryos (Attree et al. 1990). For spruce species, abscisic acid (ABA) promotes maturation, with the optimal concentration of ABA varying among species (Dunstan et al. 1988; Hakman and von Arnold 1988; Attree et al. 1990; Roberts et al. 1990 a). Therefore, our second objective was to examine the ABA requirements for maturation and to determine if these requirements are affected by the genetics of the material, both at the clonal and family levels.

Germination of conifer somatic embryos is often precocious or uneven. This can be partly corrected by partial

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desiccation of mature somatic embryos prior to placing them on germination medium (Roberts et al. 1990 b). Proper germination, resulting in a well-balanced shoot/root ratio, is essential for successful establishment of emblings in soil. Our third objective is to determine the genetic controls within and between families in the germination process.

Materials and methods

Plant material

The embryogenic cultures used were obtained from explants excised from seeds of 30 full-sib families from six-parent diallel crosses (Park et al. 1993). This resulted in 1 700 embryogenic lines (clones), representing all 30 families. Of these clones, 747, again representing all the families, were retained for subsequent bulking-up, storage, maturation, germination and cryopreservation experiments.

Bulking-up

Six to fourteen clones from each of the 30 families, to a total of 329 clones, were bulked-up by subculturing embryogenic tissue at 2-week intervals over an 8-week period. The medium used for that purpose was half-strength Litvay medium(½ LM) with 2,4-D as described by Park et al. (1993). Initially, there were two Petri plates for each clone with each plate containing nine tissue clumps, each about 1 cm in diameter. Each clump that had grown enough to be divided at the time of subculture was cut into about 1-cm-diameter pieces. Thus the number of plates per clone, each with nine clumps of tissue per plate, increased during subsequent subcultures. The rate of growth (GROW) of each clone was determined by counting the number of plates per clone obtained in the 8-week proliferation period.

Storage

For each clone, one plate with nine healthy clumps was put in storage at four temperatures (5, 10, 15 and 25 °C) for three durations (1, 2 and 3 months). A total of 2 525 plates was used for 329 clones (6–14 clones for each family).

After exposure to each storage temperature and period, the cultures were placed on fresh initiation medium for 6 weeks and then subcultured every 2 weeks. Then the condition (COND) of each clump was rated, using six subjective categories from 1, being most healthy (non-necrotic), to 6, being dead. The percent survival (SURV) for each clone was calculated from the number of clumps that had started growing again at the end of the 6-week period.

Maturation

The maturation experiment was carried out with clumps that had been stored for 1 month and that had started growing vigorously again during the 6-week post-storage recovery period. A total of 312 clones was available for the maturation experiment. The clumps were transferred from initiation medium containing kinetin to maturation media. Five maturation media containing ½ LM with 0, 12.5, 25, 37.5 and 50 M of abscisic acid (ABA) were prepared. For the first 2 weeks, these media contained 6% sucrose and 0.4% Gelrite. For the remainder of the maturation cycle, they contained 3.4% sucrose. For each of the 312 clones, one clump was placed on maturation medium with each of the five ABA concentrations. They were kept at 25 °C under fluorescent light (16 h 55 mol $m^{-2}s^{-1}$) and subcultured every 2 weeks on medium with the same ABA concentration. Five weeks after the initial transfer to ABA-containing medium, mature embryos were picked from the clumps and counted for each clone and ABA treatment. The count included normal mature somatic embryos (NMSE), as well as precociously germinating somatic embryos (PGSE) but not grossly malformed embryos. The normal mature somatic embryos have clearly developed, but non-elongated, cotyledons. Precociously germinating somatic embryos have green cotyledons that are elongating. The total number of mature somatic embryos (TMSE) is the sum of normal mature and precociously germinating somatic embryos (TMSE = NMSE + PGSE).

Germination

Germination experiments were carried out using both normal mature and precociously germinating somatic embryos from each clone. These embryos were removed from each of the five different ABA media, except in the case of a few clones where such embryos were not available from all media. The germination medium was quarter-strength Litvay's medium but with half strength Fe, KNO_3, and $MnSO_4 \cdot H_2O$. The sucrose concentration was reduced to 2% and the Gelrite concentration to 0.21%. Prior to placing the normal mature somatic embryos, picked from cultures on maturation medium, onto the germination medium, they were partially desiccated by placing them on millipore filters in 6-well dishes. Three of the wells contained a millipore filter with about 15 embryos, the other three wells contained sterile water. The 6-well dishes were sealed with Parafilm and placed in the dark at 25 °C for 3 weeks. At the end of the 3 weeks, the filters with embryos were transferred to germination medium. The precociously germinating somatic embryos picked from the maturation media were placed on germination medium without desiccation. Since desiccation is used to stimulate germination, it was deemed not to be required for the precociously germinated embryos. A record was kept for each clone regarding what ABA treatment they had received during maturation and whether they were NMSE or PGSE. In the statistical analysis, we considered each clone, ABA treatment and maturation-type combination as one sampling unit. The number of embryos per sampling unit varied (1–100 +). Of the 894 sampling units, 537 were normal (NMSE) and 357 were precociously germinating mature embryos (PGSE).

The embryos were examined after 2, 3, and 4 weeks to determine the germination and rooting rate. Numbers of the following type classes were recorded: normally rooted somatic embryos (NORM), germinating embryos with elongating cotyledons and a hypocotyl but without elongating roots (SHOOT), misshapen germinating embryos (MISS), and dead (DEAD) embryos. Embryos with a minimal hypocotyl, or root, a hypocotyl with suppressed cotyledons, and roots only, were considered as MISS.

Cryopreservation

Embryogenic tissue of 551 clones, cultured on initiation medium containing kinetin, was readied for cryopreservation using a modified version of the protocol described by Charest et al. (1993). For each clone, about 1 g of actively growing tissue was placed in an Erlenmeyer flask with 7 ml of half-strength Litvay's liquid medium, with 2% sucrose and 0.4 M sorbitol. The flasks were shaken on a rotary platform shaker at 100–115 rpm for 20–24 h. The flasks were then placed on ice and 3 ml of a cold DMSO solution was added in six successive 0.5-ml aliquots over a 30-min period. The DMSO solution was prepared as follows: 30 ml of a stock containing 15 ml of full-strength Litvay medium and 15 ml DMSO was added to 60 ml of half-strength Litvay medium with 0.4 M sorbitol. This created a final solution that contained half-strength Litvay, 0.27 M sorbitol and 16.6% (v/v) DMSO. After the flasks had been left on ice for 30 min to equilibrate, tissue from each flask was distributed in 1-ml aliquots over eight cryogenic vials (i.e., eight replicates/clone). The vials were placed in retaining canes (four vials per cane). The canes were suspended in a freezer cooled to 0 °C. After a 10-min holding period, they were cooled to –40 °C at a rate of approximately –0.33 °C per min and held at –40 °C for 10 min. The canes were then immersed in liquid nitrogen (–195 °C). To test survival and recovery, one vial from each of the eight cryopreserved for each clone was, after 1 day in liquid nitrogen, thawed in a 37–40 °C water bath until complete-

ly thawed (after about 2 min). The thawed embryogenic tissue was poured onto sterile filter paper disks. These disks were placed on initiation medium and, after 24 h, transferred to fresh initiation medium. Once the tissue began to grow, it was considered to have survived cryopreservation.

Statistical models and analysis

The growth-rate data of the embryogenic cultures (GROW), obtained during the bulking-up phase, were subjected to an analysis of variance using the following linear model:

$$Y_{ijk} = \mu + g_i + g_j + s_{ij} + m_i + m_j' + r_{ij} + e_{ijk} \quad (1)$$

where

- Y_{ijk} is the observation for k-th clone of the family resulting from crossing between the i-th and j-th parents;
- μ is the overall mean;
- g_i (g_j) is the general combining ability (GCA) effect of the i-th female (j-th male) parent;
- s_{ij} is the specific combining ability (SCA) effect involving the i-th and j-th parents;
- $m_i(m_j)$ is the maternal effect of the i-th (j-th) female parent, such that $m_i = -m_i'$;
- r_{ij} is the reciprocal effect involving the reciprocal crosses of the i-th and j-th parents; and
- e_{ijk} is the random error component containing the effect of k-th clone within the ij-th family.

All terms in the model, except the overall mean, were considered as random effects. The computations, including the calculations of the expected mean squares, were carried out using a computer program, DIALL (Schaffer and Usanis 1969).

Data from the storage experiment, i.e., the condition of recovery (COND) and the percent of survival (SURV), were subjected to analysis of variance, using the model:

$$Y_{hklm} = \mu + F_h + C_{hk} + T_l + D_m + FT_{hl} + FD_{hm} + TD_{lm} + e_{hklm} \quad (2)$$

where

- Y_{hklm} is the observation on the k-th clone of h-th family stored at the l-th temperature for the m-th period;
- μ is the overall mean;
- F_h is the h-th family effect, h = 1, 2, ... , 30;
- C_{hk} is the k-th clone within the h-th family effect, k = 1, 2, ..., 14;
- T_l is the l-th storage temperature effect, l = 1, 2, 3, 4;
- D_m is the m-th duration effect; m = 1, 2, 3;
- FT_{hl} is the interaction effect of h-th family and the l-th storage temperature;
- FD_{hm} is the interaction effect of h-th family and the m-th storage duration;
- TD_{lm} is the interaction effect of the l-th storage temperature and m-th duration;
- e_{hklm} is the random error component.

The number of normal mature somatic embryos (NMSE), precociously germinating somatic embryos (PGSE) and total mature somatic embryos (TMSE) was analyzed using the model:

$$Y_{hkl} = \mu + F_h + C_{hk} + A_l + FA_{hl} + e_{hkl} \quad (3)$$

where

- Y_{hklm} is the number of maturing somatic embryos of the k-th clone within the h-th family for l-th ABA treatment;
- A_l is the l-th ABA treatment effect, l = 1, 2, ..., 5;
- FA_{hl} is the interaction effect involving h-th family and l-th ABA treatment;
- e_{hkl} is the random error component; and all other terms are as defined previously.

The percentage of somatic embryos that were normal (NORM), had germinated and had formed a shoot but no root (SHOOT), were misshapen (MISS) or were dead (DEAD) was analyzed using the model:

$$Y_{hklm} = \mu + F_h + C_{hk} + A_l + M_m + FA_{hl} + FM_{hm} + e_{hklm} \quad (4)$$

where

- Y_{hklm} is the observation for k-th clone of the h-th family resulting from the l-th ABA treatment that produced m-th maturation-type;
- M_m is the m-th maturation-type effect, m = 1, 2;
- FA_{hl} is the interaction effect of the h-th family and l-th ABA treatment;
- FM_{hm} is the interaction effect of the h-th family and m-th maturation-type;
- e_{hklm} is the random error; and all other terms are as defined previously.

All the counted and rating data, i.e., the number of plates used to assess GROW, the condition of recovery from storage (COND), and the numbers of NMSE and PGSE, were transformed by taking the square roots of the data [$\sqrt{(x + 0.5)}$] before analysis of variance to improve normality (Sokal and Rohlf 1981), while the percentage data, i.e., SURV, NORM, MISS and DEAD, were transformed by taking the arcsine values of the square roots of the percentages ($\sin^{-1}\sqrt{\%/100}$). For all analyses of variance, families and clones within families were considered to be random effects while the treatment effects were considered to be fixed effects. Computations of analyses of variance for models (2)–(4), including the computations of the expected mean squares, were performed using the computer program, RUMMAGE (Bryce 1980). The variance components for all random effects terms were estimated. The standard deviations of the estimates were calculated according to Anderson and Bancroft (1952). Approximate significance tests for all sources of variation in the models were performed by comparing expected mean squares (Satterthwaite 1946l; Tietjen 1974) at the 5% probability level. Subsequent use of the term "significant" indicates statistical significance.

The sums of squares due to family effects in these models were further partitioned by using the model (1) [see details in our earlier paper (Park et al. 1993)]. Therefore, variance due to families (σ^2_F) includes twice the variance due to GCA, the variance due to SCA, and the maternal and reciprocal effects; i.e., $\sigma^2_F = 2\,\sigma^2_g + \sigma^2_s + \sigma^2_m + \sigma^2_r$. Similarly, total phenotypic variance is estimated as the sum of the estimated variance components from the linear models.

Results

Family means

Family means for the characters studied, including the initiation data from our earlier paper (Park et al. 1993), are presented in Table 1. Since family effects were considered to be random in the analyses, comparisons of the means were not carried out. The range of the means, however, indicates variability among families.

Growth of embryogenic tissue

The growth rate (GROW) of the embryogenic tissue during the 8-week bulk-up period varied significantly among families. Error variance (σ^2_E), which contains variance due to clones within families (σ^2_C) was the largest component of the variation, i.e., 62.3% of the total variance (Table 2). The variance due to general combining ability of parents ($\sigma 2^2_g$) was 19.3%, and that due to reciprocal effects (σ^2_r)

Table 1 Family means for somatic embryo initiation percentage (INIT), growth rate (GROW), percent survival (SURV) and condition of recovery (COND) from storage, numbers of normal (NMSE), precociously germinating (PGSE), and total mature (TMSE) somatic embryos, and percentages of normal (NORM), shoot only (SHOOT), misshapen (MISS) and dead (DEAD) embryos during germination

Parents		INIT[a]	GROW	SURV	COND	NMSE	PGSE	TMSE	NORM	SHOOT	MISS	DEAD
Female	Male											
DNR-27	GPC-02	22.8	7.5	74.1	3.6	7.6	8.8	16.4	12.5	41.1	26.0	20.4
DNR-27	MRL-03	42.9	3.4	78.2	3.5	11.4	14.6	26.0	16.6	39.7	28.8	15.2
DNR-27	GPC-03	24.9	6.0	86.6	3.1	3.3	11.3	14.6	29.6	32.5	15.2	22.7
DNR-27	GPC-15	38.5	5.5	89.0	2.9	4.0	6.7	10.7	14.3	33.1	30.4	22.1
DNR-27	MRL-11	43.9	9.5	66.9	3.7	3.1	8.6	11.7	15.0	56.0	29.0	0.0
GPC-02	DNR-27	23.2	4.3	65.7	3.9	4.8	7.8	12.6	20.6	30.2	32.9	16.2
GPC-02	MRL-03	27.9	7.2	56.2	3.8	10.4	22.7	33.1	17.8	36.5	30.8	15.0
GPC-02	GPC-03	3.8	8.0	64.8	3.6	3.8	7.7	11.5	14.3	29.3	56.3	0.0
GPC-02	GPC-15	15.0	7.7	70.0	3.4	2.7	5.0	7.7	36.1	34.2	15.3	14.3
GPC-02	MRL-11	32.2	11.2	47.2	4.2	2.9	4.4	7.3	39.1	37.7	8.2	15.0
MRL-03	DNR-27	39.7	4.5	73.2	3.5	5.5	15.9	21.4	30.6	44.3	21.1	3.9
MRL-03	GPC-02	17.0	3.9	72.6	3.1	2.9	15.3	18.2	17.2	37.0	30.6	15.2
MRL-03	GPC-03	42.2	6.5	81.9	3.2	7.9	12.9	20.8	33.0	33.8	10.1	23.1
MRL-03	GPC-15	45.5	4.4	82.9	3.1	12.9	11.5	34.4	16.2	32.3	20.7	31.0
MRL-03	MRL-11	39.8	4.1	65.6	3.8	7.2	23.5	30.7	23.1	37.3	20.9	18.8
GPC-03	DNR-27	54.1	4.0	91.5	2.7	4.4	11.7	16.1	36.6	31.4	23.6	8.4
GPC-03	GPC-02	22.0	6.1	86.3	3.1	2.9	9.2	12.1	12.8	31.0	43.3	12.9
GPC-03	MRL-03	47.0	2.6	91.8	2.8	4.7	11.7	16.4	16.1	37.9	36.4	14.4
GPC-03	GPC-15	46.1	4.2	90.8	2.7	4.5	6.7	11.2	25.8	25.8	39.1	9.4
GPC-03	MRL-11	40.0	8.9	76.9	3.2	2.3	5.6	7.9	8.0	91.5	0.0	0.0
GPC-15	DNR-27	22.6	2.1	76.0	3.5	6.1	11.0	17.1	33.1	29.0	23.4	14.5
GPC-15	GPC-02	3.3	4.5	90.1	2.5	3.7	16.6	20.3	26.7	42.1	26.2	4.8
GPC-15	MRL-03	43.4	3.9	88.0	2.8	5.5	14.9	20.4	23.7	42.3	18.6	15.4
GPC-15	GPC-15	8.9	3.3	100.0	2.4	6.8	4.7	11.5	14.2	27.1	49.4	9.4
GPC-15	MRL-11	44.9	9.0	70.1	3.5	3.2	2.6	5.8	3.4	96.6	0.0	0.0
MRL-11	DNR-27	21.1	7.5	70.1	3.2	13.0	32.3	45.3	34.3	23.3	15.1	28.0
MRL-11	GPC-02	15.2	5.6	64.3	3.6	4.7	12.4	17.1	16.4	35.7	28.7	19.2
MRL-11	MRL-03	54.6	4.5	64.0	3.9	9.5	23.4	32.9	22.5	29.6	28.2	20.1
MRL-11	GPC-03	35.6	6.0	92.6	2.0	3.2	29.0	32.2	13.4	29.6	15.7	41.2
MRL-11	GPC-15	24.1	4.1	73.8	3.3	6.9	10.4	17.3	25.1	35.1	25.5	14.5
Overall mean		32.9	6.7	74.4	3.3	5.7	11.7	17.4	22.2	35.7	26.3	15.8

[a] Somatic embryo initiation data from Park et al. (1993)

was also relatively large (13.0%). The variance due to specific combining ability (σ^2_s) and maternal effect (σ^2_m) were not important.

Storage

The condition (COND) of cultures after 6 weeks of subculture following storage was significantly influenced by the duration of storage. The overall mean COND score deteriorated from 2.6 to 4.0, to 4.7 after 1, 2, and 3 months of storage, respectively. At 5, 10, 15, and 25 °C, the mean values for COND were 3.4, 3.2, 3.4 and 3.5, respectively, but the differences among these were not significant and showed no trend. Among families, the mean COND score varied from 4.2 to 2.0 (Table 1).

Most of the genetic variance in COND was accounted for by the variances due to GCA of parents and clones within families, amounting to 9.9% and 9.5% of the total phenotypic variance, respectively (Table 2). The variances due to SCA, maternal and reciprocal effects were negligible. The family treatment interaction variances, i.e., σ^2_{FT} and σ^2_{FM}, were significant but relatively small. The largest variance, however, was due to random error. This variance includes all the higher-order interactions not accounted for by the linear model.

The survival of cultures following storage (SURV) was significantly influenced by storage temperature, duration,

Table 2 Estimated variance components (one standard deviation) and their percentage[a] of total phenotypic variance for growth rate (GROW) during the bulk-up phase and for condition of recovery (COND) and survival (SURV) in a storage experiment

Variance component[b]	GROW		COND		SURV	
	Estimate	%	Estimate	%	Estimate	%
$2\sigma^2_g$	0.1122 (0.0367)*	19.3	0.0118 (0.0033)*	9.9	0.0382 (0.0103)*	12.4
σ^2_s	0.0185 (0.0216)	3.2	–0.0005 (0.0006)	0.0	–0.0028 (0.0007)	0.0
σ^2_m	0.0130 (0.0181)	2.2	0.0007 (0.0006)	0.6	0.0027 (0.0016)*	0.9
σ^2_r	0.0756 (0.0392)*	13.0	0.0004 (0.0009)	0.3	–0.0013 (0.0011)	0.0
σ^2_C	–		0.0113 (0.0025)*	9.5	0.0251 (0.0054)*	8.2
σ^2_{FT}	–		0.0017 (0.0009)*	1.4	0.0075 (0.0028)*	2.4
σ^2_{FD}	–		0.0031 (0.0015)*	2.6	0.0164 (0.0050)*	5.3
σ^2_e	0.3628 (0.0295)	68.9	0.0904 (0.0028)	75.7	0.2180 (0.0068)	70.8
Total	0.5821	100.0	0.1194	100.0	0.3079	100.0

[a] Negative variance component estimates considered to be zero in the calculation of percentages
[b] σ^2_g, σ^2_s, σ^2_m, σ^2_r, σ^2_C, σ^2_{FT}, σ^2_{FD}, and σ^2_E are variance components due to general combining ability, specific combining ability, maternal, reciprocal, and clones within family, family × storage temperature interaction, family × storage duration interaction, and random error, respectively
* Significant at the 0.05 level

Table 3 Estimated variance components (one standard deviation) and their percentages[a] of total phenotypic variance for normally maturing somatic embryos (NMSE), precociously germinating somatic embryos (PGSE) and total mature somatic embryos (TMSE)

Variance component[b]	NMSE		PGSE		TMSE	
	Estimate	%	Estimate	%	Estimate	%
$2\sigma^2_g$	0.0832 (0.0306)*	3.5	0.3812 (0.1273)*	7.7	0.4922 (0.1596)*	7.8
σ^2_s	–0.0019 (0.0262)	0.0	0.0400 (0.0782)	0.8	0.0225 (0.0876)	0.4
σ^2_m	–0.0002 (0.0128)	0.0	0.0221 (0.0324)	0.4	0.0278 (0.0393)	0.4
σ^2_r	0.0405 (0.0444)	1.7	0.0391 (0.0805)	0.8	0.0336 (0.0969)	0.5
σ^2_C	0.8224 (0.0967)*	34.3	2.1299 (0.2215)*	42.9	2.7374 (0.2857)*	43.2
σ^2_{FA}	0.0557 (0.0259)*	2.3	0.1277 (0.0464)*	2.6	0.1622 (0.0593)*	2.6
σ^2_e	1.3945 (0.0595)	58.2	2.2296 (0.0952)	44.8	2.8592 (0.1220)	45.1
Total	2.3963	100.0	4.9696	100.0	6.3349	100.0

[a] Negative variance component estimates considered to be zero in the calculation of percentages
[b] σ^2_{FA} is variance component due to family×ABA interaction. All other symbols defined as previously
* Significant at the 0.05 level

and the interaction between these two. Survival declined from 88.0% for storage at 25 °C to 63.5% at 5°C. Similarly, survival declined from 89.1% for 1 month of storage to 54.3% for 3 months' storage. The mean survival rate varied from 47.2% to 100% among families (Table 1). A regression analysis indicated that there was a significant linear increase in survival of the cultures with increasing temperature [SURV = 56.9 + 1.276 (Temperature), $R^2 = 0.97$]. Cultures can be stored at 25°C for about 1 month without noticeable loss in the survival and quality ratings, while considerable deterioration occurred after 2 months of storage. However, temperature had little effect on subsequent, eventual recovery of the cultures. Surviving cultures, even if rated as poor at the end of the storage period, recovered eventually. This recovery often required several subculture cycles.

The largest genetic component of variance in SURV was the variance due to parental GCA (12.4%) followed by the variance between clones within families (8.2%) and to maternal effects (0.9%), while those due to SCA and reciprocal effects were negligible. Variances due to family treatment interactions were also significant (Table 2).

Maturation of somatic embryos

Estimated variance components for the number of normal (NMSE), precociously germinating (PGSE) and total (TMSE) mature somatic embryos are presented in Table 3. Variance due to parental GCA was significant, accounting for 3.5 to 7.8% of the total phenotypic variance. But most of the genetic variance was accounted for by that due to clones within families, from 34.3 to 43.2% of total variance. The variances due to SCA, maternal and reciprocal effects were small and not significant. Variance due to family × ABA interactions was also small but statistically significant.

ABA had a significant effect on NMSE and PGSE, and TMSE compared to no ABA. For the normal mature so-

Table 4 Effects of ABA as indicated by mean (standard error) numbers of normal mature (NMSE), precociously germinating (PGSE) and total mature (TMSE) somatic embryos, and percentages of normal germination (NORM) and germination with shoots only (SHOOT) at varying ABA levels

ABA levels (μM)	Maturation			Germination	
	NMSE	PGSE	TMSE	NORM	SHOOT
0	2.9 (0.3)	6.6 (0.7)	9.5 (0.8)	20.2 (2.2)	40.6 (2.9)
12.5	5.3 (0.5)	13.6 (1.1)	18.9 (1.4)	25.7 (1.9)	36.9 (2.0)
25	7.6 (0.8)	13.7 (1.1)	21.3 (1.6)	19.8 (1.7)	39.6 (2.3)
37.5	4.1 (0.5)	14.1 (1.2)	18.2 (1.4)	20.3 (1.9)	35.5 (2.4)
50	8.5 (0.8)	10.4 (0.9)	18.9 (1.4)	24.4 (1.9)	28.3 (2.2)
Mean	5.7 (0.3)	11.7 (0.5)	17.4 (0.6)	22.2 (0.9)	35.7 (1.0)

Table 5 Mean percentages (standard error) for germination with normal roots (NORM), with shoots only (SHOOT), misshapen embryos (MISS), and dead (DEAD) with respect to maturation types

Maturation type	NORM	SHOOT	MISS	DEAD
NMSE	20.3 (1.5)	31.2 (1.9)	9.3 (1.2)	39.2 (2.2)
PGSE	23.4 (1.0)	39.3 (1.2)	37.5 (1.4)	0.2 (0.2)
Overall mean	22.2 (0.9)	35.7 (1.0)	26.3 (1.1)	15.8 (1.1)

matic embryos (NMSE), the optimal ABA level was not distinct; for the TMSE, it was at 25 μM. At that concentration, the mean number of TMSE was 21.3 while that of the no-ABA control was 9.5 (Table 4).

Germination of somatic embryos

During the 4-week germination period, 22.2% of the total mature somatic embryos produced normal roots (NORM), 35.7% produced normal shoots but no roots (SHOOT), 26.3% became misshapen (MISS) and 15.8% died (DEAD) (Table 5).

ABA treatment levels used during the maturation had a significant effect on normal germination (NORM) although actual differences were relatively small, and varied from 19.8 to 25.7% (Table 4). Furthermore, there was no obvious trend in percentages of NORM at increasing levels of ABA treatment. ABA had a significant effect on shoot-only germination (SHOOT). Again, a clear trend with concentration levels of ABA was not obvious.

Whether the embryos were NMSE or PGSE at the beginning of germination also had a significant effect on NORM and SHOOT although the difference was small (Table 5). Both maturation types (NMSE and PGSE) had similar percentages of NORM, but a slightly higher percentage of the PGSE produced SHOOT. However, both maturation types were quite different with respect to MISS and DEAD. There was a significant difference between the mean percentages of MISS, i.e., 37.5% for PGME vs 9.3% for NMSE. The NMSE had a higher percentage of DEAD (39.2%) while that for PGSE was negligible (0.2%) (Table 5). This suggests that NMSE may be sensitive to the desiccation treatment.

Most of the genetic variance in NORM was due to variation among clones within families, amounting to 17.9% of the total variance (Table 6). None of the components of the family variances, i.e., GCA (σ^2_g), SCA (σ^2_s), maternal (σ^2_m) and reciprocal (σ^2_r) variances, were significant. However, a significant family × maturation-type interaction variance (σ^2_{FM}), 10.8% of the total variance, was found. For SHOOT, variance due to clones within families (σ^2_C) was the only significant genetic source of variation amounting to 12.1% of the total variance. The variation due to interactions of families and maturation types was significant, amounting to 7.4% (Table 6). Similarly, σ^2_C was the only significant source of genetic variance in MISS, amounting to 22.1% of the total variance (Table 6). Again, for DEAD, σ^2_C was the significant source of genetic variance, amounting to 16.1% of the total variance. The variance due to interactions of family and maturation type was almost as large (13.2%) as that due to clones within family (Table 6).

Cryopreservation

Of the 551 clones cryopreserved, 457 (82.9%) were successfully defrosted and repropagated initially. Of the 94 clones that did not survive, 54 could not be tried again because the non-cryopreserved part of each of these clones was not in good enough condition to be used again. However, 40 were successfully reintroduced in cryo-storage, defrosted and repropagated. Culture quality appeared to be the main factor determining survival in cryopreservation. Genetics probably played no major role in survival of the clones.

Discussion and conclusion

The interpretation of the various variance components in this paper is similar to that in the discussion of the initiation of SE (Park et al. 1993). It is evident that genetics played a major role during all phases of somatic embryogenesis. Maturation, like initiation, is under additive genetic control. However, the GCA variance ($2\sigma^2_g$) contribution declined from 41.7% during the initiation phase (Park et al. 1993) to 7.8% during maturation (TMSE), and to 1.5% during normal germination (NORM) (Table 7). Similarly, the variances due to SCA, maternal and reciprocal effects declined during maturation and germination. Consequently, for germination none of the family components of variance (i.e., σ^2_g, $\sigma 2^2_s$, σ^2_m, and σ^2_r) were significant. The variance due to clones (σ^2_C) cannot be estimated for the initiation phase for the simple reason that clones do not yet exist at the beginning of the initiation phase. During the bulk-up phase (GROW), the clones were considered as rep-

Table 6 Estimated variance components (standard deviation) and their percentages[a] of total phenotypic variance for germination with normal roots (NORM), with shoots only (SHOOT), misshapen embryos (MISS) and dead (DEAD)

Variance[b] component	NORM		SHOOT		MISS		DEAD	
	Estimate	%	Estimate	%	Estimate	%	Estimate	%
$2\sigma_g^2$	0.0024 (0.0018)	1.5	–0.0060 (0.0016)	0.0	–0.0020 (0.0018)	0.0	–0.0018 (0.0009)	0.0
σ_s^2	0.0012 (0.0031)	0.8	0.0049 (0.0056)	2.3	0.0047 (0.0052)	2.9	–0.0011 (0.0030)	0.0
σ_m^2	0.0004 (0.0010)	0.2	0.0041 (0.0030)	1.9	0.0003 (0.0010)	0.2	0.0028 (0.0018)	1.4
σ_r^2	0.0000 (0.0032)	0.0	0.0007 (0.0039)	0.3	–0.0018 (0.0035)	0.0	–0.0056 (0.0025)	0.0
σ_C^2	0.0287 (0.0064)*	17.9	0.0259 (0.0090)*	12.1	0.0363 (0.0084)*	22.1	0.0318 (0.0094)*	16.1
σ_{FA}^2	–0.0006 (0.0028)	0.0	–0.0002 (0.0042)	0.0	0.0004 (0.0031)	0.2	–0.0023 (0.0033)	0.0
σ_{FM}^2	0.0172 (0.0073)*	10.8	0.0159 (0.0083)*	7.4	0.0043 (0.0041)	2.6	0.0260 (0.0103)*	13.2
σ_e^2	0.1100 (0.0064)	68.8	0.1626 (0.0094)	76.0	0.1183 (0.0069)	72.0	0.1368 (0.0079)	69.3
Total	0.1599	100.0	0.2141	100.0	0.1643	100.0	0.1974	100.0

[a] Negative variance component estimates considered to be zero in the calculation of percentages
[b] σ_{FM}^2 is the variance component due to family × maturation type interaction. All other symbols defined as previously
* Significant at 0.05 level

Table 7 Percentages of genetic components of variances relative to total phenotypic variances during the initiation of embryogenic tissue (INIT), growth rate of embryogenic tissue during the bulk-up phase (GROW), total mature somatic embryos (TMSE), and germination of somatic embryos with normal roots (NORM)

Variance component	INIT[a]	GROW	TMSE	NORM
$2\sigma_g^2$	41.7	19.3	7.8	1.5
σ_s^2	4.4	3.2	0.4	0.8
σ_m^2	7.8	2.2	0.4	0.2
σ_r^2	14.8	13.0	0.5	0.0
σ_C^2	–	[b]	43.2	17.9
σ_e^2	3.3	62.3[b]	45.1	68.8

[a] Initiation data averaged over treatment combinations from Park et al. (1993)
[b] Variance due to clones are confounded with experimental error since the clones are considered as replicates during analysis
–, Data not available since clones were developed during the initiation

licates for statistical analysis; the error variance (σ^2_e), therefore, contains variance due to clones. This variance was high. The variance due to clones declined during the maturation phase (TMSE) and declined still further during normal germination (NORM) (Table 7). This drastic reduction in genetic variances from initiation to germination suggests that different sets of genes are responsible for the initiation, maturation and germination phases. This, of course, is not unexpected. There was no correlation between the percentages of SE initiation and the different maturation and germination characters. There was also no correlation between the various maturation and germination characters. This again suggests that different gene sets operate during each phase.

For all the SE characters examined, variance due to clones (σ_C^2) was significant in all cases and was often the largest of the genetic components of variance, although it declined from bulking-up (GROW) to germination. This component (σ_C^2) was translated into the remainder of the total genetic variance, after covariance of full-sibs was removed, following the additive-dominance-epistasis genetic model described by Mullin and Park (1992). According to this model, total genetic variance (σ_G^2) consists of additive (σ_A^2), dominance (σ_D^2) and epistatic (σ_I^2) variances, i.e., $\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2$, which is equivalent to $2\sigma_g^2 + \sigma_s^2 + \sigma_C^2$ when expressed in terms of the linear analysis model components. Since σ_g^2 and σ_s^2 are interpreted as being one-quarter of σ_A^2 and of σ_D^2 respectively, σ_C^2 contains the epistatic variance (σ_I^2), which is estimated as $\sigma_C^2 - 2\sigma_g^2 - 3\sigma_s^2$. Estimated genetic components of variances under the additive-dominance-epistasis model for various phases of the experiments are presented in Table 8. With the exception of SURV and COND, the magnitude of epistatic variance (σ_I^2) was large. For maturation characters, i.e., NMSE, PGSE and TMSE, σ_I^2 was consistently the largest genetic variance ranging from 63.8 to 81.7% of σ_G^2, while additive variance (σ_A^2) ranged from 18.3 to 30.3%. Dominance variance (σ_D^2) during maturation was consistently small, ranging from 0.0 to 6.3%. The composition of genetic variances among the three germination types varied drastically. For NORM, epistatic variance (σ_I^2) was large (70.2%) with an equal magnitude of additive (σ_A^2) and dominance (σ_D^2) variances (14.9% each). For both SHOOT and MISS, additive variance was lacking. Dominance variance was larger than epistatic variance for SHOOT, while epistatic variance was larger for MISS; however, the difference was only about 10%.

Epistatic variance is difficult to estimate accurately without additional information on gene action, allele frequencies and numbers of loci involved. Therefore, the estimates presented here must be viewed cautiously. In addition to the usual assumptions required for interpretation of the genetic model (see Mullin and Park 1992), two further assumptions apply. Firstly, when present, epistasis is due primarily to interactions involving more than two or three loci. Where this is not the case, estimates of σ_A^2 and σ_D^2 may be contaminated seriously by a fraction of epista-

Table 8 Estimated genetic components of variance (standard deviation) during bulk-up, storage, maturation and germination phases

Variance[a]	Estimate	%	Estimate	%	Estimate	%
	Bulk-up and storage experiment					
	GROW		SURV		COND	
σ^2_A	0.2244 (0.5408)	45.5	0.0764 (0.0004)	100.0	0.0236 (0.0000)	95.9
σ^2_D	0.0740 (0.0019)	15.0	–0.0112 (0.0000)	0.0	–0.0020 (0.0000)	0.0
σ^2_I	0.1951 (0.7388)	39.5	–0.0047 (0.0000)	0.0	0.0010 (0.0073)	4.1
σ^2_G	0.4935 (0.7363)	100.0	0.0605 (0.0213)	100.0	0.0226 (0.0071)	100.0
	Maturation of somatic embryos					
	NMSE		PGSE		TMSE	
σ^2_A	0.1664 (0.0037)	18.3	0.7624 (0.0648)	29.9	0.9844 (0.1019)	30.3
σ^2_D	–0.0076 (0.0027)	0.0	0.1600 (0.0245)	6.3	0.0900 (0.0307)	2.8
σ^2_I	0.7449 (0.1388)	81.7	1.6287 (0.4110)	63.8	2.1777 (0.5026)	66.9
σ^2_G	0.9037 (0.1174)	100.0	2.5511 (0.3464)	100.0	3.2521 (0.4372)	100.0
	Germination of somatic embryos					
	NORM		SHOOT		MISS	
σ^2_A	0.0048 (0.0000)	14.9	–0.0120 (0.0000)	0.0	–0.0040 (0.0000)	0.0
σ^2_D	0.0048 (0.0000)	14.9	0.0196 (0.0001)	53.3	0.0188 (0.0001)	43.7
σ^2_I	0.0227 (0.0118)	70.2	0.0172 (0.0193)	46.7	0.0242 (0.0181)	56.3
σ^2_G	0.0323 (0.0080)	100.0	0.0248 (0.0111)	100.0	0.0390 (0.0105)	100.0

[a] σ^2_A, σ^2_D, σ^2_I, and σ^2_G are additive, dominance, epistatic and total genetic variances, respectively. Negative estimates are considered to be zero for calculation of percentages

sis and σ^2_I will be underestimated by a corresponding amount. Secondly, it is assumed that "C-effects", defined by Lerner (1958) as "non-random environmental variances common to members of subgroups within a population", are negligible or absent. If these assumptions are not met, estimates of variance may be biased (Mullin et al. 1992). The extent of the contribution of C-effects to among-clone variance is difficult to quantify without further experimentation.

It is, however, interesting to note the different magnitudes of σ^2_I among different phases of SE as well as within each phase. For instance, among the three germination types, there was a drastic reduction in σ^2_I for SHOOT and MISS when compared with NORM. Such change in the magnitude of genetic variances suggests changes in gene expression. One may speculate that three or more genes, or groups of genes, are responsible for germination of somatic embryos, one gene, or group of genes, being responsible for the development of cotyledons, another for the development of the hypocotyl and another for the development of roots. When all the genes are turned on, we observe normally germinated embryos; when not all genes are turned on properly during germination, malformed embryos will result. If we assume that a greater number of genes will interact when all genes are turned on, it would follow that a larger proportion of epistatic variance will result than when some genes are turned off.

Mayer et al. (1991), working with *Arabidopsis*, found mutations in nine major genes that affect the body organization of embryos. They found four main classes of pattern deletions: the first was an apical deletion with the gene mutation affecting the cotyledons and apical meristem; the second was a central deletion with the gene mutation affecting the hypocotyl; the third was a basal deletion with the gene mutation deleting both hypocotyl and root; the fourth was a terminal deletion with the gene mutation deleting the root and reducing or eliminating the cotyledons. All these abnormalities occurred in those of our germinating embryos which were categorized as misshapen (MISS). This suggests that, with the culture protocols we used, one or more major genes are either not turned on, or not turned on consistently, wherever abnormalities occurred.

We have shown that with the protocols used, genetic variability among and within families played a major role in the initiation, maturation and germination of somatic embryos in white spruce. It would be of interest to determine if, after protocol improvement in future experiments, the same genetic patterns are maintained or if improved culture conditions will change gene expression. If with improved protocols gene expression is changed more in some families than in others, a genetic analysis similar to the one described here could result in entirely different among- and within-family genetic patterns.

Clonal forestry, defined as deployment of tested clones, has many advantages over conventional forestry (Libby and Ahuja 1993). However, with the traditional cloning method of rooting of cuttings, it is not widely practised with conifers, because maturation affects the propagation capacity of the donor clones and the performance of ramets (Bonga and von Aderkas 1993). Somatic embryogenesis in conjunction with cryopreservation can circumvent maturation problems. As shown here, presumed-juvenile embryogenic tissue is easily cryopreserved in liquid nitrogen during the clonal testing, thus providing practical possibilities for arresting maturation. It is also encouraging that we obtained emblings from all 30 families used in the experiment.

In our earlier paper (Park et al. 1993), we showed that there is a strong genetic effect during the initiation of embryogenic tissue. In our current paper, we show that the genetic effects in bulking-up, maturation and germination are less strong. With declining genetic control during maturation and germination, it is the initiation phase that can be most effectively manipulated by breeding programs. Therefore, there is only a limited opportunity to breed for improved maturation and germination. When clones produce many emblings, these can be used for mass propagation. When clones produce only a few emblings, mass propagation by SE is not possible; however, the few emblings produced by these clones can be mass propagated by rooting of cuttings. Thus, SE still has the advantage of being able to maintain clones in a juvenile state in cryopreservation while long-term field testing of these same clones is conducted. Alternative clonal propagation schemes based solely on rooting of cuttings lack this distinct advantage.

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