Relationship between field performance, family, embryo morphology, and isozyme heterozygosity, and in vitro reactivity in jack pine

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Abstract: The influence of field performance, family, embryo morphology, and isozyme heterozygosity level on in vitro reactivity of *Pinus banksiana* Lamb. was evaluated on embryos from five superior families, five inferior families, and a mixed seed lot. Embryo length, number of cotyledons, and isozyme heterozygosity were determined for each embryo. Seed germination and fresh weight were determined on a family level. On average, superior families showed higher percentages of embryos that formed buds in vitro. Within each performance class, the analysis based on initial number of embryos revealed differences among families for the percentage of green embryos and embryos with adventitious buds and shoots. When calculations were based on green embryos only, i.e., excluding embryos that remained white, there were no differences among families. Thus, the overall in vitro potential of a family appears to be strongly dependent upon the capacity of embryos to turn green. On a per family basis, seed germination was positively correlated with most in vitro characters, with the exception of mean shoot length per shoot-forming embryo. Small embryos had a lower probability of producing buds and shoots, and embryos with three cotyledons showed a higher mortality than embryos with four or more cotyledons. No significant relationships were observed between heterozygosity level and in vitro reactivity, with analyses performed on green embryos only.

Résumé : L'influence de la performance au champ, de la famille, de la morphologie des embryons et de leur degré d'hétérozygotie sur la réactivité in vitro de *Pinus banksiana* Lamb. a été évaluée sur des embryons de cinq familles supérieures, de cinq familles inférieures et d'un lot de graines mélangées. La longueur de l'embryon, le nombre de cotylédons et le degré d'hétérozygotie enzymatique ont été mesurés sur chaque embryon; le taux de germination et le poids frais des graines l'ont été au niveau familial. Les familles supérieures ont eu un pourcentage d'embryons formant des bourgeons in vitro plus élevé que les familles inférieures. Au sein de chaque classe de performance, lorsque les calculs étaient basés sur l'ensemble des embryons mis en culture, des différences entre les familles ont été observées dans le pourcentage d'embryons verts et d'embryons avec bourgeons et tigelles adventifs. Lorsque les pourcentages sont calculés sur les embryons verts uniquement, i.e., excluant ceux qui restent blancs, les différences entre les familles ne sont plus significatives. Ces résultats suggèrent que le potentiel in vitro d'une famille dépend grandement de la capacité de ses embryons à verdir. Sur une base familiale, le taux de germination est corrélé positivement avec presque tous les paramètres de réactivité in vitro, sauf la longueur moyenne des tiges par embryon. Les petits embryons avaient une plus faible probabilité de produire des bourgeons et des tigelles, et les embryons avec trois cotylédons ont subi une mortalité supérieure à ceux qui en avaient quatre ou plus. Aucune relation significative n'a été observée entre le degré d'hétérozygotie des embryons et leur réactivité in vitro, lors d'analyses effectuées sur les embryons verts seulement.

Introduction

In conifers, variation in explant response in vitro is a common phenomenon in both organogenic (Cheng 1977; von Arnold 1982; Patel and Thorpe 1984; Noh et al. 1988; Bergmann and Stomp 1992, 1994) and embryogenic systems (Webb et al. 1989; Becwar et al. 1990; Tremblay 1990; Ekberg et al. 1993). For example, Canary Island pine (*Pinus canariensis* Sweet et K. Spreng.) was reported to exhibit considerable variation in adventitious bud and shoot formation, and shoot elongation among embryos, and even among cotyledons from the same embryo (Martinez Pulido et al. 1990). In black spruce (*Picea mariana* (Mill.) BSP) both callus and embryogenic tissue formation varied among explants and families (Cheliak and

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Klimaszewska 1991). In jack pine (*Pinus banksiana* Lamb.), adventitious shoots have been successfully induced in vitro on excised cotyledons (Karnosky and Diner 1984) and whole embryos (Chesick and Bergmann 1991; Lemay 1993; Harry and Thorpe 1994). Karnosky and Diner (1984) reported considerable inter-embryo variation in shoot formation, with values ranging from zero to 28 shoots per embryo. Experiments in our laboratory have given similar results, with jack pine shoot numbers ranging from zero to 18 per embryo (C.H. Briand, unpublished). Explant heterogeneity may cause a lack of repeatability among experiments in vitro (Go et al. 1993) and make the development and optimization of protocols for plantlet formation extremely difficult. Numerous studies have shown that morphogenesis can be influenced by the genotype of the explant, the physiological status of the donor plant, and the tissue culture environment (Bornman 1983; Törmälä 1990).

Apart from observation of genotypic variation, genetic component of in vitro reactivity has rarely been analysed. One of these studies, performed on white spruce (*Picea glauca* (Moench) Voss), showed that the initiation of somatic embryogenesis was under strong additive genetic control, with a high narrow-sense heritability (Park et al. 1993). In the field, a relationship between radial growth or growth variability and heterozygosity was found in some forest tree species (Mitton and Grant 1980; Knowles and Grant 1981; Bush and Smouse 1991). More specifically in jack pine, a relationship between growth, biomass production, and heterozygosity was expressed at a juvenile stage, in stressed environments (Govindaraju and Dancik 1986, 1987). These observations raise the question whether a relationship exists between heterozygosity and in vitro performance.

To gain some insight on factors affecting adventitious organogenesis in jack pine embryos, we have studied both phenotypically superior and inferior families. The objectives were to define (1) whether embryos from superior families have a better in vitro potential than embryos from inferior families, (2) whether germination rate and morphological characteristics of embryos are correlated with in vitro response, and (3) whether the isozyme heterozygosity of an embryo influences its in vitro response.

Material and methods

Plant material

Seeds from five superior and five inferior open-pollinated jack pine families collected in the Abitibi-Témiscamingue region of Quebec, Canada (between 46°56′ and 48°06′N, and 77°16′ and 78°52′W), were obtained from the Ministère des Ressources naturelles du Québec (MRN). Families had been classified as superior or inferior on the basis of height growth during field trials. Growth measurements were taken on trees after 10 years (R. Beaudoin, MRN, unpublished) and superior families had significantly greater height growth ($P = 0.0001$). A mixed seed lot (Berry 87) was obtained from Les Serres de Guyenne, Abitibi, Quebec. Seedlings from this seed lot have routinely been used for reforestation. All seeds were stored in the dark at 4°C for respectively 5 (Berry 87), 10 (families 82 038 and 83 279), or 14 (all other families) years, until the beginning of the study. Seed fresh weight (FW) was measured to the nearest 0.01 mg with a Mettler AJ100 electronic balance (26–40 seeds per family). Germination rates were determined as follows. Twenty-five seeds from each family were placed on moistened filter paper in Petri plates (100 × 15 mm), and kept in the dark at room temperature. The number of germinated seeds (radicle visible) was recorded after 3 weeks.

In vitro culture

Seeds were sterilized according to the following schedule: (1) 95% ethanol for 2 min, (2) 6% sodium hypochlorite (full-strength Javex) containing a few drops of Tween 80 for 30 min, (3) 70% ethanol for 15 min, (4) 3% sterile hydrogen peroxide for 15 min, and (5) three rinses in sterile deionized water (10 min each). The seeds were then imbibed overnight in sterile 0.6% Javex in the dark. The embryos were aseptically excised and plated horizontally on basal media (ca. 10 mL) in 60 × 15 mm Petri plates, which were then sealed with Parafilm M. Forty embryos were placed in culture for each family and for the mixed seed lot (four per Petri plate, 10 replicates). The length of each embryo was measured to the nearest 0.1 mm using a Wild-Leitz dissecting microscope equipped with an ocular micrometer.

The basal media consisted of the mineral salts and vitamins of Schenk and Hildebrandt (1972) at one-half strength, 1.5% sucrose (w/v), 10 μM 6-benzylaminopurine (BAP), and 0.7% Difco Bacto agar (w/v). The Na–Fe–EDTA stock solution was prepared according to Steiner and Van Winden (1970). The pH was adjusted to 5.7 prior to autoclaving (20 min at 121°C and 103.3 kPa). After 3 weeks on the induction medium, the embryos were transferred to BAP-free medium containing 0.2% neutralized activated charcoal (w/v). After a further 3 weeks, the embryos were transferred twice to BAP- and activated charcoal-free medium (two 21-day passages, from the 6th to 12th week). To allow for shoot elongation and development, 100-mL baby food jars (with Magenta B-Cap closures) containing ca. 25 mL of medium were used for the final passage (from the 9th to 12th week). All cultures were maintained at 26 to 31°C (shelf level) in a tissue culture chamber. Illumination was from Vita-Lite fluorescent tubes, with a photon flux of ca. 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (shelf level) and a photoperiod of 16 h.

The number of cotyledons per embryo was recorded after 3 weeks in culture (cotyledons reflexed and separated at this time). Cotyledon counts were made only on green embryos, as the cotyledons of embryos that remained white did not reflex, making an accurate count difficult. The number of (1) dead, (2) white, and (3) green embryos was recorded after 3 and 12 weeks in culture. As none of the embryos scored as white at 3 weeks turned green, we believe they would have remained nonresponsive for the entire 12 weeks. They were therefore removed after 6 weeks to avoid mortality and frozen for subsequent electrophoretic analysis. The number of green embryos with adventitious buds was recorded after 3 weeks in culture, while the number of green embryos with adventitious shoots (stem ≥2.0 mm in length), the number of shoots per embryo, and shoot length (mean per embryo) were determined after 12 weeks in culture. Proportions (based on 40 explants per family initially placed in culture) of green embryos at 3 and 12 weeks, of embryos with buds after 3 weeks, and of embryos with shoots after 12 weeks were computed on a per Petri plate basis, the experimental unit being a group of four embryos. Proportions of green embryos with buds (3 weeks) and of green embryos with shoots were also calculated (i.e., excluding dead and white embryos). The mean number of shoots and the mean shoot length were computed based on the shoot-forming embryos only.

Electrophoresis

White embryos (6 weeks), and green embryos with or without shoots (12 weeks) were stored at –70°C. They were ground in Feret's extraction solution (Feret 1971), containing 10 μL NADP (2 $mg \cdot mL^{-1}$). Eight enzyme systems were assayed using cellulose acetate gels (76 × 76 mm Titan III) with a Tris–glycine buffer (pH 8.5), using the methodology of Hebert and Beaton (1989). The enzyme systems were as follows: aconitase (ACO), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6P), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), and 6-phosphoglucomate dehydrogenase (6PG).

Table 1. *P*-values and percent variance (in parentheses) from separate two-level nested ANOVA of jack pine seed and embryo characteristics.

Character	Between performance classes	Among families within performance classes	Error
Seed FW	0.494	<0.001	—
	—	(35.6)	(64.4)
Embryo length	0.682	<0.001	—
	—	(35.7)	(64.3)
Cotyledon number[a]	0.383	0.004	—
	—	(6.1)	(93.9)
Average level of heterozygosity[a]	0.255	0.692	—
	—	(1.1)	(98.9)

Note: The Berry 87 seed lot is not included in calculations.
[a]Excluding white embryos.

The loci for each enzyme system were numbered sequentially from the most anodal to the most cathodal. Little enzyme activity could be detected in the white embryos; thus, they were excluded from further analysis. The level of heterozygosity for each embryo was calculated as number of heterozygous loci ÷ number of loci scored × 100. These percentages were used to define the average level of heterozygosity of the families.

Statistical analysis

The influence of field performance (superior or inferior) and family on seed fresh weight, embryo length, number of cotyledons, average level of heterozygosity, and the in vitro response indicators was explored using nested ANOVA. The in vitro response indicators expressed in proportions were standardized with logit transformations (logit = $\log(p/1-p)$), while untransformed data were used for shoot number and shoot length. In all ANOVA, performance was treated as a fixed effect, while families within performance was treated as a random effect and *F*-values were based on type I sums of squares, using Satterthwaite's approximation (Zar 1984).

To compare germination rates between superior and inferior performance classes, the Mann–Whitney test was used. To evaluate the effect of family seed FW, germination rate, embryo length, and cotyledon number on in vitro response, correlation coefficients were computed. The influence of embryo morphology (embryo length and cotyledon number) and heterozygosity level on in vitro response was ascertained using log–linear models (when more than two factors were considered), χ^2 analysis, Student's *t*-tests, and the Kruskal–Wallis test. Embryo length was converted into three classes, each representing one-third of the observed range: small (≤2.68 mm), medium (2.69 to 3.24 mm), and large (≤3.25 mm). Individual locus heterozygosity was converted into two classes, representing the presence or absence of heterozygous loci. Furthermore, the data from embryos with one or more heterozygous loci were pooled and compared with data from homozygous embryos. Statistical analyses were performed using SAS (SAS Institute Inc. 1988) and Minitab (Minitab Inc. 1992).

Results

Seed and embryo characteristics

Jack pine seeds weighed between 1.13 and 5.94 mg, with family means ranging from 2.77 to 3.96 mg. The excised embryos, placed in culture, were between 2.12 and 3.80 mm in length, with family means from 2.82 to 3.23 mm. Green embryos bore three to six cotyledons (mode = 4), with family means ranging from 3.5 to 4.3. There was a weak positive correlation between embryo length and the number of cotyledons per embryo (r = 0.198, $P < 0.001$). Average family seed FW was positively correlated with embryo length (r = 0.749, P = 0.008).

These characters did not vary significantly between the superior and inferior performance classes. There was, however, significant variation among families within a performance class for each character (Table 1). Families accounted for approximately 36% of the variation in seed FW and embryo length, but only 6% of the variation in cotyledon number of green embryos (Table 1). In all cases, the error term accounted for the majority of the variance. Seed germination ranged from 64 to 100% in the superior families and from 20 to 80% in the inferior ones (Table 2). Eighty percent of the seeds from the mixed seed lot (Berry 87) germinated. On average, germination was higher in the superior performance class (90.4%) than in the inferior one (55.2%) (P = 0.028, Mann–Whitney test).

Nested ANOVA revealed that the average level of isozyme heterozygosity of green embryos did not vary significantly between superior and inferior performance classes or among families within a performance class (Table 1). With the exception of *ACO1*, less than 8% of embryos were heterozygous at any of the loci scored, with *PGI2* and *PGI4* completely homozygous for all embryos (Table 3). Overall, 34% of the embryos were heterozygous for at least one locus, while 66% were homozygous for all loci scored. For those embryos that exhibited heterozygosity, on average 14.8 ± 3.9% of their loci were heterozygous. There was no correlation between the level of embryo heterozygosity and embryo length (r = –0.012, P = 0.830) or the number of cotyledons per embryo (r = –0.027, P = 0.656). On a per family basis, heterozygosity showed no correlation with germination (r = 0.089, P = 0.795) or seed FW (r = 0.240, P = 0.478).

Influence of performance class and family on in vitro response

After 3 and 12 weeks in culture, superior families consistently showed a higher percentage of green embryos than the inferior families (Table 2). Nested ANOVA revealed no significant differences between performance classes, while significant differences were observed among families within performance classes (Table 4). Families accounted for the largest amount of variation at 3 weeks (55%) and a considerable amount of variation at 12 weeks (36%).

On average, superior families had a higher percentage of embryos with buds after 3 weeks than inferior families (Table 2). When this percentage was evaluated relative to the initial number of embryos (40 per family), it differed significantly between superior and inferior performance classes (P = 0.053) as well as among families within performance classes ($P < 0.001$) (Table 4). When dead and white embryos were excluded, bud formation did not differ significantly between performance classes or among families. Regarding the characters related to shoot formation, a significant difference was found only among families for the percentage of embryos with shoots, when calculations included dead and white embryos (Tables 2 and 4).

Using the standard deviation as a measure of variability, the mixed seed lot Berry 87 exhibited no more variation in in vitro response than many of the half-sib families (Table 2). Furthermore, when the families and the mixed seed lot (Berry 87) were ranked for each in vitro response variable, the mixed seed

Table 2. Means and standard deviations[a] (in parentheses) for the response of jack pine families in vitro.

Family	% germination	% green embryos[b] (3 weeks)	% embryos[b] with buds (3 weeks)	% embryos[c] with buds (3 weeks)	% surviving green embryos[b] (12 weeks)	% embryos[b] with shoots (12 weeks)	% embryos[c] with shoots (12 weeks)
Superior							
4002	92	100.0 (0.0)	90.0 (12.9)	90.0 (12.9)	92.5 (16.9)	57.5 (33.4)	62.5 (33.9)
7605	64	52.5 (32.2)	45.0 (30.7)	82.4 (33.4)	40.0 (24.1)	27.5 (21.9)	66.7 (44.1)
8663	100	92.5 (12.1)	82.5 (23.7)	88.3 (19.3)	77.5 (21.9)	60.0 (26.9)	77.5 (26.1)
11304	96	95.0 (10.5)	95.0 (10.5)	100.0 (0.0)	77.5 (32.2)	42.5 (35.5)	50.9 (35.2)
83279	100	97.5 (7.9)	97.5 (7.9)	100.0 (0.0)	95.0 (10.5)	40.0 (31.6)	40.8 (31.3)
Group mean	90.4 (15.1)	87.5 (23.8)	82.0 (26.7)	92.3 (18.3)	76.5 (29.2)	45.5 (31.4)	59.7 (35.3)
Inferior							
4801	20	27.5 (14.2)	22.5 (18.4)	77.8 (44.1)	20.0 (15.8)	12.5 (13.2)	64.3 (47.5)
4807	68	65.0 (31.6)	60.0 (26.9)	94.4 (11.0)	62.5 (29.5)	45.0 (19.7)	75.9 (23.7)
5008	52	72.5 (14.2)	62.5 (17.7)	86.7 (17.2)	62.5 (17.7)	27.5 (21.9)	45.0 (37.7)
8601	56	37.5 (27.0)	27.5 (24.9)	70.8 (36.5)	25.0 (26.4)	15.0 (24.2)	50.0 (44.7)
82038	80	97.5 (7.9)	75.0 (28.9)	76.7 (27.7)	72.5 (32.2)	42.5 (35.5)	55.5 (34.1)
Group mean	55.2 (22.5)	60.0 (32.3)	49.5 (30.9)	81.5 (29.2)	48.5 (32.5)	28.5 (26.7)	58.1 (37.2)
Mixed seed lot							
Berry 87	80	100.0 (0.0)	90.0 (12.9)	90.0 (12.9)	80.0 (23.0)	55.0 (25.8)	67.5 (31.3)

[a]Standard deviations were calculated on a Petri plate basis.
[b]Including dead and white embryos.
[c]Excluding dead and white embryos.

Table 3. Number of jack pine embryos scored (*N*) and family and global proportions of heterozygous embryos (%) for the loci for six isozymes.

	ACO1		*PGM1*		*PGM2*		*G6P1*		*G6P2*		*ADH3*		Avg. level of heterozygosity		
Family	*N*[a]	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	SD
Superior															
4002	21	28.6	7	0	37	0	35	0	24	0	36	8.3	37	3.2	6.5
7605	11	45.5	16	0	16	0	16	0	6	0	13	23.0	16	6.8	7.1
8663	25	28.0	31	0	31	0	31	6.5	11	0	29	6.9	31	4.9	8.3
11304	22	27.3	31	16.1	31	0	30	6.7	13	0	29	3.4	31	6.4	9.5
83279	22	45.5	38	2.6	38	0	38	0	17	0	36	2.8	38	4.3	6.5
Global	101	33.7	153	3.9	153	0	150	2.7	71	0	143	7.0	153	4.8	7.6
Inferior															
4801	6	33.3	8	0	8	0	8	0	4	0	8	13.0	9	4.6	7.0
4807	17	23.5	24	0	24	4.2	23	0	15	0	20	5.0	24	3.4	6.0
5008	11	36.4	25	4.0	25	0	25	0	16	6.3	24	4.2	25	3.7	6.1
8601	5	20.0	10	0	10	0	10	0	4	0	9	11.0	11	2.8	6.3
82038	23	26.1	29	3.4	29	0	29	3.4	13	0	29	6.9	29	4.6	7.3
Global	62	27.4	96	2.1	96	1.0	95	1.0	52	1.9	90	6.7	96	3.9	6.5
Mixed seed lot															
Berry 87	22	54.5	33	3.0	33	0	32	6.3	19	5.3	30	10.0	33	7.7	7.4
All seeds pooled	185	34.1	282	3.2	282	0.4	277	2.5	142	1.4	263	7.2	282	4.8	7.3

[a]The sample size varies among isozymes, as some embryos could not be scored accurately for some isozymes.

lot ranked in the top one-third for all characters measured with the exception of shoot length (rank: 6/10, one tie).

On a per family basis, seed germination exhibited strong positive correlations with the percentage of green embryos after 3 and 12 weeks in culture ($r = 0.874$, $r = 0.870$, respectively; $P < 0.001$), the percentage of embryos with buds after 3 weeks (based on initial number of embryos: $r = 0.892$, $P = 0.001$; based on green embryos only: $r = 0.630$, $P = 0.038$), the percentage of embryos with shoots (based on initial number of embryos: $r = 0.821$, $P = 0.002$), and the number of shoots per shoot-forming embryo ($r = 0.666$, $P = 0.025$), but was not correlated with the percentage of embryos with shoots based on

Table 4. *P*-values and percent variance (in parentheses) from separate two-level nested ANOVA of the response of jack pine in vitro.

Character	Between performance classes	Among families within performance classes	Error
% green embryos	0.0944	<0.001	—
(3 weeks)[a]		(54.8)	(45.2)
% embryos with buds	0.0530	<0.001	—
(3 weeks)[a]	—	(43.5)	(56.5)
% embryos with buds	0.0923	0.3843	—
(3 weeks)[b]	—	(0.8)	(99.2)
% surviving green embryos	0.0752	<0.001	—
(12 weeks)[a]	—	(35.9)	(64.1)
% embryos with shoots	0.1096	0.0118	—
(12 weeks)[a]	—	(14.1)	(85.9)
% embryos with shoots	0.8883	0.1077	—
(12 weeks)[b]	—	(8.0)	(92.0)
Mean no. of shoots	0.3867	0.3664	—
per shoot-forming embryo	—	(0.9)	(99.1)
Mean shoot length	0.7730	0.1182	—
	—	(4.3)	(95.7)

Note: The Berry 87 seed lot was excluded from these calculations.
[a]Including dead and white embryos.
[b]Excluding dead and white embryos.

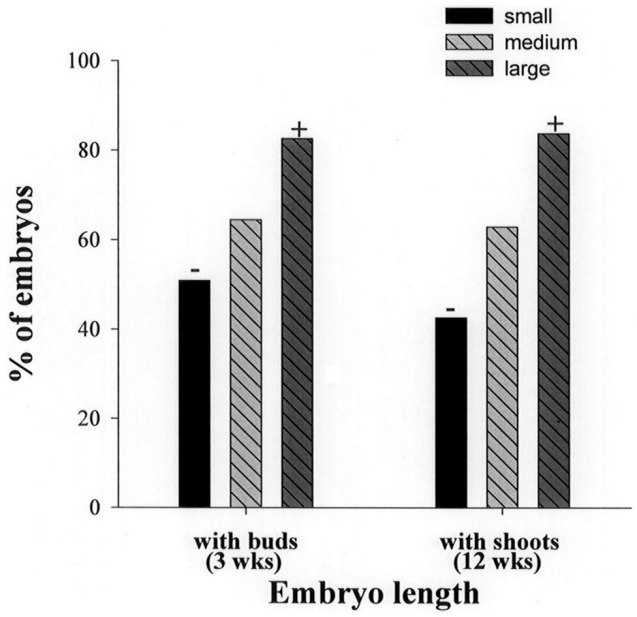
green embryos only ($r = –0.0226$, $P = 0.947$). No significant correlations were found between family mean seed FW and any of the in vitro response variables.

Influence of seed and embryo characteristics on in vitro response

There was a significant positive correlation between embryo length and embryo state (dead, white, green, green with buds or green with shoots) after 3 and 12 weeks ($r = 0.274$ and $r = 0.306$, respectively; $P < 0.001$). To further investigate the relationship between embryo length and in vitro response, log–linear models were constructed in order to take into account the effects of performance and family. When controlling for these two factors, there was a significant effect of embryo length on bud and shoot formation: small embryos were found to have a lower probability of both bud and shoot formation ($P = 0.001$ and $P = 0.003$, respectively; Fig. 1). The effects of embryo length on shoot formation became nonsignificant when only those embryos that had formed adventitious buds after 3 weeks were used in the analysis ($P = 0.068$).

When evaluating the effects of cotyledon number, we could not take performance class or family into account, as cotyledon numbers were only obtained for green embryos. As stated earlier, however, little of the variation in cotyledon number could be attributed to either performance class or families within a performance class (Table 1). Despite a lower level of bud formation (3 weeks) on green embryos with only three cotyledons, χ^2 analysis indicated that bud formation was independent of cotyledon number ($P = 0.109$). Green embryo survival at 12 weeks, however, varied with cotyledon number ($P = 0.001$), with survival depressed for those with only three cotyledons. For those embryos that survived until the end of the experiment, shoot formation was found to be independent of cotyledon number ($P = 0.097$). Both the number of shoots per shoot-forming embryo ($P = 0.960$) and shoot length ($P = 0.128$) were also unrelated to cotyledon number (Kruskal–Wallis test).

Fig. 1. Relationship between embryo length and adventitious bud (3 weeks) or shoot (12 weeks) formation in jack pine. –, a significant deficiency of embryos with buds or with shoots; +, a significant excess at $P < 0.01$, based on log–linear analysis.



For each polymorphic locus, χ^2 tests were computed to evaluate the relationship between isozyme heterozygosity and in vitro response. The frequency of heterozygous embryos observed for any particular locus was not high enough to take into account the effects of performance class and family (Table 3). The percentage of embryos with buds (3 weeks),

Table 5. Comparison of in vitro reactivity of homozygous and heterozygous jack pine embryos: proportion of embryos with buds (3 weeks) and shoots (12 weeks).

(A) Embryos with buds.

	Homozygous embryos		Heterozygous embryos		Test	
Isozyme locus	Total no.	% with buds	Total no.	% with buds	χ^2	p
ACO1	122	97.52	63	93.65	1.62	0.189
PGM1	273	95.24	9	100.00	0.86	0.353
PGM2	281	95.37	1	100.00	0.10	0.758
G6P1	270	95.19	7	100.00	0.68	0.409
G6P2	140	95.70	2	100.00	0.17	0.677
ADH3	244	95.50	19	100.00	0.19	0.194
Global[a]	191	94.24	93	95.70	0.27	0.600

(B) Embryos with shoots.

	Homozygous embryos		Heterozygous embryos		Test	
Isozyme locus	Total no.	% with shoots	Total no.	% with shoots	χ^2	p
ACO1	122	64.75	63	58.73	0.64	0.423
PGM1	273	60.44	9	55.56	0.09	0.770
PGM2	281	39.86	1	100.00	1.02	0.314
G6P1	270	61.11	7	28.57	2.96	0.085
G6P2	140	62.14	2	50.00	0.12	0.729
ADH3	244	60.25	19	63.16	0.06	0.802
Global[a]	191	60.21	93	59.14	0.03	0.863

[a]For the global class, an embryo with one or more heterozygous loci was considered as heterozygous.

percentage of shoot-forming embryos (12 weeks) (Table 5), and number of shoots per shoot-forming embryo (data not shown) were all independent of heterozygosity status at any particular locus. It should be noted that at the 0.10 level of significance, however, embryos heterozygous for *G6P1* were found to be less likely to form shoots than embryos homozygous at this locus. Despite a general trend towards higher average values for the heterozygous embryos, differences were not significant for either shoot number or length.

Discussion

The influence of genotype and family on in vitro traits has been reported for other pine species (for review see Bergmann and Stomp 1994). However, generally speaking, very few studies on conifers have taken into account the performance of the mother tree. Go et al. (1993) reported that embryos from two fast-growing provenances of *Pinus caribaea* Morelet var. *hondurensis* produced nodules earlier on their cotyledonary surface, developed shoots earlier from induced buds, and produced more shoots per surviving embryo at lower BAP concentrations when compared with embryos from two slow-growing provenances. On the other hand, the response in terms of the frequency of embryos producing buds was similar in all provenances. Our study showed a relationship between mother-tree field performance and organogenic potential. In fact, the percentage of jack pine genotypes producing buds differed significantly between performance classes, with a group mean of 82% in superior families compared with 49.5% in inferior families. On a practical level, these results are of interest, since a high percentage of reactive genotypes is desirable for in vitro production of selected tree families on a large scale.

Within a performance class, in vitro response differed significantly between families for all parameters evaluated on the initial number of embryos, i.e., greening as well as bud and shoot formation. When bud and shoot formation were compared based on green embryos only, there were no significant differences. These results indicate that families differed in their capacity to produce green embryos, but once an embryo had turned green, the probability of bud and (or) shoot production did not differ significantly between families. The overall in vitro potential of a family, in terms of the percentage of its genotypes being amenable to produce adventitious buds or shoots, was thus strongly dependent upon the capacity of embryos to turn green. This capability was shown to be related to embryo length, which differed significantly among families. Thirty-six to 55% of the variation in the proportion of green embryos and the proportion of embryos with adventitious buds was explained by the family term in the nested ANOVA. This suggests a maternal effect on these in vitro response characters, given the fact that all members of each family are half-sibs (same mother, pollen donor unknown).

Among the measured seed and embryo characters, germination level was the only one differing significantly between superior and inferior performance classes. Moreover, on a per family basis, germination was positively correlated with all in vitro characters, except mean shoot length per shoot-forming embryo. Seed germination has been previously shown to be positively correlated with induction of embryogenic callus in *Picea glauca* (Tremblay 1990), while in organogenic systems of nine pine species, no correlation was found between the probability of superior in vitro performance and that of successful germination (Bergmann and Stomp 1992). In *Pinus radiata* D. Don, the range of seed germination frequencies for 31 families was very similar to the range of frequencies of greening of embryos after 3 days in culture, but not to the frequency of healthy green embryos after 6 days (Bergmann

and Stomp 1994). In our study, with the exception of family 5008, germination and greening percentages followed similar tendencies, and germination was more strongly correlated with percentages evaluated on initial number of embryos put in culture than with the percentage of embryos with buds or shoots based on green embryos only. This suggests that the potential of an embryo to go through all stages of in vitro reactivity may be influenced by factor(s) that are also involved with successful germination.

No relationship was observed between the heterozygosity level of families and in vitro response. Little enzyme activity could be detected in white embryos, suggesting that these embryos could have been in a different physiological stage or possibly dormant (Weeden and Wendel 1989). Thus, we could not ascertain whether a relationship exists between heterozygosity and the capacity of greening of the embryos for the eight enzymatic systems studied. In a parallel study, the average level of heterozygosity of these families was determined for the same eight enzymatic systems on embryos extracted from the seeds after 24 h of imbibition. For 8 of 10 families, the average levels of heterozygosity estimated from the imbibed embryos were between 1- to 4.5-fold lower than those estimated from the green embryos subpopulation. This suggests that determination of the level of heterozygosity in green embryos resulted in an overestimation of the average level of heterozygosity of the family. We must also consider the possibility that some isozyme forms that were not detected under normal germination conditions could have been expressed under in vitro conditions. The relationship between heterozygosity and developmental events is complex, and studies have shown inconsistent results (Knowles and Mitton 1980; Mitton and Grant 1980; Giles 1984; Govindaraju and Dancik 1987; Strauss 1989, 1991; Sherry and Lord 1996). Concerning organogenetic response of the green embryos, no relationship was found with the level of heterozygosity at any particular locus, except for the lower propensity of embryos heterozygous at *G6P1* to produce shoots. Heterozygosity level, therefore, cannot be used as a marker for selecting families with high in vitro potential.

Embryo length was the main factor influencing the fate of an embryo under in vitro conditions. The longer the embryo, the higher the probability it would undergo greening, survive until the 12th week, and produce buds and shoots. Once an embryo had formed buds, however, its length had little impact on its capability to produce shoots. Thus, the significant relationship observed between embryo length and shoot-forming potential appears to ensue from the one between embryo length and bud-forming potential. Presumably, longer embryos were more vigorous than smaller embryos, either from genotypic superiority or from a phenotypic advantage of having reached a more advanced development stage in the seed. In addition, absorption of nutrients might be favoured in longer embryos, through a greater contact surface with the media.

Cotyledon number affected the survivorship of green embryos at 6 and 12 weeks but not their organogenic potential. Embryos with three cotyledons produced buds at a frequency comparable to that of embryos with four to six cotyledons, but a higher number died later. This might reflect a better performance of the latter in absorbing nutrients, a higher number of cotyledons potentially increasing the contact surface with the media. Other factors such as the amount of DNA per nucleus could also be involved in these differing survivorship levels. In germinating embryos of three jack pine families, including two (8663 and 11304) used in the present study, total DNA amount per nucleus was shown to decrease with an increasing number of cotyledons (Wyman et al. 1997). The nucleotypic hypothesis (Bennett 1972) proposed that variation in the amount of DNA may have developmental and adaptive importance. It is possible that development under in vitro conditions might be subjected to such nucleotypic effects.

Conclusions

Our study showed a relationship between mother-tree field performance and organogenic potential. Under the in vitro culture protocol used, superior jack pine families had a higher frequency of embryos with adventitious buds than inferior families. Within a performance class, differences among families were also observed in the capacity of the embryos to turn green and produce buds or shoots. When only the green embryos were considered, however, no differences among families in the ability to produce buds or shoots were observed, suggesting that family in vitro potential is strongly dependent upon the capacity of an embryo to turn green. Embryo length was shown to have a significant effect on bud and shoot production, with small embryos showing a lower organogenic potential. In addition, the number of cotyledons appeared to affect the survival of embryos through the course of the experiment. No significant relationships were observed between heterozygosity level and in vitro reactivity, with analyses performed on responsive (green) embryos only.

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