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Overcoming the challenges of family and genotype representation and early cell line proliferation in somatic embryogenesis from control-pollinated seeds of *Pinus radiata*.

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Abstract

The principal aim of this investigation was to test improved methods for initiation of embryogenic cell lines developed with open-pollinated seeds, on control-pollinated material, and furthermore to improve early cell line proliferation prior to cryopreservation. A total of 20 control-pollinated seed families, many with unrelated parents, were tested. Three cone collections were made between 15 December 2008 and 5 January 2009. Two zygotic embryo explant-preparation techniques were tested; embryos with retained megagametophytes, and excised embryos. Initiation medium was a modified Litvay medium (Glitz). Following initiation, growth of embryogenic tissue was tested on two proliferation media; a modified Litvay medium (Glitz2) and a modified Verhagen and Wann medium (BLG1). Initiation was obtained from both explant-preparation techniques. The best initiation treatment used excised embryos, with 52% of all explants from all collections and all families giving rise to proliferating embryogenic tissue. At the optimum collection time for each of the families, this treatment resulted in a range of 44% – 93% initiation success with a mean of 70% per family. Continued proliferation of initiated cell lines was high with 99% of cell lines initiated from excised embryos continuing to proliferate. After 28 days of growth on the two tested media, Glitz2 and BLG1, the embryogenic mass showed mean increases of 25 and 29 fold, respectively. This represents a major improvement over our previous work.

Keywords: control-pollinated seed; excised embryo; *Pinus radiata*; proliferation; somatic embryogenesis.

Introduction

Conifer somatic embryogenesis (SE) is the primary technology enabling all conifer biotechnology products, including transgenic trees, to be prepared. However, the most important role of SE is in enabling the implementation of clonal forestry, defined as the deployment of genetically tested tree clones, preferably integrated with tree improvement programmes (Park et al., 2006). The ability to arrest physiological maturation of embryogenic tissues in cryogenic storage while field testing takes place, followed by the potential to mass propagate material at a later date, make this approach highly attractive. Progress in both tree-breeding (including gene-assisted selection) and genetic engineering has huge potential to extend the versatility of *Pinus* spp.. This potential includes not only versatility in tree production by increasing

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access to a wider range of sites and extending plantations into what is considered marginal land for commercial forestry, but also the increased versatility of the products produced from the timber (Wilcox et al., 2007; Walter et al., 2010). Encouraging results for potential commercialisation of somatic embryogenesis protocols have been reported by CellFor Inc. Canada for a number of conifer species including *P. radiata* (Sutton, 2002). The technology incorporated the use of bioreactors for liquid culture and a mini-plug system for germination of somatic embryos. It was the company's aim to have a centralised production facility that would facilitate embryos being shipped worldwide. Unfortunately, none of these protocol details (including possible initiation success) are available publicly and, to date, it has not proved economic to purchase a service for New Zealand. Other propagation protocols, such as organogenesis, also exist and have been used for radiata pine. While initiation success for radiata pine using organogenesis is around 80 – 100% (Hargreaves et al., 2004; Hargreaves et al., 2005), this method does not scale-up easily, and nor does it offer ready access to biotechnology applications, as does SE.

Somatic embryogenesis protocols are available for radiata pine (*Pinus radiata* D.Don), but the success of initiation has been low and very variable between different families, ranging from 0 to 30% of genotypes (Hargreaves et al., 2009). These levels of success are insufficient for commercial application not only for radiata pine but also for many other economically important pine species (Park et al., 2006). Therefore, increased SE initiation success is crucial. Recently, both different types of media and timing of initiation were evaluated in an attempt to improve SE initiation success in radiata pine (Hargreaves et al., 2009). A new protocol was developed and was found to be very successful, with initiation success above 50% for 19 open-pollinated families.

The commercial radiata pine seedling industry in New Zealand relies largely on control-pollinated seed rather than open-pollinated seed. A 2009 New Zealand nursery survey found that, of the 37.7 million *P. radiata* tree stocks produced, 70% originated from control-pollinated seed (cuttings 25%; seedlings 45%). The remainder (30%) were produced from open-pollinated seed (Forest Owners' Association, & Ministry of Agriculture and Forestry, 2009). New Zealand also supplies the Australian radiata pine seed market. Ideally, our results on open-pollinated seed could be extrapolated and applied directly to control-pollinated seeds. Therefore, it was necessary to obtain empirical data to confirm whether a protocol combining excised embryos with Glitz medium would also be effective using control-pollinated seed. There are also practical issues in dealing with control-pollinated seed that are not relevant to open-pollinated seed. These issues include: contamination by other organisms; lower seed set; and crosses being more likely to exhibit incompatibility (Hargreaves et al., 2009). These practical issues also provide a strong commercial imperative to undertake this particular study. Therefore, the aim of this current study was to apply our new SE initiation protocol to control-pollinated radiata pine seed.

Materials and methods

Source material

Green cones were collected from twenty control-pollinated families of elite *Pinus radiata* trees at a commercial seed orchard (Proseed) located at Amberley, North Canterbury, New Zealand. Two cones per collection were taken from each family and a collection was made on each of three dates: 15 and 29 December 2008; and 5 January 2009. Details of crosses in respect to their relatedness were as follows; two crosses had a female parent in common, four crosses had the same male parent and two further crosses had a parent in common either as a female or a male. Seeds were extracted from the cones at time of collection and sterilised according to methods previously described (Walter & Grace, 2000).

Classification of embryos

Samples of 10 sterilised seeds were taken from the combined cone sample from each of the families at each collection time and zygotic embryo development in these seeds was classified on an 8-stage scale. This scale was similar to that used by Owens and Blake (1985), but with two earlier stages, so that our stages 3 – 8 were equivalent to stages 1 – 6 of Owens and Blake (1985). At stage 1, pro-embryos had formed (visible suspensor cells, but no pronounced cleavage of the primary embryo). Stage 2 showed early cleavage polyembryony (no clearly dominant embryo, but often 4 or more embryo initials visible with some separation of suspensors). Stages 3 – 6 were "bullet" stages with clearly dominant (bullet shaped) embryos of increasing development, with the numeric score assigned to the dominant embryo (at this stage the dominant embryo had formed a bundle of suspensor tissue). *Pinus radiata* often has several embryos in various states of maturation within one seed, although the dominant embryo is usually obvious. Our Stage 7 (equivalent to Stage 5 in Owens and Blake (1985)) had a clearly developed epicotyl and at Stage 8 (equivalent to Stage 6 in Owens and Blake (1985)), a clear whorl of cotyledons was present. Empty seeds were also noted (no embryo visible in the corrosion cavity).

Tissue culture

The remaining sterilised immature seeds were aseptically removed from the seed coat. The best two

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treatments from our previous research conducted with open-pollinated seeds (Hargreaves et al., 2009) were selected and applied to the explants. Treatment 1 used the intact megagametophyte (including the developing zygotic embryo) and Treatment 2 involved dissecting-out the developing zygotic embryo and discarding megagametophytic tissue. Explants were scored for contamination and proliferation over the entire culture duration.

Initiation and proliferation

Cell lines initiated from Treatments 1 and 2 were kept separate. Tissue generated from each treatment was plated out onto Glitz medium for four weeks. This is a modified Litvay medium (Litvay et al., 1985; Walter & Grace 2000). The pH of the medium was adjusted to 5.7 prior to autoclaving (121 ºC, 0.12 MPa, 20 min). Amino acids were filter-sterilised but not pH-adjusted, and were added to the autoclaved medium. The medium was then dispensed into gamma-irradiated polyethylene Biolab Petri dishes, 90 mm diameter x 25 mm depth, supplied by ThermoFisher Scientific, Auckland. A total of 1800 explants for each treatment from the three collections were cultured. There were five replications, with six explants in each replication per treatment, per family, per collection date. Cultures were incubated in low light (5 μM/m^2/s) at 24 +/- 1 ºC.

Four weeks after initiation, cultures were aired by unsealing the Petri dishes, lifting the lids (inside a laminar flow bench) and re-sealing. Airing of cultures in this way facilitates removal of ethylene produced by the plant tissues that may build up in the culture vessel. Explants with proliferating embryogenic tissue 3 – 5 mm in diameter were registered and weighed. Registered explants were transferred to new Petri dishes containing fresh Glitz medium and were grown for two weeks. This tissue was then divided into smaller pieces and shifted to a new position on the same medium (described as an air-shift transfer) and incubated for a further two weeks. The fresh weight of the tissue was recorded at this time and a defined sample of tissue was weighed and placed in a new Petri dish with one of two types of media for further proliferation. The proliferation media were either a modified Glitz medium (Glitz2) that excluded casein hydrolysate and included an additional 950 mg of L-glutamine and 1000 mg of asparagine or BLG1 medium,which is a modified Verhagen and Wann medium (Verhagen & Wann, 1989); (Find et al., 2002); (Walter et al., 2005). The osmolality of the media were measured with a Vapro 5520 vapour pressure osmometer. This tissue was air-shifted after two weeks. In cases where proliferation had been abundant, a fresh dish of medium was used for the lumps of tissue. A final, destructive assessment of fresh weight was made after a further two weeks. The total culture period was up to 12 weeks to initiate cell lines on Glitz medium prior to registration, then a further four weeks on fresh Glitz medium followed by four weeks on either Glitz2 or BLG1 medium.

Morphology

The morphology of eight cell lines from eight families was studied after 8 weeks of post-registration culture as described above. Tissues were stained with acetocarmine, examined using a microscope (axiovert 200 inverted microscope; Zeiss, Germany) and photographed. Comparative photographs were made of whole lumps of embryogenic tissue on proliferation medium.

Data analysis

The number of replications varied. This was due to: over- or under-sampling of the target 60 seeds, per family per collection time; and/or discarding of contaminated cell lines after registration. These factors were taken into consideration as appropriate in the analyses conducted.

The data for initiation, contamination and fresh weight were analysed using PROC GLM of the SAS software package. A linear model was used that considered treatment and collection to be fixed effects. Family, replication (collection), and treatment by family interaction were considered as random effects. Treatment- and family means were compared using the Tukey multiple range test option.

Proliferation data were presented in two ways: (1) analysis of final fresh weight; and (2) as data scored using a 1 – 9 scale. This was because the final weight data had a severely skewed distribution, where a few cell lines had very high rates of proliferation. The use of scaled data was an attempt to put proliferation into a normal distribution. Low values on the scale represent poor proliferation and the high values indicate good proliferation, with a mean of around 5. The scale used was as follows:

Scale 1 = lowest 0 – 5% of total proliferation values;

Scale 2 = 5.001 – 10%;

Scale 3 = 10.001 – 25%;

Scale 4 = 25.001 – 40%,

Scale 5 = 40.001 – 60%;

Scale 6 = 60.001 – 75%;

Scale 7 = 75.001 – 90%;

Scale 8 = 90.001 – 95%; and

Scale 9 = 95.001 – 100% of total proliferation values.

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TABLE 1: Summary of full seed and mean zygotic embryo development per cross per collection (sample size = 10).

	Collection 1 (15-Dec-08)		Collection 2 (29-Dec-08)		Collection 3 (5-Jan-09)	
Family	**Full seed**	**Embryo development**	**Full seed**	**Embryo development**	**Full seed**	**Embryo development**
1	9	1.3	7	2.1	10	5.3
2	10	1.0	10	1.8	10	2.9
3	10	1.1	10	2.5	10	3.5
4	8	1.0	6	2.3	10	4.1
5	8	1.1	6	1.5	10	3.4
6	10	1.0	10	1.1	10	1.9
7	9	1.0	9	1.3	7	1.6
8	5	1.0	2	2.0	10	2.7
9	8	1.0	10	1.5	10	2.4
10	6	1.0	5	3.2	6	3.0
11	9	1.0	9	2.0	10	3.7
12	8	1.0	10	1.7	8	2.8
13	6	1.0	10	3.0	10	5.5
14	7	1.3	10	3.0	7	5.1
15	5	1.0	10	2.2	10	3.7
16	10	1.2	10	1.7	10	3.2
17	10	1.0	10	1.7	8	1.9
18	10	1.0	8	1.4	10	1.9
19	10	1.0	10	1.9	10	4.6
20	10	1.0	10	2.6	10	3.4
Mean	8.4	1.05	8.6	2.03	9.3	3.33
%	(84%)		(86%)		(93%)	

Results

Classification

All families tested produced sufficient control-pollinated seed for sampling and testing with the two described explant preparation techniques for all three collections. The average percentage of full seed increased with maturity. An average of 84% full seed was recorded for the first collection (15 December 2008), 86% full seed for the second collection (29 December 2008) and 93% for the third collection (5 January 2009), Table 1. There was some noticeable variation in full seed scores within families, across the three collection dates. The most extreme variability was in family 8, which scored 50% for Collection 1, 20% for Collection 2 and 100% for Collection 3. Observed embryo development became more variable between families as maturation increased. Late stage pro-embryos (Stage 1.6) were observed in family 7 while advanced bullet stage embryos (Stage 5.5) were obtained from family 13 at the third collection (Table 1).

Contamination

High rates of microbial contamination were observed in Treatment 1 (intact megagametophytes), Table 2. However, contamination decreased as the maturation of the explants increased, i.e. a contamination mean of 77%, 45% and 28% for all families for Collections 1 – 3 respectively. Seed and embryo dissection for each family was split between two primary operators to counter possible operator effects of contamination and inadvertent damage to either explant type. Significantly more contamination occurred in material from Collection 1 than from Collections 2 and 3 ($p \leq 0.05$). The total number of families from which intact megagametophytes were obtained were 40%, 60% and 80% for Collections 1 – 3, respectively. Family differences in contamination were large. Of explants generated from from Collection 1, only one family (family 9) had no contamination while 10 other families were 100% contaminated. Some non-contaminated explants were obtained from all families at Collection 3 with 10 families suffering 10% or less contamination.

Overall, the rate of contamination was much lower for Treatment 2 (excised embryos) compared with Treatment 1 (Tables 3 and 2, respectively). However, as with Treatment 1, contamination in Treatment 2 material decreased as the maturation of explants increased, with an mean contamination rate of 15%, 9% and 4% for all families for Collections 1 – 3, respectively. Again, as with Treatment 1, differences

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TABLE 2: Treatment 1 (Glitz + intact megagametophyte), total number of explants (seeds) sampled, explants contaminated (%) and explants initiating cell lines per collection.

	Collection 1 (15 Dec 2008)			Collection 2 (29 Dec 2008)			Collection 3 (05 Jan 2009)		
Family	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**
1	30	100 f	0 d	0	na	na	36	61 bcd	0 e
2	30	100 f	0 d	30	63 bcde	3 c	36	3a	61a
3	30	30abc	63a	30	63 bcde	23 bc	30	0a	50abc
4	30	97 f	3 cd	30	27abc	23 bc	30	0a	53ab
5	30	70 cdef	3 cd	30	100 e	0 c	12	83 d	0 e
6	30	100 f	0 d	30	77 cde	0 c	30	87 d	7 de
7	30	100 f	0 d	30	0a	33 bc	30	10a	33abcde
8	30	100 f	0 d	30	0a	0 c	18	72 cd	0 e
9	30	0a	50ab	30	0a	53ab	36	36abc	36abcde
10	30	100 f	0 d	24	42abcd	4 c	30	3a	17 bcde
11	30	100 f	0 d	30	100 e	0 c	30	0a	37abcde
12	30	100 f	0 d	30	83 de	0 c	30	67 cd	13 cde
13	30	50 bcde	27 bc	30	80 de	3 c	30	3a	43abcd
14	30	27ab	17 cd	30	17ab	13 c	30	0a	13 cde
15	18	78 def	6 cd	30	3a	0 c	30	20ab	0 e
16	30	43 bcd	17 cd	30	43abcd	30 bc	30	77 cd	7 de
17	30	100 f	0 d	30	13ab	33 bc	30	0a	47abc
18	30	100 f	0 d	30	100 e	0 c	30	87 d	7 de
19	30	60 bcdef	0 d	30	33abcd	23 bc	30	0a	27abcde
20	30	87 ef	0 d	30	0a	73a	30	3a	60a
Means[2]	588[3]	77 b	9 b	564[3]	45a	17ab	588[3]	28a	27a
LSD[4]		43	26		51	35		41	38

[1] Means with the same alphabetical letter within a column are not significantly different at $p = 0.05$ by Tukey's range test.

[2] Means with the same alphabetical letter within a row are not significantly different at $p = 0.05$ by Tukey's range test.

[3] Total explants per collection

[4] LSD for initiation = 13 for contamination; LSD = 24 for collection means

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TABLE 3: Treatment 2 (Glitz + excised zygotic embryo), total number of explants (seeds) sampled, explants contaminated (%) and explants initiating cell lines (%) per collection.

	Collection 1 (15 Dec 2008)			Collection 2 (29 Dec 2008)			Collection 3 (05 Jan 2009)		
Family	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**	**Total explants**	**Explants contam. (%)[1]**	**Cell lines initiated[1]**
1	30	10a	33 bcdef	30	17a	53abc	30	0a	53abc
2	30	7a	20 cdef	30	7a	60abc	30	0a	90a
3	30	0a	93a	30	10a	67abc	30	0a	77ab
4	24	4a	71ab	30	7a	77abc	30	0a	83ab
5	30	13a	17 def	18	11a	44abc	30	27 c	13 c
6	30	7a	17 def	30	7a	20 c	30	10abc	57ab
7	30	40 b	0 f	30	0a	33abc	24	0a	79ab
8	30	7a	0 f	21	0a	43abc	30	0a	60ab
9	30	0a	43 bcdef	30	0a	80abc	30	3ab	70ab
10	30	70 c	0 f	22	0a	73abc	30	3ab	73ab
11	24	92 c	0 f	30	60 b	23 bc	30	0a	70ab
12	30	10a	7 ef	30	10a	47abc	30	10abc	47 bc
13	24	8a	67abc	30	10a	60abc	30	0a	60ab
14	30	0a	60abcd	30	0a	60abc	27	0a	48abc
15	21	19ab	38 bcdef	30	0a	60abc	30	0a	50abc
16	30	0a	63abcd	29	10a	83ab	30	7ab	87ab
17	30	0a	27 bcdef	30	0a	70abc	30	0a	63ab
18	30	20ab	20 cdef	30	13a	40abc	30	20 bc	60ab
19	30	3a	40 bcdef	30	7a	73abc	30	0a	73ab
20	30	0a	50abcde	30	0a	93a	30	3ab	77ab
Means[2]	573[3]	15	33 b[2]	570[3]	9	58a[2]	591[3]	4	64a[2]
LSD[4]		26	49		30	60		18	42

[1] Means with the same alphabetical letter within a column are not significantly different at $p = 0.05$ by Tukey's range test.

[2] Means with the same alphabetical letter within a row are not significantly different at $p = 0.05$ by Tukey's range test.

[3] Total explants per collection

[4] LSD for initiation = 13 contamination = 11 for collection means

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TABLE 4a: *F* tests and *p* values from analysis of variance on cell lines in Treatment 1 for initiation data.

	Collection 1			Collection 2			Collection 3		
Source	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**
Rep	4	1.27	0.2906	4	0.32	0.8611	5	1.78	0.1286
Family	19	13.33	0.0001	18	9.87	0.0001	19	9.52	0.0001
Rep*Family	74	1.37	0.0290	71	1.36	0.0346	73	0.88	0.7440
Error	490			470			490		

TABLE 4b: *F* tests and *p* values from analysis of variance on cell lines in Treatment 1 for contamination data.

	Collection 1			Collection 2			Collection 3		
Source	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**
Rep	4	1.75	0.1479	4	0.14	0.9659	5	1.21	0.3130
Family	19	14.68	0.0001	18	14.68	0.0001	19	20.17	0.0001
Rep*Family	74	3.09	0.0001	71	3.26	0.0001	73	2.21	0.0001
Error	490			470			490		

in contamination occurred between families. Of Treatment 2 explants generated from Collection 1, 14 families had 10% or less contamination. By Collection 3, 18 families had 10% or less contamination.

Initiation

Two families (1 and 8) failed to initiate any cell lines from Treatment 1 material obtained at any of the three collections some other families (e.g. 5, 6, 12, 15 and 18) had very low success. Mean initiation rates for Treatment 1 increased from 9% for Collection 1 to 27% for Collection 3 (Table 2), as the developmental stage of explants increased. However, only differences between Collections 1 and 3 were significantly different ($p = 0.05$). Not surprisingly, families with high rates of contamination produced few or no initiations. Although the highest mean initiation rate was obtained from Collection 3, initiation rates varied significantly from 0 to 61% ($p < 0.001$) between families for all three collections. Tables 4a and 4b contain ANOVA p values relating to Table 2.

Initiation was significantly better with excised embryos (Treatment 2, Table 3). Initiation rates followed a similar trend to Treatment 1, with increasing numbers of initiations as the explants matured. Mean initiation rates for Treatment 2 increased from 33% for Collection 1 to 64% for Collection 3, Table 3. Also, overall mean initiations from Collections 2 and 3 were significantly better than those from Collection 1 (Table 3). Of significance was the high number of families represented at each collection; 80% in Collection 1 and 100% in Collections 2 and 3. The numbers of cell lines per family tended to be positively-skewed, particularly in Collections 2 & 3. Only families 2 and 5 had significantly different numbers of cell lines (90% and 13% respectively at Collection 3; $p < 0.001$), Table 3. Tables 5a and 5b present ANOVA p values relating to Table 3.

When the best initiation treatment, excised embryos (Treatment 2), is considered with reference to optimum (peak) collection time for each family, success was very high with an overall mean initiation of 69.9% (Table 6). Peak performance was optimal for three families at Collection 1, for eight families at Collection 2 and for nine families at Collection 3. Individual embryo development scores per family indicated an optimum embryo development for excised embryos around late stage cleavage polyembryony to early dominant embryo formation when excised embryos were combined with Glitz medium. What is also important is that often cell line numbers were good for one or both of the shoulder collections with this treatment (Table 3).

Proliferation

The total number of cell lines initiated, weighed and registered, was 298 for Treatment 1. A small number of cell lines subsequently became contaminated on the Glitz/Glitz2 or Glitz/BLG1 treatments (see Tables 7 and 8). At registration, initial fresh weights of tissue (mean per family) from Treatment 1 varied

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TABLE 5a: *F* tests and *p* values from analysis of variance on cell lines in Treatment 2 for initiation data.

	Collection 1			Collection 2			Collection 3		
Source	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**
Rep	4	0.60	0.6660	4	1.04	0.3941	4	0.81	0.5225
Family	19	8.53	0.0001	19	3.21	0.0002	19	4.28	0.0001
Rep*Family	72	1.78	0.0002	72	2.00	0.0001	75	1.05	0.3679
Error	477			474			492		

TABLE 5b: *F* tests and *p* values from analysis of variance on cell lines in Treatment 2 for contamination data.

	Collection 1			Collection 2			Collection 3		
Source	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**	**df**	***F* test**	***p* value**
Rep	4	0.45	0.7688	4	0.22	0.9264	4	1.22	0.3092
Family	19	18.60	0.0001	19	5.67	0.0001	19	4.35	0.0001
Rep*Family	72	1.18	0.1584	72	1.55	0.0046	75	1.04	0.4028
Error	477			474			492		

TABLE 6: Summary of Treatment 2 (Glitz + excised zygotic embryo) detailing family, collection number, embryo development at peak initiation, number of explants tested, and % of cell lines at peak initiation plus number and % of initiated cell lines that continued to proliferate.

Family	Collection No.	Embryo development	No. Explants tested	No. Cell Lines Initiated	% Initiation	No. Cell Lines proliferated on Glitz at 28-days[1]	% Cell Lines proliferated
1	2	2.1	30	16	54	16	100
2	3	2.9	30	26	87	26	100
3	1	1.1	30	28	93	28	100
4	3	4.1	30	25	84	25	100
5	2	1.5	18	8	44	8	100
6	3	1.9	30	17	57	17	100
7	3	1.6	24	19	79	19	100
8	3	2.7	30	18	60	18	100
9	2	1.5	30	24	80	24	100
10	3	3.0	30	22	73	22	100
11	3	3.7	30	20	67	20	100
12	2	1.7	30	14	47	13	93
13	1	1.0	24	16	67	16	100
14	1	1.3	30	18	60	12	67
15	2	2.2	30	18	60	14	78
16	3	3.2	30	27	90	26	96
17	2	1.7	30	21	70	21	100
18	3	1.9	30	18	60	16	89
19	2	1.9	30	22	73	21	95
20	2	2.6	30	28	93	28	100
Total			576	405		390	
Mean	2.3	2.18		20	69.9		95.9

[1] All initiated cell lines included late registrations, not grown on and a few that became contaminated (a small number did not subsequently generate Glitz 28-day weights).

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from 30 mg to 280 mg. By the end of the first 28-day proliferation phase, the maximum final fresh weight obtained for Family 7 was 29 times the initial weight. It should be noted that families 11 and 14 had higher ratios based on their initial fresh weights (Table 9). Of families that expressed proliferation, only Family 6 had a final fresh weight lower than its initial weight.

Proliferation rates for the second 28-day-period for Treatment 1 were extremely high in some cases. For example, after 28 days proliferation on BLG1, fresh weight from family 13 increased 203 fold. Increase in fresh weight ranged from 10 to 488 fold on BLG1 and from 10 to 217 fold on Glitz2 (Table 9). In most cases, family means for cell lines showed more fresh weight gain on BLG1 media than on Glitz2 (Tables 7 & 9).

The total number of cell lines initiated, weighed and registered, was 878 for Treatment 2. A small number of cell lines subsequently became contaminated on the Glitz/Glitz2 or Glitz/BLG1 treatments (see Tables 7 and 8). At registration, initial fresh weights of tissue (mean per family) for Treatment 2 varied from 80 – 240 mg. By the end of the first 28-day proliferation phase, increases in fresh weight varied from 2 to 17 fold (Table 11). After 28 days proliferation on the Glitz2 test proliferation medium, Treatment 2 samples on showed 7 to 28 increases in fresh weight. Treatment 2 samples grown for 28 days on BLG1 medium exhibited a wider range in increases — from 7 to 39 (families 2 and 4 respectively: Table 11).

In general, proliferation of cell lines from Treatment 2 (excised embryos) was high and showed less variation than observed in Treatment 1. However, the relative sample size is important and more cell lines resulted from Treatment 2 than from Treatment 1 (Table 8). The cell lines obtained from Treatment 1 gained significantly more fresh weight than those obtained from Treatment 2 on either of the two proliferation test media (Glitz2 p = 0.018, BLG1 p = 0.03). However, enough tissue for subsequent cryopreservation was generated from both treatments (Tables 7 & 10).

Morphology

Differences within any given cell line were observed in embryogenic masses from Treatments 1 and 2 after culture on either Glitz2 or BLG1 medium. Embryogenic masses grown on Glitz2 medium tended to be dense in appearance, with few defined suspensor cells. By comparison, tissue obtained following proliferation on BLG1 medium was often glassier in appearance with suspensor strands clearly distinct. When this tissue was moved, it clearly held more moisture than tissue grown on Glitz2 medium. This difference is illustrated in the left and right images respectively for two cell lines (Figures 1a & 2a). Fewer embryo initials were visible in samples of stained tissue from masses proliferated on BLG1 medium than on Glitz2 medium. Masses proliferated

TABLE 7: Treatment means for proliferation data for cell lines represented on both Glitz2 and BLG1

Treatment no.	Total no. explants tested	No. cell lines tested	Overall survival (%)	Proliferation					
				% of initial weight			1 – 9 scale		
				Glitz	Glitz2	BLG1	Glitz	Glitz2	BLG1
1	1740	274	15.7	1923a	3573a	5493a	5.15a	5.40a	5.29a
2	1734	873	50.3	1218 b	2459 b	2941 b	4.83 b	4.79 b	4.77 b
Least Significant Difference				282	521	939	0.33	0.40	0.44

TABLE 8: Survival of cell lines that were initiated and tested on Glitz2 and/or BLG1

Treatment no.	Glitz2		BLG1	
	No. Cell lines	Survival (%)	No. Cell lines	Survival%
1	298	88.3	308	88.3
2	878	98.7	880	98.8

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TABLE 9: Treatment 1 (intact megagametophyte), summary of proliferation data for fresh weight at initiation (mg) and following 28 days' culture on Glitz; sample weight (mg) prior to culture on Glitz2 or BLG1 and following 28 days' culture on Glitz2 or BLG1 (proliferation at day 28 is measured as a percentage of initial fresh weight); and scaled proliferation data for Glitz, Glitz2 and BLG1.

Family	Total explants	Cell lines initiated[1]	Initial weight Glitz	28-day Glitz	Initial weight Glitz2	28-day Glitz2	Initial weight BLG1	28-day BLG1	Scaled data[2] 28-day Glitz	Scaled data 28-day Glitz2	Scaled data 28-day BLG1
1	66	0	na[3]	na	na	na	na	na	na	na	na
2	96	23	90	1126	120	2045	120	2149	5.14	4.41	4.09
3	90	41	100	1467	120	2451	120	2408	4.46	4.82	4.23
4	90	24	70	1100	100	6051	120	10086	4.59	6.50	6.41
5	72	1	60	na	na	na	na	na	na	na	na
6	90	2	120	-38[4]	40	8710	20	9779	1.00	9.00	9.00
7	90	20	100	2957	150	1677	160	2789	5.76	4.18	4.82
8	78	0	na	na	na	na	na	na	na	na	na
9	96	44	100	1940	160	3489	140	4437	5.22	5.55	5.74
10	84	6	280	2205	200	2366	200	2180	6.33	4.33	4.33
11	90	11	70	2472	120	9323	120	6212	6.09	6.00	5.36
12	90	4	50	881	120	6627	130	6623	3.50	7.50	7.50
13	90	22	110	962	130	3237	110	22299	3.80	5.42	5.47
14	90	13	70	2627	150	1612	150	1636	5.45	4.36	4.09
15	78	1	30	141	20	1901	30	3232	3.00	5.00	5.00
16	90	16	160	1202	140	3989	150	4005	5.00	5.81	5.75
17	90	24	190	2492	160	2830	150	2725	6.21	5.42	4.83
18	90	2	30	179	40	3216	20	8339	2.50	6.00	7.00
19	90	15	110	2555	140	3329	140	3233	6.00	5.54	5.29
20	90	40	130	2781	140	4276	120	7537	5.34	6.16	6.34

[1] Cell lines initiated that then continued to proliferate (a small number of cell lines became contaminated or were late registrations).

[2] Scale applies a normal distribution to fresh weights. Low values on the scale of poor proliferation and high values are high proliferation, with a mean of around 5.00.

[3] No seed was available for sampling.

[4] Family 6: Cell lines had a small amount of surviving tissue after initial fresh weight (subsequent growth was good).

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TABLE 10: F tests and p values from analysis of variance on all data

Source	df	Proliferation % of initial						Proliferation 1 – 9 scale					
		Glitz		Glitz2		BLG1		Glitz		Glitz2		BLG1	
		F test	*p* value	*F* test	*p* value	*F* test	*p* value	*F* test	*p* value	*F* test	*p* value	*F* test	*p* value
Coll	2	4.63	0.0160	0.71	0.4965	1.67	0.2001	4.48	0.0179	0.95	0.3932	3.53	0.0381
Rep(coll)	13	0.15	0.9998	0.96	0.4922	1.83	0.0347	0.29	0.9938	0.65	0.8113	1.39	0.1565
Treat	1	2.50	0.1197	5.88	0.0183	5.22	0.0300	0.39	0.5366	5.47	0.0241	9.27	0.0044
Fam	19	2.31	0.3827	1.14	0.3907	-1.22		1.34	0.2757	2.07	0.2350	7.26	0.3704
Fam*Treat	16	0.58	0.8679	1.60	0.1266	0.37	0.9736	1.29	0.2849	0.92	0.5624	0.87	0.6050
Coll*Fam*Treat	17	1.93	0.0126	0.61	0.8799	24.02	0.0001	1.42	0.1173	2.22	0.0038	2.87	0.0001
Coll*Fam	34	0.92	0.5938	1.86	0.1059	0.44	0.9787	1.96	0.0725	0.97	0.5530	0.46	0.9721
Error	1146												

on BLG1 also demonstrated increased maturation compared with those from Glitz 2 medium, as indicated by the size of both the embryo and suspensor cells. These differences are illustrated in the left and right images respectively for two cell lines (Figures 1b & 2b). Conversely, Glitz2 medium gave rise to multiple small embryo initials with smaller suspensors. These observed differences in embryo-initial complexity were consistent across all the families tested, although there was some variation within a given range. An example of the differences in the initial size of mature embryos between family 9, cell line 16 and family 12, cell line 6 is shown in Figures 1b and 2b, respectively.

Discussion

Earlier work with *Pinus radiata* using 10 control-pollinated families reported that at an optimum collection time, 33% of explants gave rise to embryogenic tissue, with a range by family of 13.6% to 87% (Smith et al., 1994). These authors did not present detailed data, however. Recent research published for open-pollinated *P. radiata* shows considerable improvement in initiation using a combination of excised embryos with Glitz medium, with an mean of 70% of all explants forming embryogenic tissue and a range of 47% – 97% across 19 families at the optimum collection time (Hargreaves et al., 2009).

Our results with 20 control-pollinated families show a similar success rate to that obtained with open-pollinated families using the same initiation method. For control-polinated seed, a mean of 70% of all explants formed embryogenic tissue with a range of 44% – 93% at the optimum collection time (three collections over four weeks). It should be noted however, that using the zygotic embryo stage as an indication of optimum time for embryogenic cell line initiation has limitations. Observations with the excised embryos indicate that it is often not the dominant (and 'scored') embryo that initiates the cell mass but rather the less differentiated embryos in association with that embryo (Hargreaves et al., 2009). Von Aderkas et al. (1991) give a thorough description of larch (*Larix leptolepis, L. decidua, L. occidentalis* and *L*. x *eurolepsis*) embryogeny both in *vivo* and *in vitro* and have observed embryoids developing from cells of the suspensors. Small centres of cytoplasmically dense cells develop among the suspensor cells and give rise to organised meristematic centres, themselves producing suspensors (Von Aderkis et al., 1991).

We have assumed until now that we have genetic homogeneity in resultant cell lines. However, we cannot be sure that the *Pinus radiata* cell lines generated in our present study are from one fertilisation event. In a study of *Pinus taeda* L, Becwar et al. (1991) showed that it was possible to initiate embryogenic tissue from

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TABLE 11: Treatment 2 (excised zygotic embryo), summary of proliferation data for fresh weight at initiation (mg) and following 28 days' culture on Glitz; sample weight prior to culture on Glitz2 or BLG1 and following 28 days' culture on Glitz or BLG1(proliferation at day 28 is measured as a percentage of initial fresh weight); and scaled proliferation data for Glitz, Glitz2 and BLG1.

Family	Total explants	Cell lines initiated[1]	Initial weight Glitz	28-day Glitz	Initial weight Glitz2	28-day Glitz2[2]	Initial weight BLG1	28-day BLG[2]	Scaled data[3] 28-day Glitz	Scaled data 28-day Glitz2[2]	Scaled data 28-day BLG1[2]
1	90	42	130	1388	140	2444ab	140	2000 bcde	5.27	5.00abcde	4.32 cdef
2	90	51	110	1228	160	1270 b	170	1299 de	5.21	3.74 ef	3.34 fg
3	90	71	150	900	120	2378ab	150	2611 abcde	4.14	4.49abcdef	4.53 cdef
4	84	65	100	1167	130	3248ab	130	5229a	4.90	5.33abcd	6.02ab
5	78	17	130	803	100	2947ab	100	2700abcde	4.29	5.29abcd	5.00abcde
6	90	28	160	1062	140	1269 b	150	1727 cde	4.54	3.86 def	3.96 defg
7	84	29	200	1331	160	790ab	160	1987 bcde	5.11	4.39abcdef	4.18 defg
8	81	27	80	924	90	601ab	80	2037 bcde	4.56	4.42abcdef	4.41 cdef
9	90	58	120	1324	130	2388ab	130	2867abcde	5.07	4.86abcde	4.84abcde
10	82	38	160	1307	150	3861a	150	5034ab	4.97	5.03abcde	5.11abcde
11	84	28	130	1038	130	4005a	120	4302abcd	4.59	5.77ab	5.73abc
12	90	30	150	1526	140	1679ab	140	3001abcde	5.19	4.30 bcdef	5.04abcde
13	84	52	200	635	130	2256ab	130	3193abcde	3.88	4.79abcde	4.67 bcdef
14	87	49	130	2374	180	1673ab	180	2481abcde	5.45	4.09 def	4.53 cdef
15	81	41	110	1028	130	1137 b	130	1120 e	4.63	3.09 f	2.83 g
16	89	69	240	1073	160	3405ab	170	3153abcde	4.67	5.68abc	5.35abcd
17	90	48	190	1443	170	2325ab	160	2347abcde	5.35	4.85abcde	4.46 cdef
18	90	36	110	1253	110	1591ab	130	1583 cde	4.88	4.26 cdef	3.85 efg
19	90	56	160	1422	140	2385ab	140	2779abcde	5.13	5.15abcde	5.04abcde
20	90	66	190	1066	150	3627ab	140	4679abc	4.77	5.86a	6.20a
LSD						2578		3158		1.48	1.43

[1] Cell lines initiated that then continued to proliferate (a small number of cell lines became contaminated or were late registrations).

[2] Means with the same alphabetical letter in a column are not significantly different at $p = 0.05$ by Tukey's range test.

[3] Scale applies a normal distribution to fresh weights. Low values on the scale of poor proliferation and high values are high proliferation, with a mean of around 5.00.

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TABLE 12: *F* tests and *p* values from analyses of variance on treatment 2 proliferation data.

		28-day Glitz		28-day Glitz2		28-day BLG1		Scaled data 28-day Glitz		Scaled data 28-day Glitz2		Scaled data 28-day BLG1	
Source	**df**	***F* test**	***p* value**	***F* test**	***p* value**	***F* test**	***p* value**	***F* test**	***p* value**	***F* test**	***p* value**	***F* test**	***p* value**
Coll	2	2.99	0.0665	1.78	0.2014	0.29	0.7561	5.45	0.0102	5.31	0.0138	0.97	0.4002
Rep(coll)	12	0.92	0.5290	0.86	0.5865	1.23	0.2588	0.79	0.6615	0.52	0.9034	1.12	0.3369
Fam	19	0.87	0.6131	2.30	0.0140	5.25	0.0001	1.12	0.3703	3.54	0.0005	7.72	0.0001
Coll*Fam	34	2.29	0.0001	1.07	0.3698	0.70	0.8998	2.29	0.0001	1.63	0.0141	1.15	0.2619
Error	805												

more than one zygotic embryo of a *P. taeda* seed and that the resulting cell lines may be genetically different. Several biotechnology companies (e.g. Arborgen and CellFor) have undertaken field trials with *Pinus* species, and to date, there have been no reports of significant problems with genetic fidelity within clones.

The explant preparation technique used here for control-pollinated *Pinus radiata* and the recently published work with open-pollinated *P. radiata* (Hargreaves et al., 2009) suggest that both maternal and paternal influences may be reduced when this initiation method is employed. Further research is required to examine the specific influences of both parents in relation to the influence on cell-line initiation. Research with *P. taeda* L. indicated a positive influence on initiation with selection of female parents and in some cases a favourable male (MacKay et al., 2006). Work with eight control-pollinated crosses of *P. pinaster* (Ait) found that the probability of explants producing embryogenic tissue depended strongly on the pollen parent (Lelu-Walter et al., 2006). The results presented here for control-pollinated *P. radiata* clearly indicate the potential for the megagametophyte to exert a negative effect upon initiation success. While this could be considered evidence of a maternal effect, does not, on its own, establish that different mothers exert a different maternal influence in *P. radiata*. Unpublished preliminary work with *P. patula* Scheide. et Deppe by the current authors indicates a possible beneficial nutritive effect from culture with the megagametophyte. Comparing the same treatments (Glitz media with megagametophytes or dissected embryos, Hargreaves et al., 2009) between open-pollinated and control pollinated seed the contamination means across all collection times increased from 32.3% to 50% for the megagametophyte treatment respectively and from 5.9% to 9.3% for dissected-out embryos. No seeds were recorded as being empty (no embryo in corrosion cavity) in the data obtained from 19 families of open-pollinated seed. In contrast 13% of the 20 control-pollinated families sampled for this research showed empty seeds. This difference could be due to the methodologies employed in control-pollinated vs open-pollinated seed production. In most cases, the contamination was overt (possibly megagametophytes without embryos were decomposing and it would be relatively simple to transfer this to other megagametophytes without our knowledge). Less frequently, the contamination observed was more subtle, appearing almost as exudates growing only in connection with the megagametophyte. Horgan (1987) in earlier work with mature organogenic *P. radiata* shoot material, isolated from the field, reported persistent problems with a bacterial contaminant throughout the culture period. The process of bagging receptive female cones for up to 8 weeks, for control-pollinations, may create an environment which increases the number of opportunistic contaminants present in the developing seed. In previous research, with open-pollinated

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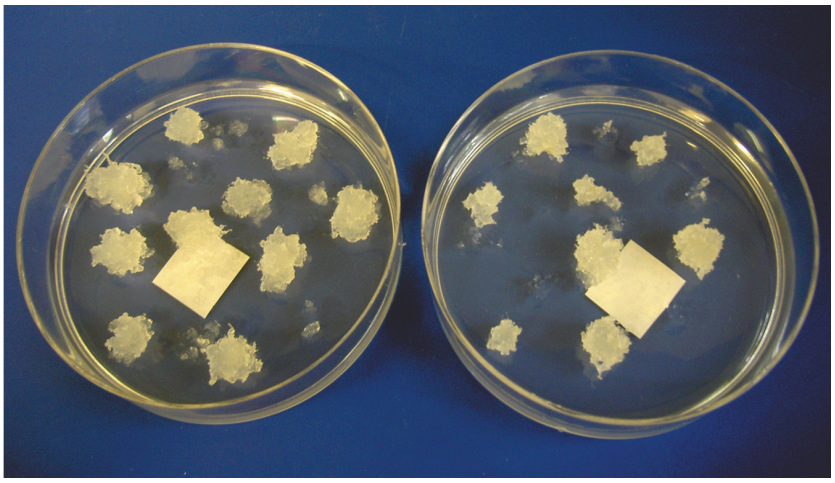

FIGURE 1a: Comparison of *P. radiata* family 9 cell line 16, on proliferation media BLG1 (Left) and Glitz2 (right) (Petri dish 90 mm x 25 mm depth).

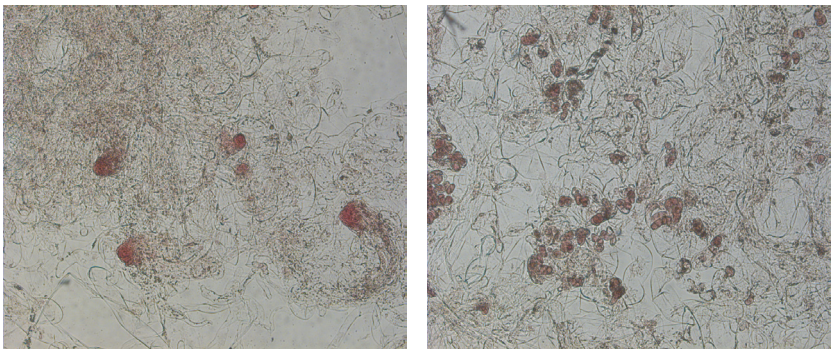

FIGURE 1b: Comparison of *P. radiata* family 9 cell line 16, stained with acetocarmine from BLG1 (Left) and Glitz2 (right).

seed, it was discussed that some of the negative effect of contamination may be contributed to by megagametophyte death during the culture period and thus demise of the zygotic embryo initials (Hargreaves et al., 2009). This suggestion runs counter to Mackay et al. (2006) who suggested that the presence of the megagametophyte might extend the influence of the mother tree into culture to a greater degree and thus account for the importance of maternal effects. Perhaps this is the case, but the maternal effects may be inhibitory rather than being positive. However, when proliferation data is considered alone, it does appear that the megagametophytes confer a benefit as the surviving cell lines showed greater fresh weight gains on Glitz and then on Glitz2 or BLG1 (Table 7). This was despite the fact that the megagametophyte was removed from the cell mass at registration. It may be that only vigorous embryogenic tissue is able to escape the effects of the dying megagametophyte tissues in comparison to the excised zygotic embryos. A subset of the proliferation data presented here was analysed from crosses where representation from both initiation treatments was high. When the most vigorous cell lines were compared for fresh weight mass they were not significantly different between initiation treatments (data not shown). This seems to support the above observation with regard to a selection for more vigorous lines in the megagametophyte treatment.

Lelu-Walter et al. (2006) used a similar approach of combining excised embryos with a modified Litvay medium to achieve high levels of cell line initiation in *Pinus pinaster*, testing 8 control-pollinated families with up to 100% of explants producing embryogenic cell lines. These results for *P. pinaster* and now for *P. radiata* indicate that these species may no longer be considered recalcitrant, at least for the initiation phase, compared to other *Pinus* spp. (Bonga et al., 2010; Park et al., 2006).

The proliferation protocols used after initiation resulted in significant improved capture of cell lines compared to previous methods used for *Pinus radiata* which resulted in only 49% of lines showing fresh weight gain (Hargreaves et al., 2009). In the earlier work, a liquid suspension method was used and may

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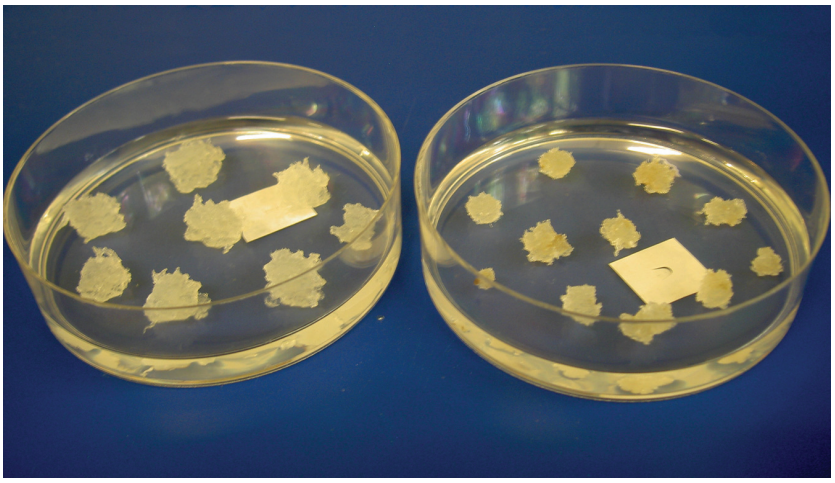

FIGURE 2a: Comparison of *P. radiata* family 12 cell line 6 on proliferation media BLG1 (Left) and Glitz2 (right) (Petri dish 90 mm x 25 mm depth).

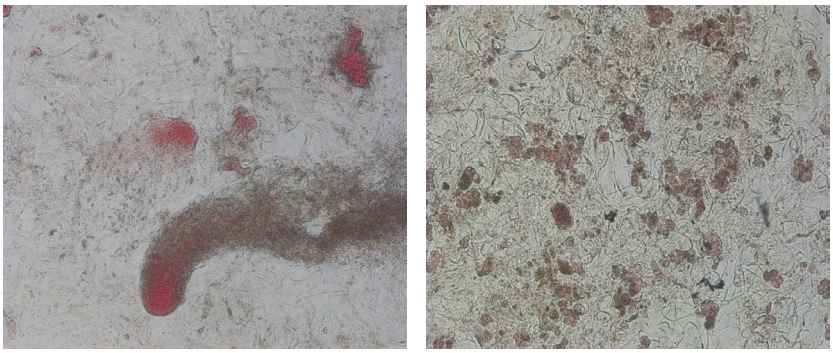

FIGURE 2b: Comparison of *P. radiata* family 12 cell line 6 stained with acetocarmine from BLG1 (Left) and Glitz2 (right)

have contributed to the subsequent poor growth (Hargreaves et al., 2009). Aronen et al. (2009) reported significantly improved proliferation using a suspension method compared to a lump method for cell lines of *P. sylvestris* L. The suspended lines had an mean of 23.9 +/- 1.2, times the initial weight in 6 weeks compared to 9.1 +/- 0.5, times the original weight for the tissue lumps (clumps). The *P. sylvestris* lines originated from intact megagametophytes and there were no significant differences in the proliferation of lines originating from different donor trees. The results reported here, using a lump (clump) method show an mean proliferation of 36 or 54 times the original weight (using either Glitz2 or BLG1 medium, respectively) after 4 weeks of culture from *P. radiata* initiated from megagametophytes (274 cell lines tested). With cell lines originating from excised embryos, the lump method resulted in an mean proliferation of 25 or 29 times the original weight (Glitz2 and BLG1 respectively) after 4 weeks (873 lines tested). There were some significant differences in proliferation between the 20 families tested ($p \leq 0.01$) (Tables 9 and 11). Even more growth may be possible if successful suspension protocols are developed for early *P. radiata* callus proliferation, as clearly there would be an improvement of nutrient contact with all cells in comparison to a lump of tissue.

The initiation medium Glitz is not as good for rapid proliferation when compared to either Glitz2 or BLG1 media, Table 7. Earlier work with *Pinus radiata* indicated that EDM6 medium was not good for initiation or early proliferation of embryogenic masses when tissue was transferred directly to it from Glitz medium (Hargreaves et al., 2009). EDM6 Medium has significant differences in overall composition from Glitz and this may be too big a contrast for tissue at the early proliferation stage, despite its obvious suitability for continued maintenance and proliferation of established cell lines (Walter & Grace 2000, Walter et al., 2005). The two proliferation media (Glitz2 and BLG1) tested in our study also have marked differences in major, minor and vitamin stocks, and osmolality, though amino acids and plant growth regulators are very similar. These two media have been found to be of benefit when cell lines were not proliferating well on EDM6 (L. Grace, C. Reeves, J. Find unpublished data). The osmolality

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of Glitz2 medium is 130 mmol/kg compared with 110 mmol/kg for BLG medium. This could explain some of the differences in growth and morphology observed. Even though proliferation was higher on BLG1 medium, the differences in embryo complexity between the two test media, both visual and as revealed by staining, may have important implications for sustained *de novo* cleavage polyembryony, cryopreservation and genetic engineering. It is clear both from the results presented here and previously for *P. radiata* (Hargreaves et al., 2009) and research with a range of other conifer species, that the initiation rate gradually diminishes as the zygotic embryo matures (Bonga et al., 2010). This decrease in embryogenic activity also occurs in *de novo* somatic embryogenesis (Bonga et al., 2010). Testing such observations empirically with *P. radiata* is now possible. Operationally, transferring *P. radiata* to either Glitz2 or BLG1 media shortly after initiation will result in sufficient cell mass for cryopreservation and subsequent maturation studies.

Some of the variation in data presented here for full seeds and zygotic embryo development at each collection for each family, prior to initiation into culture may be attributed to cone effects and the relatively small sample size (2 cones, 10 seeds per collection, per family). Cones were sometimes collected from different ramets of the same mother trees and may have had different canopy positions, with size and number of cones in any whorl also contributing to within family variation.

Previous research with *Pinus radiata* and other *Pinus* species has indicated a strong genetic influence on initiation success using current SE technologies (Klimaszewska et al., 2007; Lelu-Walter et al., 2006; MacKay et al., 2006). Deployment progress has been restricted by the generally low percentage of families that can be established as cell lines by current SE techniques. However, the results presented here for control-pollinated seeds from a genetically diverse selection indicate that it is possible to provide laboratory conditions that significantly improve initiation success across all families.

Conclusions

The results presented here demonstrate that improved initiation and early cell line proliferation methodologies can be successfully applied to control-pollinated seed from a commercial New Zealand radiata pine seed orchard. This is an important breakthrough of likely high benefit to the forest industry. Peak initiation rates of 70% for all families were equivalent to those achieved with open-pollinated seed using an equally large test population of elite crosses. Importantly, fewer control-pollinated cone collections than open-pollinated collections (three versus five) were required to achieve the same results for initiation. This confirms the interaction of specific zygotic embryo maturation stages with subsequent *in-vitro* initiation performance (late-stage and cotyledonary zygotic embryos were not sampled).

Significant improvements were made in early cell line proliferation with 94% of all initiated cell lines continuing to proliferate on both test media (Glitz2 and BLG1) compared to only 49% in previous results with open-pollinated seed.

Beneficial implications for clonal forestry practices include easier identification of successful collection times in commercial seed orchards and reduction of associated labour costs (fewer cones and collections required). Important cost savings in the laboratory phases can be made with fewer explant dissections to ensure representative numbers of genotypes per crosses.

Rapid post-initiation cell line proliferation (as shown on both Glitz2 and BLG1) results in fewer subcultures being required before tissue is available for cryopreservation and subsequent maturation treatments to produce plants for field testing.

The success using control-pollinated seed with initiation and early proliferation of tissues should have positive implications for application to commercial clonal forestry operations. The potential improvements include improved family representation through improved genotype capture, earlier cryopreservation and subsequent maturation studies, and facilitating planting stock production for field trials in the second winter following initiation, thus enabling rapid deployment of new genetic combinations. Future work will focus on the potential significance of the differences in embryo morphology observed between the two proliferation media (Glitz2 and BLG1) in respect to subsequent cryopreservation and somatic embryo maturation stages.

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