Plant Cell, Tissue and Organ Culture **29**: 247–255, 1992.

Optimization of bud induction in cotyledonary explants of *Pinus canariensis*

Carolina Martinez Pulido[1], Indra S. Harry & Trevor A. Thorpe*
*Plant Physiology Research Group, Department of Biological Sciences, University of Calgary, Calgary, Alta., Canada T2N 1N4 ([1]present address: Departamento de Biología Vegetal, Universidad de la Laguna, Tenerife, Spain) (*requests for offprints)*

Received 22 August 1991; accepted 18 December 1991

Key words: Canary Island pine, cotyledonary explants, explant age, induction, liquid pulse treatments, *Pinus canariensis*

Abstract

A method is described for using liquid pulsing as an alternative to the conventional induction protocol for *Pinus canariensis*. Using Day 0 and Day 3 explants, the best exposure time was 8 h and 4 h respectively, in a non-buffered 100 μM N^6-benzyladenine solution, followed by culture on half-strength Bornman's medium containing 3% sucrose and 0.8% Difco Bacto^R agar. With this procedure, 97% of the cotyledonary explants produced about 14 buds/explant. These results were comparable to a 14-day induction period on full-strength Bornman's medium containing 10 μM N^6-benzyladenine.

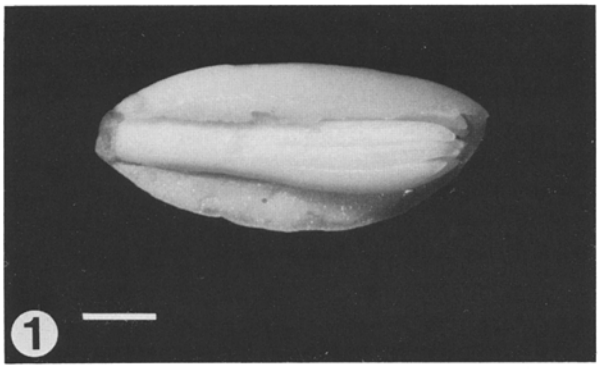
Introduction

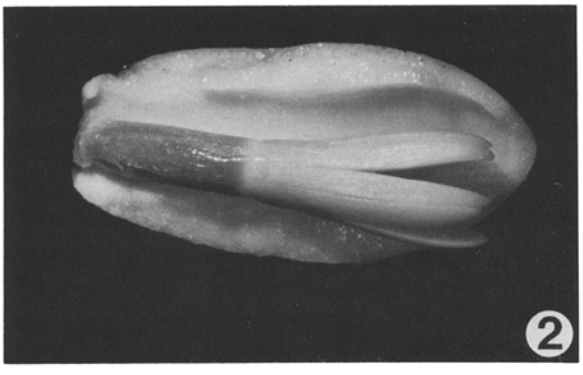
The concentration and mode of application of plant growth regulators are among the most important factors influencing morphogenic response in *in vitro* cultured conifer tissues (David 1982; Bornman 1983; Vogelmann et al. 1984).

Traditionally, plant growth regulators are incorporated into liquid or agar-solidified medium. However, explants can be exposed to or 'pulsed' in high concentrations of phytohormones for short periods of time. According to Bornman (1983) a pulse induction method was more effective for *Picea abies*, as both the number of buds increased and the response time was reduced. Von Arnold & Eriksson (1985) also found that adventitious buds of this species developed faster after exposure to a high concentration of N^6-benzyladenine (BA) in an aqueous solution, and that the variation among different experiments was lower as compared to explants cultured on an agar-solidified medium supplemented with BA.

The overall aim of this study was to determine whether a similar number of adventitious buds per cotyledon could be produced by using liquid pulse treatments instead of the conventional method of induction for *Pinus canariensis* Sweet ex. K. Spreng. The wood obtained from this species is very hard and useful for construction. A micropropagation protocol using cotyledons cultured on an agar-solidified basal medium containing phytohormones has been previously developed (Martinez Pulido et al. 1990). Therefore, our current objectives were as follows:

- to determine the shortest pulse time required to produce adventitious buds on newly isolated (Day 0) and 3-day-old germinated (Day 3) explants using a non-buffered BA solution;
- to compare the effect of pulsing Day 0 and Day 3 embryos in both a non-buffered and buffered BA solution;


1

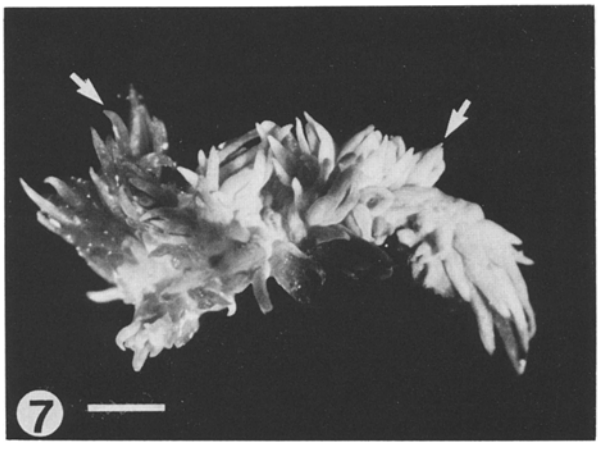

2


3


4


5


6


7


8

- to determine the shortest and best induction time required to produce adventitious buds on Day 3 cotyledonary explants using an agar-solidified medium;
- to compare these findings with the previously established protocol for *P. canariensis* (Martinez Pulido et al. 1990).

Materials and methods

Plant material

Seeds of *P. canariensis* were collected by Instituto para la Conservación de la Naturaleza from open-pollinated trees in natural stands in Gran Canaria (Canary Islands, Spain). These were sterilized and dissected as described by Martinez Pulido et al. (1990).

Two types of explants, those from newly dissected or Day 0 (Fig. 1) embryos and from Day 3 germinated embryos (Fig. 2), were utilized. For germination, a portion of the megagametophyte was removed and the remaining section containing the embryo, was plated on water agar containing 1% sucrose and solidified with 0.8% Difco-Bacto^R agar. This method eliminated differences in cotyledon quality and response which could arise if some cotyledons were in contact with the medium and others were not. After three days of germination, intact embryos of cotyledons were used as explants. Germination plates were incubated at 30–40 μmol m^{-2} s^{-1} at 27 ± 1°C under a 16-h photoperiod provided by Sylvania Gro-Lux F40712 Gro-WS lights. Experimental material was maintained under similar conditions, but the photon flux rate was increased to 80–100 μmol m^{-2} s^{-1}.

Culture medium

The mineral salts, Media for Conifer Micropropagation, described by Bornman (1983) were used either at full or half-strength (MCM or 1/2MCM). These formulations were supplemented with sucrose at 3% (w/v), myo-inositol and asparagine each at 100 mg l^{-1}, nicotinic acid and thiamine-HCl each at 5 mg l^{-1} and pyridoxine-HCl at 0.5 mg l^{-1}. Difco-Bacto^R agar was added at 0.8% (w/v) and media were adjusted to pH 5.7–5.8 before autoclaving. Activated charcoal (0.05%; Sigma No. C4386) was added prior to autoclaving. BA, when incorporated into agar-solidified medium was added at a final concentration of 10 μM before pH adjustment. MCM mineral salts containing 10 μM BA was used for induction, 1/2MCM without phytohormones was used for bud development, and 1/2 MCM containing 0.05% activated charcoal was used for bud elongation. Standard Petri plates (100 × 15 mm) were used for the first two stages, and deeper dishes (100 × 25 mm) for elongation.

Bud induction and development

For pulsing experiments, embryos were treated in an aqueous non-buffered or buffered solution, pH 5.5–5.6, containing 100 μM BA. These treatments were carried out by immersing the whole embryos in a 25 ml aliquot of the filter-sterilized BA solution in 50-ml Erlenmeyer flasks; these flasks were then placed on a gyrotory shaker (50 rpm), kept in a 24 ± 1°C culture room. When buffered, the BA solution was made up with 25.5 ml of 0.1 M-citric acid and 74.5 ml of 0.1 M-trisodium citrate, pH 5.5 (Dawson et al. 1986). Day 0 and Day 3 embryos were pulsed in a non-buffered 100 μM BA solution for short time periods of 5, 10, 15, 20, 25 and 30 min; in addition, both types of explants were treated in a non-buffered and buffered BA solution for 1, 2, 3, 4, 6, 8 and 16 h. After the induction pulse, treated embryos were blotted on sterile filter paper, and excised cotyledons were plated

←

Figs 1 & 2. Embryos of *Pinus canariensis* just after dissection (*1*), and after a 3-day germination period (*2*); the portion of the megagametophyte with the embryo was placed on water agar for germination. *Figs 3–8.* Cotyledons of *P. canariensis* after various induction treatments and development on 1/2MCM medium. *Figs 3 & 4* Day 0 (*3*) and Day 3 explants (*4*) pulsed for 0, 5, 10, 15, 20, and 30 min (left to right) in a non-buffered 100 μM BA solution after 8 weeks.
Figs 5 & 6. Day 0 (*5*) and Day 3 (*6*) explants pulsed for 0.5, 1, 2, 3, 4, 6, 8 & 16 h (left to right) in a non-buffered 100 μM BA solution after 8 weeks. *Fig. 7.* A Day 3 cotyledon treated for 4 h in a non-buffered BA solution, and cultured on 1/2MCM for 8 weeks. *Fig. 8* A Day 3 cotyledon cultured for 14 days on MCM agar-solidified medium containing 10 μM BA, and for 6 weeks on 1/2MCM; arrows show developing buds. (Scale bars = 10 mm for Figs 1–6 and 2 mm for Figs 7 & 8).

horizontally on the surface of plates with bud development medium. After 3–4 weeks of culture, the explants were transferred to bud elongation medium with 0.05% (w/v) activated charcoal.

In another series of experiments, cotyledons from 3-day-old embryos were cutivated on agar-solidified bud induction medium containing 10 μM BA. Explants were cultured for 1, 2, 3, 4, 6, 8, 10 and 14 days, after which they were transferred to phytohormone-free bud development medium (1/2MCM), and after one month to elongation medium. This conventional method was used as a control to compare the advantages and disadvantages of using a liquid pulsing method for induction.

Bud formation and data analysis

After 4 weeks of culture on elongation medium, numbers of buds were estimated by examining the explants under a stereoscopic microscope. Only structures which had defined whorls of needles were counted as buds (see arrows Figs. 7 & 8). The number of adventitious buds per cotyledon was counted, and the standard error (S.E.) calculated. One factor or one-way analysis of variance and the Scheffé test were used to compare means. The Bud Forming Capacity (BFC) index was calculated as follows:

$$\text{BFC index} = (\text{average number of buds per cotyledon}) \times (\%\ \text{cotyledons forming buds}) \div 100$$

This index was useful for it incorporated the number of cotyledons producing buds and the average number of buds per cotyledon. The combination of these two factors gives a more realistic determination of the efficacy of a given treatment.

Results

Liquid pulse treatments

Day 0 and Day 3 explants: 5–30 min
If explants were not treated with BA, they elongated and no adventitious buds were formed (see Figs 3 and 4). In general, when both Day 0 and Day 3 explants were exposed to BA for short periods, adventitious buds formed near the tips of the cotyledons and these elongated rapidly. This pattern was observed in all experiments when BA treatments were not optimal. Even a 5 min exposure to 100 μM BA solution was sufficient for bud initiation. Using this time, about 54.3% of cotyledonary explants formed an average of 3.1 buds (Table 1). These values, as well as the percentage of cotyledons forming buds, increased with longer exposure times.

An important finding of this experiment was that over 80% of Day 0 explants formed adventitious buds after a 15 min pulse. Conversely, only 38.9% of Day 3 cotyledons (BFC < 1) pulsed for 15 min formed buds (Table 1). Again, the percentage of cotyledons forming buds, and the BFC indices increased with longer exposure times and after a 30-min pulse, more than 66% of Day 3 cotyledons formed buds. Therefore, with Day 3 explants, between 20–25 min of exposure to 100 μM BA solution was necessary to produce results similar to Day 0 explants pulsed for only 5–10 min. When the exposure times were less than 30 min, Day 0 explants were more responsive.

Day 0 explants:1–16 h
Cotyledons from newly isolated embryos pulsed for 8 or 16 h in a non-buffered 100 μM BA solution, produced the highest number of buds (11) per cotyledon (Table 2). In addition, over 95% of pulsed cotyledons formed buds after a 2–8 h exposure to BA (Fig. 5); shorter or longer times of exposure lowered the percentage of cotyledons forming buds. In contrast, when the pulsing solution was buffered, the highest number of buds/cotyledon (10.0 and 10.3) were obtained when the induction treatments were between 4 and 6 h (Table 2). The BFC indices were similar (9.1 and 9.3, respectively). Response declined slightly when the explants were kept for 16 h in a buffered solution. Only 80% of the cultured cotyledons formed buds; these were crowded on the explants, and it was impossible to recognize individual structures.

Overall, the best result for Day 0 explants was obtained when the pulsing solution was not buffered. The BFC index was 11.0 after 8 h, as compared to 9.3 after 6 h obtained in a buffered solution (Table 2).

Table 1. Effect of pulsing newly isolated (Day 0) and 3-day-old (Day 3) embryos of *Pinus canariensis* for short periods of time in a buffered aqueous solution containing 100 μM BA, and culturing excised cotyledons on development medium.[1]

Pulse time (min)	Day 0[2]			Day 3[2]		
	% cotyledons forming buds	Average no of. buds/cotyledon after 8 wk ± SE	BFC[3] index	% cotyledons forming buds	Average no. of buds/cotyledon after 8 wk ± SE	BFC[3] index
5	54.3	3.1 ± 0.5a[4] (1–8)[6]	1.7	11.4	1.0 ± 0a[5] (1)	0.1
10	74.3	4.7 ± 0.5ab (1–12)	3.5	25.7	3.8 ± 0.8b (1–11)	1.0
15	81.5	3.3 ± 0.4a (1–8)	2.7	38.9	3.0 ± 0.6b (1–6)	1.2
20	90.4	6.8 ± 0.4b (2–15)	6.1	41.7	3.4 ± 0.6b (1–8)	1.5
25	89.4	7.8 ± 0.4b (3–13)	6.9	44.1	3.4 ± 0.6b (1–8)	1.5
30	83.9	7.4 ± 0.6b (3–16)	6.2	66.7	4.4 ± 0.8b (1–11)	2.9

[1]1/2MCM (Bornman 1983) was used for development.
[2]Experiments were repeated three times using 17 cotyledons per treatment.
[3]BFC (bud forming capacity) = (avg no. of buds/cotyledon) × (% cotyledons forming buds) + 100.
[4&5]Means with the same letter are not significantly different at $p = 0.05$ using Scheffé's multiple comparison procedure.
[6]Range of buds per cotyledon.

Table 2. Effect of pulsing newly isolated embryos (Day 0) of *Pinus canariensis* in a non-buffered and buffered aqueous solution containing 100 μM BA, and culturing excised cotyledons on development medium.[1]

Pulse time (h)	Non-buffered[2]			Buffered[2]		
	% cotyledons forming buds	Average no. of buds/cotyledon after 8 wk ± SE	BFC[3] index	% cotyledons forming buds	Average no. of buds/cotyledon after 8 wk ± SE	BFC[3] index
1	87.5	7.1 ± 0.6a[4] (2–14)[6]	6.2	93.5	6.1 ± 0.5a[5] (2–15)	5.7
2	95.9	7.5 ± 0.5a (3–16)	7.2	94.3	9.1 ± 0.6b (3–16)	8.6
3	96.1	10.4 ± 0.4b (4–18)	10.0	95.0	9.7 ± 0.6b (3–19)	9.2
4	95.4	10.8 ± 0.5b (6–16)	10.3	91.0	10.0 ± 0.5b (3–18)	9.1
6	95.9	8.1 ± 0.4a (3–14)	7.8	90.6	10.3 ± 0.7b (3–20)	9.3
8	95.4	11.5 ± 0.5b (6–23)	11.0	85.7	9.7 ± 0.6b (5–14)	8.3
16	81.2	11.9 ± 0.4b (10–15)	9.7	80.0	7	–

[1]1/2MCM (Bornman 1983) was used for development.
[2]Experiments were repeated three times using 17 cotyledons per treatment.
[3]BFC (bud forming capacity) = (avg no. of buds/cotyledons) × (% cotyledons forming buds) + 100.
[4&5]Means with the same letter are not significantly different at $p = 0.05$ using Scheffé's multiple comparison procedure.
[6]Range of buds per cotyledon.
[7]Buds were not demarcated by whorls of needles, and were impossible to count.

Day 3 explants: 1–16 h

The numbers of adventitious buds produced were higher when 3-day-old cotyledons were used. For example, the highest average number of buds/cotyledon (14.1) was obtained when the exposure time in a non-buffered induction solu-

Table 3. Effect of pulsing embryos of *Pinus canariensis* germinated for three days (Day 3) in a non-buffered and buffered aqueous solution containing 100 μM BA, and culturing excised cotyledons on development medium.[1]

	Non-buffered[2]			Buffered[2]		
Pulse time (h)	% cotyledons forming buds	Average no of. buds/cotyledon after 8 wk ± SE	BFC[3] index	% cotyledons forming buds	Average no. of buds/cotyledon after 8 wk ± SE	BFC[3] index
1	93.6	6.4 ± 0.4a[4] (2–11)[6]	6.0	95.6	6.1 ± 0.4a[5] (2–13)	5.8
2	91.1	10.8 ± 0.9b (3–24)	9.8	83.3	8.7 ± 0.9ab (3–21)	7.2
3	94.7	12.2 ± 0.6bc (7–20)	11.5	91.1	10.6 ± 0.5bc (4–19)	9.6
4	97.3	14.1 ± 0.8c (7–28)	13.7	94.1	11.2 ± 0.6bc (6–18)	10.5
6	95.3	12.8 ± 0.9bc (4–26)	12.2	93.0	11.8 ± 0.5c (6–20)	11.0
8	94.7	10.6 ± 0.6b (5–17)	10.0	86.4	10.0 ± 0.5bc (4–17)	8.6
16	91.3	12.2 ± 0.7bc (2–24)	11.1	80.0	9.0 ± 0.5ab (3–20)	7.2

[1]1/2MCM (Bornman 1983) was used for development.
[2]Experiments were repeated three times using 17 cotyledons per treatment.
[3]BFC (bud forming capacity) = (avg no. of buds/cotyledon) × (% cotyledons forming buds) + 100.
[4&5]Means with the same letter are not significantly different at $p = 0.05$ using Scheffé's multiple comparison procedure.
[6]Range of buds per cotyledon.

tion was 4 h (Table 3); in contrast, after 4 h, an average of 10.8 buds was produced on Day 0 explants (Table 2). Over 90% of cotyledons formed buds after a one-hour pulse, and the best response was obtained when explants were treated for 3–6 h (Fig. 6). Conversely, when the induction solution was buffered, an average of 11.2 to 11.8 buds/cotyledon was obtained after 4 to 6 h exposure (BFC 10.5 and 11.0, respectively). At all exposures, more than 80% of explants tested formed buds, with the lowest percentage obtained after 16 h (Table 3).

Like Day 0 explants, 3-day-old cotyledons pulsed in a non-buffered 100 μM BA solution, produced a higher number BFC (13.7) after 4 h than when treated in a buffered BA solution (BFC 11.0 after 6 h of treatment). If 3-day-old cotyledons were kept in a non-buffered solution for times shorter than 3 h or longer than 6 h, the average number of buds per cotyledon declined, but these averages were higher than those obtained when the solution was buffered.

Comparing Day 0 and Day 3 cotyledons (Tables 2 and 3), the highest average number of buds per cotyledon (14.1) and BFC (13.7) was obtained when Day 3 cotyledons were kept in a non-buffered solution for 4 h (Fig. 7). This treatment also produced the highest percentage of cotyledons (97.3) forming buds.

Induction on agar medium

Results using agar induction and Day 3 explants are presented in Table 4. Cotyledons cultured on BA-containing medium for one day appeared slightly swollen and shorter than Day 0 cotyledons, and about 25% were slighlty nodulated. However, no buds were visible after 8 weeks. After two days on BA-containing medium, about one-third of the explants formed an average of 2 buds per cotyledon.

The average number of buds increased with longer periods of culture on BA-containing medium approaching a maximum at 14 days (Fig. 8). Using this treatment, over 13 adventitious buds were produced per cotyledon. Longer times of culture on induction medium produced higher numbers of buds, but these do not elongate normally (see Martinez Pulido et al. 1990).

Selected data from induction on agar-solidified BA medium and liquid pulsing for Day 3 explants are compared in Table 5. The number of

Table 4. Effect of culturing cotyledons from 3-day-old embryos of *Pinus canariensis* on agar-solidified induction medium containing 10 μM BA for various time periods.[1]

Induction time[2] (days)	% cotyledons forming buds	Average no. of buds/cotyledon after 8 wks ± SE	Range	BFC index[3]
0	0	0	0	0
1	0	0	0	0
2	32.5	1.9 ± 0.2a[4]	1–5	0.6
3	54.5	3.0 ± 0.3ab	1–10	1.6
4	70.5	4.0 ± 0.3abc	1–11	2.8
6	83.3	5.2 ± 0.5bc	1–17	4.3
8	86.2	6.9 ± 0.5cd	1–14	5.9
10	92.1	8.9 ± 0.6d	1–18	8.1
12	94.0	11.8 ± 0.6de	2–18	11.1
14	97.7	13.4 ± 0.6e	3–31	13.1

[1]MCM (Bornman 1983) was used for induction medium.
[2]Experiments were repeated three times using 17 cotyledons per treatment.
[3]BFC (bud forming capacity) = (avg no. of buds/cotyledon) × (% cotyledons forming buds) + 100.
[4]Means with the same letter are not significantly different at $p = 0.05$ using Scheffé's multiple comparison procedure.
[5]Range of buds per cotyledon.

Table 5. Comparison between the average number of adventitious buds produced on Day 3 cotyledonary explants of *Pinus canariensis* cultured on agar-solidified medium containing 10 μM BA or pulsed in a 100 μM BA solution.

	Agar treatment		Pulse treatment		
Time (days)	Average no. of buds/cotyledon	BFC[1]	Time (min)	Average no. of buds/cotyledon	BFC[1]
3	3.0	1.6	25	3.4	1.5
4	4.0	2.8	30	4.4	2.9
8	6.9	5.9	60	6.4	6.0
10	8.9	8.1	120	10.8	9.8
12	11.8	11.1	180	12.2	11.5
14	13.4	13.1	240	14.1	13.7

[1]BFC (bud forming capacity) = (avg no. of buds/cotyledon) × (% cotyledons forming buds) + 100.

buds produced with a 3-day induction on BA-containing medium was comparable to those produced after about 25 min liquid pulse, and 8 days was comparable to a 60 min pulse. The results obtained from a 14-day induction on BA-containing medium was similar to a 3–4 h pulse (Figs 7, 8); both the average number of buds and the BFC were comparable. These results demonstrate the efficiency of using liquid pulsing in a non-buffered BA solution for adventitious bud production on 3-day-old cotyledons of *Pinus canariensis*.

Discussion

There have been various attempts made to compare the conventional and liquid pulse methods of exposing explants to phytohormones. The two main advantages of the latter are that the induction period can be reduced from days or weeks to hours, and secondly, there is a concurrent reduction in the costs of material and labour.

Pulsing methods have been attempted with *Picea abies* (Bornman 1983; Von Arnold & Eriksson 1985; Von Arnold et al. 1988), *Pinus strobus* (Webb et al. 1988), and *Picea engelmannii* (Patel & Thorpe 1986) with varying degrees of success. Vacuum infiltration was also attempted with the aforementioned spruce species. Not surprisingly, results from these methods have been conflicting, and positive results are reported only for *Picea abies* using both excised cotyledons and whole embryos. Basically, our data using *P. canariensis* showed that pulse treatments were as effective as using the conventional

induction methods, and that a non-buffered solution was more effective than a buffered BA solution. Except for a few variations, these results were similar to those obtained by Von Arnold & Eriksson (1985). For example, these authors concluded that a 2 h pulse at pH 5.5 was optimal regardless of whether the BA solution was buffered or not. The results in our experiments, perhaps due to the larger explant size, suggest that pulsing Day 3 cotyledons in a non-buffered solution for 4 h produced superior results. These results were consistent even though the initial pH 5.6 of the pulsing solution increased to pH 7.0 after 8 h.

Of interest also, is that pre-culturing embryos of Norway spruce for longer than 48 h followed by pulsing, resulted in a decreased bud formation capacity. However, if these embryos were sectioned before the BA treatment, the BFC was partially restored suggesting that BA uptake increased with wounded tissues (Von Arnold & Eriksson 1985). Our best results, based on a 3-day-germination period, suggest that for *P. canariensis*, a 72 h pre-culture period did not reduce the effectiveness of cytokinin uptake. However, this pre-culture requirement has to be established for each species. There were other advantages to using 3-day-old explants. For example, Day 0 cotyledons were excised immediately after embryos were isolated from the megagametophytic tissue; at this stage, explants can be easily damaged or become partially dessicated during the culturing period. Also, it was easier to select viable embryos after germination.

Tissues of Day 0 and Day 3 explants were competent when subjected to the appropriate organogenic stimuli. It is interesting to note that both responded similarly when the induction signal was lacking or insufficient (Figs 3–6). Shoots were formed only at the tips of cotyledons which were pulsed for short periods, and bud formation was better on Day 0 material if pulsing was less than 30 min. Buds were present along the length of the whole Day 0 explant after 30 min induction, but only at the tips of Day 3 explants pulsed for the same time period (Figs 2, 3). A similar pattern of differentiation *in vitro* has been reported for *Pseudotsuga menziesii* (Winton & Verhagen 1977), *Picea abies* (Janson & Bornman 1980), *Pinus radiata* (Biondi & Thorpe 1982) and *Pinus ponderosa* (Ellis & Bilderback 1989).

According to Ellis & Bilderback, cotyledons mature acropetally, and most likely the more responsive tips of Day 0 and Day 3 cotyledons were able to take up and translate the signals faster; however, longer induction periods elicited a meristematic response over the entire length of competent explants. It has been established that BA inhibits elongation and maturation, and induces bud formation (Ellis & Bilderback 1989; Villalobos et al. 1984). However, the mechanisms involved are still unknown, and a series of factors like cytokinin concentration, time of exposure, degree of explant maturation, and other environmental factors like pH, temperature, light etc., which could influence cytokinin uptake, have to be combined to re-direct competent tissue into meristematic nodule formation, and eventually into *de novo* bud development.

Canary Island pine exhibits a high degree of genotypic variability between individual embryos (Martinez Pulido et al. 1990). For instance, when Day 0 cotyledons were treated in a non-buffered BA solution for 30 min and 8 h, the range of buds produced were between 6–23, and 3–16, respectively (Tables 1, 2). For *Picea abies*, Von Arnold & Eriksson (1985) found that the variation among embryos was lower when the explants were treated in a BA solution compared to induction on agar-solidified medium supplemented with BA. Also, Bornman (1983) reported that variation (presumably in number of buds per explants) can be reduced by using a short duration pulse treatment. Our results clearly demonstrate that explants have varying genetic potential, and equally high or low numbers of adventitious buds can be produced regardless of the induction methods used.

In conclusion, explants of *P. canariensis* can be pulsed in an aqueous non-buffered BA solution without any reduction in the number of *de novo* buds produced. Both Day 0 and Day 3 explants can be used; however, there is a slight reduction in the BFC of Day 0 material. In the experiments described here, data were collected and the explants were discarded; however, under *in vitro* conditions, this species grows remarkably well, and adventitious shoots can be maintained and re-multiplied successfully for an indefinite

period. Therefore, if a commercial-scale production is undertaken, the associated costs of the conventional induction process as well as an additional 3-day-germination period will have to be carefully evaluated.

Acknowledgements

Carolina Martinez Pulido acknowledges with gratitude the receipt of a fellowship from the Government of the Canary Islands to complete this study. This research was supported by a Natural Sciences and Engineering Research Council of Canada grant to Trevor A. Thorpe.

References

Biondi S & Thorpe TA (1982) Growth regulator effects metabolite changes and respiration during initiation in cultured cotyledon explants of *Pinus radiata*. Bot. Gazette 143: 20–25

Bornman CH (1983) Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. Physiol. Plant. 57: 5–16

David A (1982) *In vitro* propagation of gymnosperms. In: Bonga JM & Durzan DJ (Eds) Tissue Culture in Forestry (pp 72–108). Martinus Nijhoff, Dordrecht

Dawson R, Elliot D, Elliot W & Jones K (1986) Data for Biochemical Research. Clarendon Press, Oxford

Ellis D & Bilderback D (1989) Temporal competence of embryonic *Pinus ponderosa* cotyledons to form multiple buds *in vitro*. Amer. J. Bot. 76: 348–355

Jansson E & Bornman CH (1981) *In vitro* initiation of adventitious structures in relation to the abscision zone in needle explants of *Picea abies*: Anatomical considerations. Physiol. Plant. 53: 191–197

Martinez Pulido C, Harry IS & Thorpe TA (1990) *In vitro* regeneration of plantlets of Canary Island pine (*Pinus canariensis*). Can. J. For. Res. 20: 1200–1211

Patel KR & Thorpe TA (1986) *In vitro* regeneration of plantlets from embryonic and seedling explants of Engelmann spruce (*Picea engelmannii* Parry). Tree Physiol. 1: 289–301

Villalobos VM, Oliver MJ, Yeung EC & Thorpe TA (1984) Cytokinin-induced switch in development in excised cotyledons of radiata pine cotyledons cultured *in vitro*. Physiol. Plant. 61: 483–489

Vogelmann TC, Bornman CH & Nissen P (1984) Uptake of benzyladenine in explants of *Picea abies* and *Pinus sylvestris*. Physiol. Plant. 61: 513–517

Von Arnold S & Eriksson T (1985) Initial stages in the course of adventitious bud formation on embryos of *Picea abies*. Physiol. Plant. 64: 41–47

Von Arnold S, Alsterborg E & Walles B (1988) Micromorphological studies of adventitious bud formation on *Picea abies* embryos treated with cytokinin. Physiol. Plant. 72: 248–256

Webb DT, Flinn BS & Georgis W (1988) Micropropagation of eastern white pine (*Pinus strobus* L.). Can. J. For. Res. 18: 1570–1580.

Winton L & Verhagen S (1977) Shoots from Douglas-fir cultures. Can. J. Bot. 55: 1246–1250.