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ORIGINAL ARTICLE

A combined pathway of somatic embryogenesis and organogenesis to regenerate radiata pine plants

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Abstract This study describes for the first time in *Pinus* genus a plant regeneration system via a combined pathway of somatic embryogenesis and organogenesis from immature seeds of radiata pine. Somatic embryos were obtained from embryogenic line 2162 of *Pinus radiata* D. Don on EDM basal medium containing 60 μM ABA and 6% sucrose. The explants used for organogenesis experiments were either freshly collected somatic embryos or somatic embryos germinated for 1 week. Germination medium was half-strength LP medium, supplemented with 0.2% activated charcoal. Different induction periods and BA concentrations were assayed for shoot induction. After induction treatments, explants were elongated on the same medium used for germination stage. Rooting medium was quarter-strength LP medium supplemented with three different auxin treatments: 1.5 mg L^{-1} 1-naphthalene acetic acid (NAA), 1.5 mg L^{-1} indole-3-butyric acid (IBA) and 1 mg L^{-1} IBA with 0.5 mg L^{-1} NAA (MIX). The effect of the photon flux (120 mmol m^{-2} s^{-1} and darkness) in the first week of the explants in the rooting media was also tested. This methodology could offer an alternative to overcome some problems associated with somatic embryogenesis such as the seasonality of embryogenic tissue (ET) initiation or a low embryo production from the ET, a particularly important issue in the case of genetically transformed ETs.

Keywords Auxin · Cytokinin · Micropropagation · *Pinus radiata* · Somatic embryos

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Introduction

The level of domestication in forest trees is significantly lower than for agricultural plants. However, there is a great potential for improvement through genetic breeding. Using this type of technologies, considerable genetic gain has been achieved in plantation forest trees such as *Pinus radiata*. This conifer is grown mainly in New Zealand, Chile, Australia, South Africa and Spain. Native to California, this species shows superior growth rates, and many improvements to its genetic value have been made over the past 50 years (Walter 2004). Although conventional seed orchards provide genetically improved seeds, traditional breeding strategies combined with in vitro vegetative propagation have shown advantages such as additional genetic gain achieved by capturing non-additive genetic variation, as well as the speed with which clones may be introduced to meet market goals and the ability to program diversity into a clonal plantation (Park et al. 1998).

In vitro vegetative propagation from physiologically mature tissue (even as young as 1-year-old in some species) tends to be quite difficult and can result in changes in the attributes of the resulting plants. In Neiker-Tecnalia, our research group has successfully achieved adult clonal propagation in pine species (De Diego et al. 2008, 2010; Cortizo et al. 2009), but sometimes changes in the attributes of the resulting plants have been observed and the reinvigoration of the material has been transitory in in vitro conditions. On the other hand, vegetative propagation from physiologically juvenile tissue has been successful in a number of conifer species (Bergmann and Stomp 1992; Moncaleán et al. 2005); this phenomenon has led many organizations to focus on production of elite families through juvenile tissue propagation in the near term, either

for operational use or for clonal tests, while they continue to research methods of selected mature tree propagation (Talbert et al. 1993).

The two main in vitro techniques used routinely for plant micropropagation are somatic embryogenesis (SE) and organogenesis (Giri et al. 2004). As for in vitro embryogenesis, organogenesis has been used as a model system to study the structural, physiological, and molecular bases of development. Studies on organogenesis and SE have shown the fundamental role of plant growth regulators in in vitro culture (Jiménez 2005); this basic understanding has greatly contributed to the extension of tissue culture for commercial applications (Stasolla and Thorpe 2010).

In this sense, propagation via SE is an effective method in propagating elite plants when it is combined with other technologies, such as cryopreserving the embryogenic tissue (ET) and selecting elite clones in field tests (Park 2002). Due to the importance of this technology, SE in pines has been widely reported in the past few years (Klimaszewska et al. 2007; Lelu-Walter et al. 2008). In the majority of these reports, the induction of ET is carried out from immature seeds. The problem is that the competence window for this type of explant is narrow, lasting around 4 weeks (MacKay et al. 2006; Yildirim et al. 2006); after that, the initiation decreases to very low rates. In an attempt to overcome the narrow competence window, ET initiation has been also obtained from mature embryos in *P. lambertiana* (Gupta and Durzan 1986), *P. strobus* (Garin et al. 1998; Klimaszewska et al. 2001) and *P. taeda* (Tang et al. 2001), but at low frequencies making this approach unfeasible for large-scale production (Klimaszewska et al. 2007).

However, the major bottleneck in *Pinus* SE is maturation of the ET; in several pine species, the somatic embryos obtained feature abnormal morphology and, when they resemble their zygotic counterparts, they appear in a very low numbers (Choudhury et al. 2007; Carneros et al. 2009). During the maturation phase of SE, the embryos attain physiological and biochemical attributes that enable subsequent germination and conversion. Cotyledonary mature somatic embryos germinate with vigour and establish plants at a high frequency. The current reality is that having some genotypes with low embryo quality or number results in low germination frequency, and makes large-scale production of the genotypes too expensive and therefore eliminates these genotypes from production. Consequently, the number of genotypes that can be candidates for clonal forestry decreases (Davis and Becwar 2007). In vitro organogenesis is not restricted to a certain moment of the year as SE initiation, and the material obtained can be multiply and rooted when demanded, with no detrimental effect on the explants produced.

This study presents a novel approach to overcome some problems associated with SE such as the aforementioned seasonality of ET initiation or low embryo production due to limited success in maturation and germination steps. The approach presented here consists on the development of a combined SE and organogenesis protocol. For this purpose, the effect of different initial explants, BA concentrations and induction periods and culture conditions were assayed in somatic embryos of *Pinus radiata* D. Don.

Materials and methods

Plant material

Pinus radiata ET was obtained from immature megagametophytes cultured on EDM basal medium (Walter et al. 1998) with a combination of 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 μM 6-benzylaminopurine (BA). Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} Gelrite® were added. Medium was autoclaved at 121°C for 20 min. After autoclaving, filter-sterilized solutions, of 550 mg L^{-1} L-glutamine, 525 mg L^{-1} L-asparagine, 175 mg L^{-1} L-arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline were added to the cooled medium; the pH of these solutions was also adjusted to 5.7.

Somatic embryos were obtained from embryogenic line 2162 following the protocol described by Montalbán et al. (2010). Briefly, ET was first suspended in liquid growth regulators-free EDM medium and was vigorously shaken by hand for a few seconds. Thereafter, a 5-mL aliquot containing 100 mg fresh mass of suspended ET was poured onto a filter paper disc (Whatman no.2) in a Büchner funnel. A vacuum pulse was applied for 10 s and the filter paper with the attached ET was transferred to a maturation medium. The maturation medium had the salt formulation of EDM medium, but a higher concentration of Gelrite®, 9 g L^{-1}, and was supplemented with 60 μM abscisic acid (racemic ABA; Sigma), sucrose at 6% (Sigma) and the amino acid mixture used for initiation and maintenance of the ET. Cultures were kept in darkness at 21 ± 1°C. After 12 weeks on the maturation medium, mature somatic embryos were selected and isolated from ET. The somatic embryos collected were white to yellowish, non-germinating, with a distinct hypocotyl region, and at least three cotyledons. Two types of explants were used for induction experiments: freshly collected (NG) somatic embryos, and somatic embryos germinated for 1 week (G) (Fig. 1a, b).

Germination medium was half-strength macronutrients (except for the iron) and complete micronutrients and vitamins from LP medium (Quoirin and Lepoivre 1977,

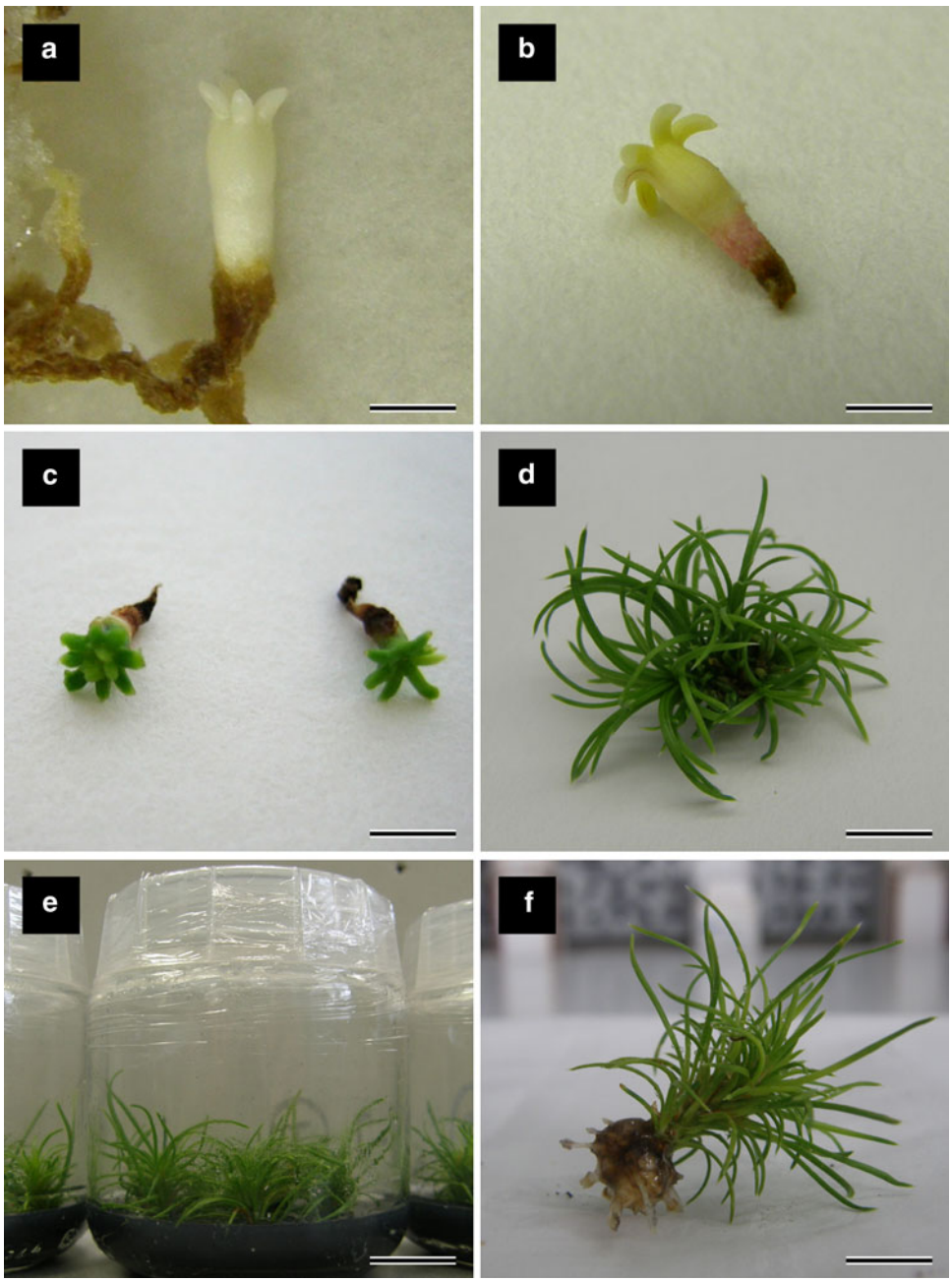

Fig. 1 Shoot induction in *Pinus radiata* somatic embryos. **a** NG embryo, *bar* 3 mm; **b** G embryo, *bar* 4 mm; **c** G embryos after 3BA4.4 treatment, *bar* 8 mm; **d** NG embryos after 3BA4.4 treatment, after 4 weeks on modified 1/2 LP lacking growth regulators, and 8 weeks on LPE, *bar* 11 mm; **e** rootable explants obtained from NG embryos after 3BA4.4 treatment, 1/2 LP lacking growth regulators, and 5 months on LPE, *bar* 22 mm; **f** explant cultured for 3 weeks on modified 1/4 LP medium supplemented with 1 mg L^{-1} IBA and 0.5 mg L^{-1} NAA (MIX), after a month on LPE, *bar* 14 mm

modified by Aitken-Christie et al. 1988) (1/2 LP), supplemented with 3% sucrose, 0.2% activated charcoal (w/v) and 10 g L^{-1} Difco Agar® granulated. Cultures were kept under dim light (40 mmol m^{-2} s^{-1}) at 21 ± 1°C.

Shoot induction

Induction media was 1/2 LP medium, supplemented with 3% sucrose, 8 g L^{-1} Difco Agar® granulated and 6-benzylaminopurine (BA). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

When NG somatic embryos were used as initial explants, different induction periods and BA concentrations were assayed: 1 μM BA for 2 weeks (2BA1), 1 or 4.4 μM BA for 3 weeks (3BA1 and 3BA4.4) and 1, 4.4 or 22 μM BA for 4 weeks (4BA1, 4BA4.4 and 4BA22). When G somatic embryos were used as initial explants, the induction treatments assayed were 1 or 4.4 μM BA for 3 weeks.

Embryos were placed on 90 × 15 mm Petri dishes containing 15 mL induction medium. Six to eight embryos per Petri dish were cultured in an inverted position with the

cotyledons immersed in the induction medium (Aitken-Christie et al. 1988).

After induction treatments, the explants were transferred to glass jars with the previously described LP medium but lacking BA for 4 weeks. Then, they were subcultured monthly on elongation medium (LPE); this medium was 1/2 LP medium lacking BA and supplemented with 0.2% activated charcoal (w/v).

The experiments were replicated two times and in each replication two to three Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were kept at 21 ± 1°C under a 16-h photoperiod of 120 mmol m^{-2} s^{-1}.

Root induction

Elongated shoots (>15 mm) were transferred to glass jars with rooting medium. Based on previous experiments, the basal medium chosen for rooting was modified LP medium with quarter-strength macronutrients except for the iron, and half-strength iron, micronutrients and vitamins, supplemented with 3% sucrose and 8 g L^{-1} Difco Agar® granulated (1/4LP). This basal medium was supplemented with three different auxin treatments: 1.5 mg L^{-1} 1-naphthalene acetic acid (NAA), 1.5 mg L^{-1} indole-3-butyric acid (IBA), and 1 mg L^{-1} IBA with 0.5 mg L^{-1} NAA (MIX). The effect of the photon flux (120 mmol m^{-2} s^{-1} and darkness) in the first week of the explants in the rooting media was also tested; the root induction experiments comprised three auxin treatments and two light regimes, a total of six combinations. The following codes were used to identify the induction media: the rooting treatment assayed (IBA, MIX or NAA) followed by the light treatment assayed the first week of the root induction period: light (L) or darkness (D). Thus, NAA-D is 1/4LP supplemented with 1.5 mg L^{-1} NAA and kept in darkness the first week.

Four shoots were cultured on each culture vessel and five glass jars per treatment were used. After 3 weeks in root induction medium, the shoots were transferred to LPE medium. Embryos were subcultured in LPE medium and kept under the same conditions described above. After a month on LPE medium, explants with and without visible roots were transferred to a wet sterile peat:perlite mixture (3:1) and acclimatized in the greenhouse under controlled conditions at 21 ± 2°C and progressively decreasing humidity.

Data collection and statistical analyses

After 3 months on LPE, the percentage of necrosed, hyperhydric embryos and embryos forming shoots (EFS) was recorded.

After 6 months on LPE, the number of shoots >3 mm per embryo, and the shoot elongation capacity (SEC) index were recorded. SEC was calculated as described by Lambardi et al. (1993):

$$\text{SEC} = (\text{average number of shoots} > 3\text{mm per embryo}) \times (\%\text{explants forming shoots}).$$

Normality was evaluated by Shapiro–Wilk normality test (Shapiro and Wilk 1965) and the equal variance of the data was analyzed by Levene's test (Brown and Forsythe 1974).

One-way analysis of variance (ANOVA) was carried out to determine differences between the shoot induction treatments for the EFS percentage, the number of shoots per embryo and the SEC index. Data for the number of shoots per embryo and the SEC index were subjected to log $(x + 1)$ transformation. Multiple comparisons were made using Duncan's post-hoc test ($p \leq 0.05$).

After a month on LPE, the percentage of shoots with roots was scored. ANOVA was carried out to determine differences among root induction treatments for the rooting percentage. Multiple comparisons were made using Duncan's post-hoc test ($p \leq 0.05$).

Results

Contamination percentages remained under 4% after all shoot induction treatments except after treatment 2BA1 (16 %) (Fig. 2). Increasing the BA concentration or the induction period decreased the number of necrosed embryos. The lowest percentage for this parameter were obtained in G embryos cultured in induction media with 1 or 4.4 μM BA for 3 weeks (11.1 and 27.8, respectively) (Fig. 2). The highest percentage of embryos that did not produce shoots and died shortly after the induction period was obtained in NG embryos cultured in induction media with 1 μM BA for 2 weeks (70.8%, Fig. 2).

Although high percentages of hyperhydricity were observed when NG somatic embryos were exposed to 1 and 22 μM BA for 4 weeks (20 and 22.5%, respectively), this was not the case for NG embryos treated with 4.4 μM BA for the same induction period (5%). Lower exposure periods did not produce hyperhydric explants (Fig. 2).

All surviving explants showed an organogenic response and produced shoots. There were significant differences for the EFS percentage between NG and G embryos (Fig. 2). When NG embryos were used as initial explants, treatments with 1 μM BA for 2 and 4 weeks presented the lowest induction percentages (12.5 and 25.0%, respectively). On the contrary, treatments with 4.4 μM BA for 3

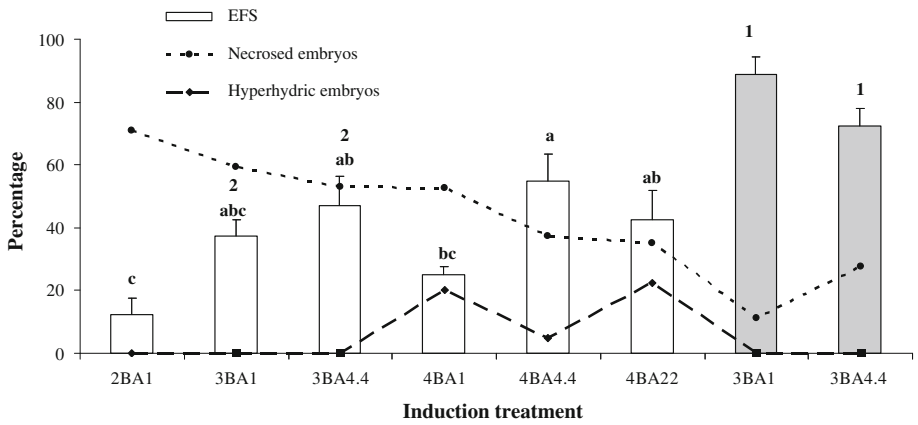

Fig. 2 *Necrosed embryos* (%), *hyperhydric embryos* (%) and embryos forming shoots (*EFS*) (%) for non-germinated (*white*) and germinated (*grey*) *Pinus radiata* somatic embryos. For the EFS percentages, *different letters* (*a*, *b*, *c*) show significant differences between different treatment in NG embryos; and *different numbers* (*1*, *2*) show significant differences between NG and G embryos for treatments with 1 or 4.4 μM BA for 3 weeks (*3BA1, 3BA4.4*) by Duncan's post hoc test ($p \leq 0.05$)

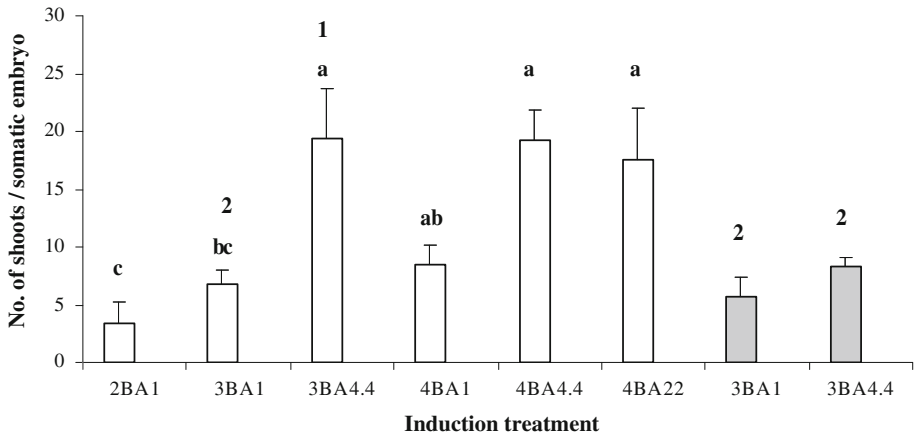

Fig. 3 Number of shoots per embryo (mean ± SE) in non-germinated (*white*) and germinated (*grey*) *Pinus radiata* somatic embryos. *Different letters* (*a*, *b*) show significant differences between different treatments in NG embryos; and *different numbers* (*1*, *2*) show significant differences between NG and G embryos for treatments with 1 or 4.4 μM BA for 3 weeks (*3BA1, 3BA4.4*) by Duncan's post hoc test ($p \leq 0.05$)

and 4 weeks showed the highest (46.9 and 55.0%, respectively). Neither the induction time nor the concentration of BA showed a clear trend for caulogenic response (Fig. 2).

When G embryos were used as initial explants, the EFS percentages obtained did not differ significantly (88.9% for treatment 3BA1, and 72.2% for treatment 3BA4.4). Comparing NG with G explants subjected to the same induction treatments (1 and 4.4 μM BA for 3 weeks), significantly better results for G embryos were observed (Fig. 2).

The lowest number of shoots in NG embryos was obtained when 1 μM BA was applied for 2 and 3 weeks to the induction medium (Fig. 3). Longer induction periods (3 or 4 weeks) or higher BA concentrations (4.4 or 22 μM) produced a significantly higher number of shoots per embryo. When NG and G embryos were compared, the number of shoots per embryo in NG explants after treatment 3BA4.4 (19.3 shoots per embryo) was significantly better than the number of shoots obtained in NG embryos after treatment 3BA1 and in G embryos after treatments 3BA1and 3BA4.4 (Fig. 3).

When the SEC value was analysed, G embryos and NG embryos from 3BA4.4 treatment presented significantly higher SEC indexes than NG embryos after treatment 3BA1 (Fig. 4). NG embryos cultured with 3BA4.4, 4BA4.4 and 4BA22 achieved the best SEC indexes (9.1, 10.2 and 7.4, respectively). Moreover, NG embryos induced with 1 μM BA for 3 or 4 weeks led to significantly higher SEC values (2.5 and 2.1, respectively) than NG embryos treated with the same concentration of BA for 2 weeks (0.4) (Fig. 4).

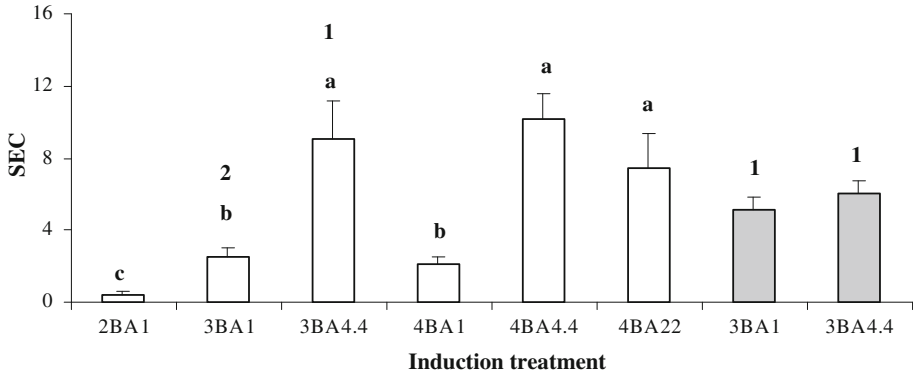

Fig. 4 Shoot elongation capacity (SEC) in non-germinated (*white*) and germinated (*grey*) *Pinus radiata* somatic embryos. *Different letters* (*a*, *b*) show significant differences between different treatment in NG embryos; and *different numbers* (*1*, *2*) show significant differences between NG and G embryos for treatments with 1 or 4.4 μM BA for 3 weeks (*3BA1, 3BA4.4*) by Duncan's post hoc test ($p \leq 0.05$)

Table 1 *Pinus radiata* rooted somatic shoots (%) after different root induction treatments: on modified 1/4 LP supplemented with 1.5 mg L^{-1} IBA, 1.5 mg L^{-1} NAA or 1 mg L^{-1} NAA with 0.5 mg L^{-1} IBA (MIX); and different light regimes for the first week of the root induction period: light (*L*) or darkness (*D*)

Rooting treatment	Rooting (%)
IBA-L	5.0 ± 3.3 b
IBA-D	5.0 ± 3.3 b
MIX-L	50.0 ± 10.0 a
MIX-D	20.0 ± 5.0 b
NAA-L	60.0 ± 12.7 a
NAA-D	25.0 ± 7.9 b

Shoots were obtained from somatic embryos after induction treatment 3BA4.4. Different letters within a column by Duncan's post hoc test ($p \leq 0.05$)

There were significant differences for the percentage of shoots with roots among root induction treatments. The percentage of explants rooted with NAA or the mixture of IBA and NAA (MIX) under a 16-h photoperiod of 120 mmol m^{-2} s^{-1} was significantly higher than the percentage of rooted explants obtained when shoots were cultured with IBA under the same light conditions (Table 1). Darkness for the first week of the root induction stage was not beneficial for the percentage of rooted explants, and these percentages were not significantly different for the different root induction treatments tested (IBA, MIX and NAA) (Table 1).

Discussion

The ability to produce morphologically and developmentally normal embryos from undifferentiated somatic cells in culture, through the process of SE, resides uniquely within the plants. Since the initial description of somatic embryo production from carrot callus cells (Steward et al. 1958), this unique developmental potential has been recognized both as an important pathway for the regeneration of plants and as a potential model for studying early regulatory and morphogenetic events in plant development (Zimmerman 1993). But, as we have pointed above, one of the major bottlenecks in conifer SE is the conversion of the ET into plants. It results, particularly in *Pinus* genus, in a limiting factor to broader application of SE in clonal propagation and genetic engineering (Klimaszewska et al. 2007).

In this study, we have tried to overcome the low frequency of plantlet regeneration often encountered in pines by means of shoot organogenesis. Our results indicate that explant type, cytokinin induction period and BA concentration significantly affect the percentage of responding somatic embryos and the number of shoots per embryo obtained. However, the success in the induction of organogenesis from somatic embryos resembled the results obtained when the same methodology was used in zygotic embryos of the same species (Montalbán 2011).

In *Pinus radiata* zygotic embryos, it has been proposed that the high bud-forming capacity of cotyledons is related to the undifferentiated state of cotyledonary cells at the time of culture (Yeung et al. 1981; Aitken-Christie et al. 1985). But in several pine species, it is a current practice to germinate whole embryos or cotyledons for a certain time, ranging from hours to days, before applying any induction treatment (Valdés et al. 2001; Villalobos-Amador et al. 2002; Hargreaves et al. 2005). In our experiments, explants germinated for 1 week before the induction treatment showed the highest percentages of EFS (88.9% for treatment 3BA1 and 72.2% for treatment 3BA4.4), whereas NG embryos did not present percentages higher than 47% for the same treatments. On the contrary, in *Pinus ayacahuite*, Saborio et al. (1997) suggested that most cotyledonary cells

of embryos kept in culture for 6 or more days might have lose ability to dedifferentiate and therefore were unable to respond to the bud induction conditions. Martinez Pulido et al. (1990) also found that explants of *Pinus canariensis* too young or too old (germinated for 1 day or 8 days) gave worse results than explants germinated for 3 days.

In our experiments, the presence of activated charcoal in the germination medium could have had a beneficial effect due to its property of adsorbing residual plant growth regulators (von Aderkas et al. 2002). Auxins and cytokinins are the two main growth regulators in plants involved in the regulation of division and differentiation and have been proved to play an important role in the induction of somatic embryogenesis (Fehér et al. 2003); later, in the SE process, the development of somatic embryos is induced by application of abscisic acid to the medium (Stasolla and Yeung 2003). The germination step before shoot induction could have made the G somatic embryos prone to an organogenic response, eliminating a growth regulator excess and preparing the explants for shoot induction.

In NG embryos, the percentage of organogenic embryos was higher when BA concentration was increased from 1 to 4.4 μM. Higher BA concentration did not give better percentages. Similar results were found in mature zygotic embryos of *Pinus halepensis* (Lambardi et al. 1993). In accordance with these authors, increasing the exposure time from 3 to 4 weeks had no significant effect on the percentage of responding embryos. In the same line, Moncaleán et al. (2005) found in *P. pinea* that increasing the exposure time from 2 to 5 weeks did not affect the percentage of responding embryos. The highest EFS percentages were obtained when G embryos were used as initial explants, independently of the BA concentration. Thus, the source of material (G vs. NG embryos) seemed to be the most critical factor; similar findings were reported by Martinez Pulido et al. (1990) in *P. canariensis* cotyledons germinated for 3 or 5 days, although these authors observed a decrease in the organogenic response when the explants were germinated for a longer period.

Hyperhydric shoots only appeared when induction treatments lasted 4 weeks, and in percentages ranging from 5 to 22%. In this sense, Capuana and Giannini (1995) observed in *P. pinea* that exposures longer than 3 weeks to BA produced shoots that showed callusing and hyperhydricity. In general terms, contamination percentages remained low.

The highest number of shoots per embryo were obtained in NG embryos induced with treatments 3BA4.4, 4BA4.4 and 4BA22 (19.3, 18.5 and 17.5, respectively). In previous experiments with *P. radiata* zygotic embryos cultured under the same conditions, we found treatment 3BA4.4 the most productive (Montalbán 2011). Biondi and Thorpe (1982) obtained similar results in germinated zygotic cotyledons of the same species, cultured for 3 weeks in medium supplemented with 5 μM BA. When these NG embryos were cultured under a lower BA concentration or for a shorter period of time, the number of shoots per embryo was significantly lower; these results are in agreement with the observations made by Moncaleán et al. (2005) in stone pine cotyledons. In our experiments, the number of shoots per embryo from G embryos was not statistically different from the values obtained in NG embryos cultured with 1 μM BA. Webb et al. (1988) also found that low BA concentration and germination for longer than 6 days negatively affected the production of shoots in zygotic cotyledons of eastern white pine. In this context, Valdés et al. (2001) reported a drop in the number of buds per cotyledon in *Pinus pinea* when the explants were germinated before the induction treatment.

The shoot elongation capacity (SEC) incorporates the EFS percentage and the number of shoots per embryo. The combination of these factors offers a realistic assessment of the efficacy of the culture conditions (Martínez Pulido et al. 1992). The lowest SEC values were found in NG somatic embryos treated with 1 μM BA. NG embryos induced with 4.4 μM BA for 3 and 4 weeks or BA at 22 μM for 4 weeks achieved the highest SEC values. In contrast, in previous experiments with *Pinus radiata* zygotic embryos, the SEC values obtained in treatments 2BA1 and 3BA4.4 were similar to the SEC index obtained in NG embryos induced with BA4.4 during 3 weeks (Montalbán 2011).

When the initial type of explant was compared, the SEC value was similar in G embryos and NG embryos, except for NG embryos induced with 1 μM BA for 3 weeks. The SEC indexes in G embryos were higher than the indexes reported by Álvarez et al. (2009) in *P. pinaster* germinated cotyledons; the reason for better values in our experiments could be the abovementioned higher organogenic response in G explants when compared with NG explants. Similar findings have been found in some angiosperms species such as red horse chestnut, *Aesculus carnea* (Zdravković-Korać et al. 2008).

Rooting percentage of shoots was significantly affected by the rooting treatment used. In accordance with our results in organogenesis from zygotic embryos, explants treated with NAA-L and MIX-L showed higher rooting percentages (60 and 50%, respectively) than explants treated with IBA (5%). In radiata pine, in vitro organogenesis is a common practice to use a mixture of IBA and NAA for rooting (Prehn et al. 2003; Hargreaves et al. 2005). NAA alone has also been reported to be effective in in vitro rooting of several pine species such as *P. ayacahuite* (Saborio et al. 1997) and *P. pinaster* (Álvarez et al. 2009).

The light regime also influenced the process: in conifers, a dark period at the beginning of the rooting stage is often

required for root formation and generally a reduction of light intensity favours adventitious root development (Brassard et al. 1996; Alonso et al. 2006). Our results are in agreement with other reports in the *Pinus* genus where rooting of explants is higher under a 16-h photoperiod (Flygh et al. 1993; Tang and Guo 2001).

Further testing on the performance of the rooted explants in the greenhouse will determine if the best treatments for in vitro rooting lead to the best acclimatisation rates or, on the contrary, the explants rooted with IBA show a better ex vitro performance as observed in radiata pine zygotic embryos (Montalbán 2011). IBA is the auxin currently used for rooting of cuttings of *P.radiata* and has been used in in vitro rooting protocols of *P. canariensis* (Martínez Pulido et al. 1994) and *P. heldreichii* (Stojičić et al. 1999).

In the last few years, several SE and organogenesis protocols have been developed in parallel instead of combining these in vitro techniques in angiosperm species such as *Manihot esculenta* (Guohua 1998), *Gossypium* spp. (Khan et al. 2006), *Aesculus carnea* (Zdravković-Korać et al. 2008) and *Hippophae rhamnoides* (Sriskandarajah and Lundquist 2009). The goal of these studies was to study the potential of both techniques, through media and growth regulator adjustments, in the regeneration of these species.

A few protocols combining SE and organogenesis have been described in angiosperms in order to maximise plant production from genetically transformed ET (Kim et al. 2009) or to regenerate endangered species (Siva et al. 2009). In conifers, there are no reports along these lines, although this methodology could overcome some problems associated with SE such as the aforementioned seasonality of ET initiation or a low embryo production obtained from the ET. The latter problem is especially important in the case of genetically transformed ETs, where the plantlet regeneration is often low (Giri et al. 2004).

We have established an efficient plant regeneration system via a combined pathway of SE and organogenesis. Once the somatic embryos are obtained, up to 19 rootable shoots can be obtained from a single embryo, these shoots presenting rooting percentages around 60%. Considering that we can obtain more than 150 embryos per 100 mg of ET (Montalbán et al. 2010), theoretically by using the described method more than 1,700 rooted shoots can be produced. Moreover, these shoots before rooting can be propagated and continuously used as a source for plant regeneration.

Furthermore, this approach facilitates the application of genetic manipulation procedures; and over time, numerous plants, representing clones, can be regenerated from a single shoot. Thus, in vitro micropropagation via induction of SE and organogenesis can be very useful for plant genetic resource management. To the best of our knowledge, this is the first attempt to report the induction of organogenesis in somatic embryos of *Pinus radiata* D. Don.

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