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Full Length Research Paper

In vitro regeneration of *Pinus brutia* Ten. var. *eldarica* (Medw.) through organogenesis

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This paper describes two *in vitro* regeneration systems through direct and indirect organogenesis in *Pinus brutia* using fascicles aseptic cultures as explants. Mechanical scarification and gibberellic acid (GA_3) were evaluated on *in vitro* seed germination. Scarification was the treatment that allowed for *in vitro* seed germination. The highest direct organogenic response was obtained in Murashige and Skoog (MS) medium containing 4.5 µM thidiazuron, whereas the highest indirect organogenesis was obtained with 9.8 µM thidiazuron and 3.4 µM paclobutrazol. The isolated shoots were rooted on MS medium supplemented with 1.70 µM indoleacetic acid. A large variation in root ability was observed among plantlets. These results suggest that both regeneration systems can be applied to the micropropagation or genetic transformation of *P. brutia*.

Key words: *Pinus brutia*, organogenesis, fascicles, paclobutrazol, thidiazuron.

INTRODUCTION

Pinus brutia Ten. var. *eldarica* (Medw.) (commonly known as Calabrician pine) is a native and restricted species in the eastern Mediterranean. Ecologically and economically speaking, it is one of the most important forest tree species in this region (Boydak, 2004). *P. brutia* is multiplied traditionally from seed and cuttings. However, seed germination is very heterogeneous, indicating changing degrees of dormancy among seeds of the same population (Ürgenç et al., 1989). Among pines, the production of rooted cuttings is very poor because of the high degree of lignification of the branches and the low rooting capacity of cuttings (Orea and Villalabos, 1990).

An ever-increasing demand for forest products has led to the development of technologies to assist traditional tree breeding programs to produce phenotypically superior and genetically improved trees. One of the most important limitations of mass production on a commercial scale is the high cost of individual plantlet production. *In vitro* regeneration can be very effective in the micropropagation and/or genetic breeding of several commercially important forest trees. Regeneration through morphogenesis has been extensively described in a number of forest micropropagation protocols in different species of the genus *Pinus*: *P. taeda* L. (Tang et al., 1998), *P. wallichiana* AB Jacks (Mathur and Nadgauda, 1999), *P. taeda* L. (Tang, 2001), *P. taeda* L. (Tang and Guo, 2001), *P. heldreichii* (Stojičić and Budimir, 2004), *P. pinea* L. (Sul and Korban, 2004), *P. virginiana* (Tag et al., 2004), *P. strobus* L. (Tang and Newton, 2005), *P. elliottii* (Tang et al., 2006), *P. sylvestris* L. (De Diego et al., 2010) and *P. radiata* (Montalbán et al., 2011).

Cotyledons, immature or mature embryos, are the most

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Table 1. Effect of scarification and GA_3 treatment on percentage of germination among *Pinus brutia* seeds (within 30 days of culture).

Treatment	GA_3 (µM)	Germination (%) (mean ± SE)
Without scarification	0.0	0.0 ± 0.0[cz]
	1.4	0.0 ± 0.0[c]
	2.8	0.0 ± 0.0[c]
	4.3	0.0 ± 0.0[c]
With scarification	0.0	51.6 ± 1.6[b]
	1.4	70.0 ± 2.8[a]
	2.8	68.3 ± 1.6[a]
	4.3	65.0 ± 2.8[a]

[z]Values with different letters are significantly different (Tukey, $p \leq 0.05$) by analysis of variance.

commonly used explants in tissue culture in the majority of coniferous species because it is difficult to induce organogenesis or somatic embryogenesis from other types of explants. Pine leaves (needles) occur in fascicles (bundles). Fascicle shoots have proven to be an ideal plant material for micropropagation. The regeneration of plants from fascicles *of P. brutia* has already been achieved (Abdullah et al., 1984, 1986, 1987). However, these protocols reported a low number of shoots per explant, and the percentage of fascicles that formed shoot buds was low. The aim of the present study was to develop two reliable protocols to enable the high-frequency induction of multiple shoot structures from aseptic fascicles through organogenesis in *P. brutia* var*. eldarica*.

MATERIALS AND METHODS

Dry seeds of *P. brutia* Ten. var. *eldarica* (Medw.) were obtained from a germplasm seed bank located in Murcia, Spain, and were used to produce the aseptic seedlings that were the source of the explants. After being washed thoroughly under running tap water for 30 min, the seeds were maintained for 48 h in water to facilitate mechanical scarification. Seeds were disinfected by immersion in ethanol at 70% (v/v) for 5 min and subsequently in a solution of commercial sodium hypochlorite (Cloralex® 6% a.i.; Distribuidora Alen S.A. de C.V., Nuevo León, México) diluted to 30% (v/v, in water) for 15 min with constant stirring, followed by three rinses in sterile distilled water. Once the seeds were sterile, different concentrations of giberelic acid (GA_3: 0, 1.4, 2.8, and 4.33 µM) combined with mechanical scarification were evaluated to induce *in vitro* germination of seeds (Table 1).

Mechanical scarification was carried out by making an incision at the top of the seed coat, with the aid of a forceps and scalpel, taking care not to damage the embryo. The medium was solidified with 0.22% (w/v) Gelrite® (Applied Bioscience Consultants and Distributor, Mumbai, India) and adjusted to pH 5.7 before autoclaving. In all treatments, the seeds were placed in Magenta boxes containing 30 ml MS (Murashige and Skoog, 1962) medium and incubated in the dark at 24 ± 2°C. Twenty-five seeds were used per treatment, distributed at a rate of five seeds per culture vessel. Upon germination, plantlets were transferred to hormone-free MS medium and maintained under a 16 h light photoperiod (40 to 50 µmol m^{-2} s^{-1}) at 25 ± 2°C.

Organogenesis induction

Fascicles (1 to 1.5 cm long) were excised from aseptic plants. These were cultivated in MS medium with different concentrations of paclobutrazol (PAC) and thidiazuron (TDZ) (Table 2). The medium was solidified with 0.22% (w/v) Gelrite® and adjusted to pH 5.7 before autoclaving. All treatments were evaluated using 25 explants distributed at a rate of 5 fascicles per Magenta box and incubated in a 16 h light photoperiod (40 to 50 µmol m^{-2} s^{-1}) at 25 ± 2°C.

Data analysis

The experiments followed a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) using a Statistical analysis system program (SAS, Version 9.1.3 for Windows). The means were compared using Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

As shown in Table 1, scarification was the determinant treatment for *in vitro* seed germination of *P. brutia.* After scarification, in the absence of GA_3 in the medium, the seed reached 51% germination. A significant increase in seed germination (70%) was observed when seeds were cultivated in medium containing 1.4 µM GA_3 (Figure 1a). However, a gradual increase in the concentration of GA_3 did not significantly change the percentage of seeds that germinated. In contrast, germination was completely prevented when seeds were not mechanically scarified, even when GA_3 was present in all treatments. Eight weeks after germination, the seedlings had reached 8 to 10 cm in height and showed a vigorous appearance, with bright green needles and abundant fascicles (Figure 1b). Scarification of seeds is a common practice in forest species, including some pine species, because it breaks seed dormancy. In *P. brutia,* scarification resulted in a significant increase in the germination of stored seeds. However, scarification in combination with GA_3 increased seed germination in this species. Embryo dormancy is thought to be due to the presence of inhibitors, especially abscisic acid (ABA), as well as the absence of growth promoters, such as GA_3. The loss of embryo dormancy is often associated with the ABA-to-GA_3 ratio.

Organogenesis induction

As shown in Table 2, the addition of PAC and/or TDZ to the culture medium influenced the behavior of the explants. There were significant differences in responses by induction treatment. Direct organogenesis induction was obtained using TDZ (4.54 and 9.08 µM), or PAC (4.4 and 6.8 µM), and a combination of PAC and TDZ (3.4 µM

Table 2. Effect of PAC and TDZ on morphogenetic response on *Pinus brutia.*

Plant growth regulator (µM)		Response (%)	Type of response	Shoots/explant (mean ± ES)
PAC	TDZ			
0	0	76	DO	2.1 ± 0.19[ez]
0	4.54	84	DO	23.3 ± 0.51[b]
0	9.08	72	DO and callus	8.0 ± 0.39[c]
0	13.62	48	Callus	0.0 ± 0.0[f]
3.4	0	80	DO	3.3 ± 0.87[e]
3.4	4.54	60	DO and callus	6.5 ± 0.21[d]
3.4	9.08	48	IO	36.5 ± 1.43[a]
3.4	13.62	36	Callus	0.0 ± 0.0[f]
6.8	0	68	DO	6.7 ± 0.26[d]
6.8	4.54	60	DO and callus	6.0 ± 0.25[d]
6.8	9.08	32	Callus	0.0 ± 0.0[f]
6.8	13.62	NR	NR	0.0 ± 0.0[f]

PAC, Paclobutrazol; TDZ, thidiazuron; DO, direct organogenesis; IO, indirect organogenesis; NR, no response. [z]Values with different letters are significantly different (Tukey, $p \leq 0.05$) by analysis of variance.

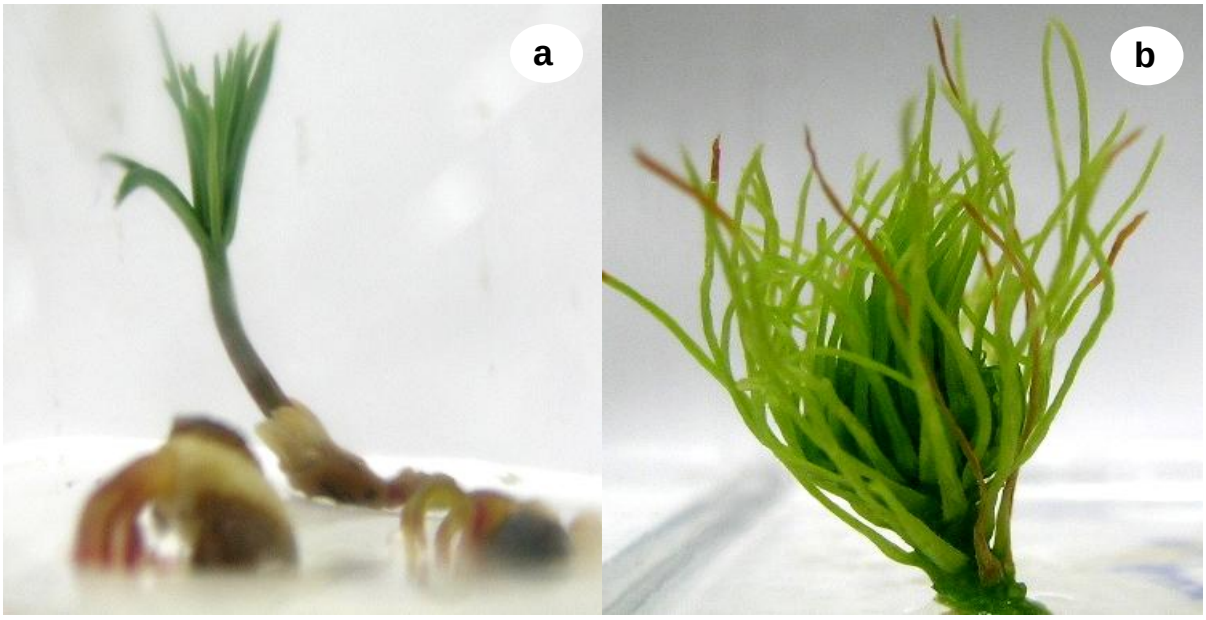

Figure 1. *In vitro* germination of *Pinus brutia.* (a) Dormancy breaking in the scarification treatment in Murashige and Skoog medium supplemented with 1.4 µM giberelic acid, (b) developing seedlings at 8 weeks germination.

PAC + 4.54 µM TDZ and 6.8 µM PAC + 4.54 µM PAC µM). The highest direct organogenic response (23.3 shoots per explant) was obtained using 4.5 µM TDZ (84%) (Figure 2a). Indirect organogenesis from callus cultures was induced on medium containing 3.4 µM TDZ + 9.08 µM PAC. This callus formed (Figure 2b) showed high morphogenetic capacity (Figure 2c), obtaining 36.5 shoots per explant. After 6 weeks, all biomass had diverged in monopolar structures that rapidly developed into shoots (Figure 2d). Two weeks later, elongated shoots showing a well-defined apex were observed (Figure 2e). Shoots (1 to 2 cm height) were transferred to rooting medium (MS) containing 1.70 µM indoleacetic acid (Figure 2f). Nevertheless, large variation in rooting ability was observed, and percent rooting was very low (about 25%).

Direct organogenesis has been reported in *P. wallichiana* AB Jacks (Mathur and Nadgauda, 1999), *P. taeda* L. (Tang and Guo, 2001), *P. heldreichii* (Stojičić and Budimir, 2004), and *P. pinea* (Sul and Sip, 2004). Mathur and Nadgauda (1999) achieved the regeneration of *P. wallichiana* through the application of TDZ (0.025 µM),

Figure 2. *In vitro* morphogenesis of *Pinus brutia. (*a) Direct organogenesis: shoot proliferation with 4.5 µM thidiazuron, (b) callus formation in medium with 3.4 µM paclobutrazol and 9.0 µM thidiazuron, (c) indirect organogenesis: high morphogenetic response of the callus formed, (d) different states during shoot development: elongation of the shoots and shoot apex formation, (e) morphogenic monopolar structures, (f) *In vitro* isolated shoots.

obtaining 5 shoots per explant on average. Indirect organogenesis has been reported in *P. taeda* L. (Tang et al., 1998) and *P. strobes* L. (Tang and Newton, 2005). Tang and Newton (2005) found that at a concentration of 6 µM TDZ, the number of shoots/g callus was 15.7.

Organogenesis in *P. brutia* has already been achieved (Abdullah et al., 1984, 1986, 1987). However, those protocols reported a low number of shoots per explant, and the percentage of fascicles that formed shoot buds was low. Micropropagation of the pine hybrid *P. brutia*

(Ten) × *P. halepensis* (Mill) using fascicle shoots was reported by Scaltsoyiannes et al. (1994), but this system permitted only the development of *in vitro* fascicles.

In the present study, the low response of explants observed with 13.62 µM TDZ concurs with that reported by Preece et al. (1991), who mentioned that at concentrations above 10 µM TDZ may be lethal during morphogenesis, leading to inhibition of callus and shoot growth. TDZ has more recently been incorporated into tissue culture media as a means of inducing regeneration because it acts as a substitute for both the auxin and cytokinin requirements of organogenesis and somatic embryogenesis in a number of plant species (Murthy et al., 1998). It has been proposed that TDZ-induced morphogenesis is related to levels of endogenous growth regulators (Murthy et al., 1995; Hutchinson et al., 1996).

Medium containing 3.4 µM TDZ + 9.08 µM PAC was able to induce callus formation (48%), but in the other treatments, shoot formation failed. According to Huetteman and Preece (1993), high concentrations of TDZ tend to stimulate callus formation in shoot proliferation in many woody species, often at the expense of axillary shoot proliferation. Similarly, it has been reported that, at concentrations higher than 1 µM, TDZ can stimulate the formation of callus, adventitious shoots, or somatic embryos (Murthy et al., 1998). TDZ induced organogenesis at much lower concentrations through a reduced dominance of the apical meristem, resulting in the formation of adventitious and/or axillary buds directly on the cultured shoot tips (Lu, 1993). Perhaps the most effective use of TDZ has been in the regeneration of woody plant species (Huetteman and Preece, 1993).

The synergism between PAC and cytokinins has also been observed in potato tissue culture (Opatrná et al., 1997), sugarcane (Lorenzo et al., 1998), pineapple (Escalona et al., 1999), and lilium (Chen et al., 2005; Thakur et al., 2006). Organogenesis is an important regeneration pathway that facilitates the *in vitro* vegetative propagation of conifers (Tang et al., 1998). Our results show the plasticity of this species to respond to the morphogenesis when treated with only TDZ (4.54 µM), or with TDZ (9.08 µM) combined with PAC (3.4 µM), forming structures both directly and indirectly, that later developed into normal shoots. Both *in vitro* regeneration systems are useful tools for the propagation of *P. brutia*, a species that is highly demanded each year to assemble the Christmas tree. These protocols are quick and easy and for their implementation, and differ in the form of inducing shoot. From the point of view of prospects, these systems will provide new possibilities for genetic improvement of this species.

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