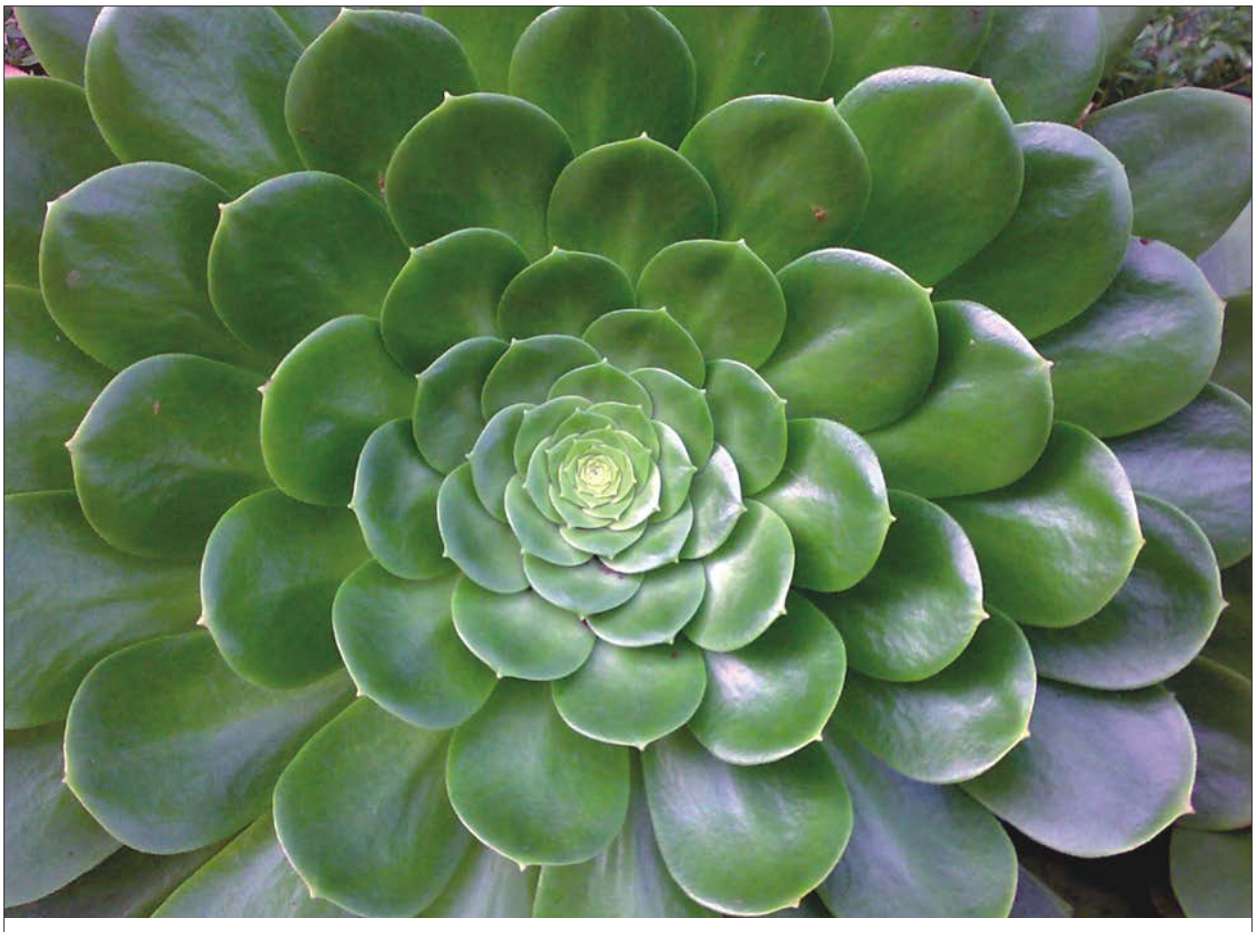

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24-epiBrassinolide Induces Somatic Embryogenesis in *Pinus wallichiana* A. B. Jacks

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Abstract: *Pinus wallichiana* A. B. Jacks (Himalayan blue pine or Bhutan pine) is one of the most recalcitrant species to *in vitro* propagation *via* somatic embryogenesis among all the Indian pines. Somatic embryogenesis is the only method that can enable us to produce large number of somatic seedlings in a short period of time to fulfill the urgent needs of afforestation programmes. Till today, no reports of somatic embryogenesis were available in *Pinus wallichiana*. To be commercially used, somatic embryogenesis technology must work with a variety of genetically diverse trees. This study highlights for the first time the successful brassinolide-mediated stimulation of embryogenesis in all the three genotypes of *Pinus wallichian* tested. 24-epiBrassinolide at 2.0 μM with 9.0 μM 2, 4-D enhanced the formation of embryogenic tissue from mature zygotic embryos on half-strength MSG basal medium. However, the frequency of somatic embryogenesis (PW145-87.4%; PW21-60.8%; PW106-91.5%) was not similar in all the three genotypes tested.

Key words: Brassinosteroids, micropropagation, Himalayan blue pine

INTRODUCTION

Pinus wallichiana AB. Jacks (Himalayan Blue pine or Bhutan pine) is one of the important indigenous pine species of India, Bhutan and Nepal. It is found in the Himalayan valleys from Kashmir to Bhutan and extending up to Arunachal Pradesh in northeastern India. Due to ever-increasing human population, natural forests of blue pine and associated trees of the region are being cleared for cultivation at an alarming rate. Somatic embryogenesis is being used for multiplying elite genotypes, although rooted cuttings are still, operationally, the most effective propagation method available for many tree species (Malabadi *et al*., 2002, 2003). However, low frequency *in vitro* plantlet regeneration of *P. wallichiana* was achieved by organogenesis from mature zygotic embryos (Konar and Singh, 1980; Bastola *et al*., 1991; Mathur and Nadagauda, 1998, 1999). No reports are available on somatic embryogenesis of Himalayan blue pine. Somatic embryogenesis is the only method that can produce large number of plants in a short period, which can fulfill the afforestation programmes for the conservation of superior genotypes for the commercial forestry (Malabadi and Nataraja, 2006a).

Brassinosteroids are a group of naturally occurring steroidal lactones that include brassinolide and its analogs. In several bioassays, they have been reported to affect cell elongation, division and differentiation of plant cells (Mandava, 1988; Sakurai and Fuijoka, 1993, 1997; Clouse *et al*., 1996; Clouse and Sasse, 1998; Fujioka *et al*., 1998; Altmann, 1999; Khripach *et al*., 2000). Others reported the initiation of embryogenic tissue in conifers, cotton, organogenesis in sweet pepper and cauliflower on media using 24-epiBrassinolide (Pullman *et al*., 2003; Wang *et al*., 1992; Franck-Duchenne *et al*., 1998; Sasaki, 2002). In various bioassays, brassinolide has been shown to be more active than, or synergistic with, auxins such as IAA or NAA (Brosa, 1999). Work with Chinese cabbage protoplasts

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has shown that 24-epiBrassinolide promoted cell division in presence of 2, 4-D and kinetin (Nakajima *et al*., 1996). Oh and Clouse (1998) demonstrated that brassinolide increased the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Ronsch *et al*. (1993) reported an improvement in the rooting efficiency and survival of Norway spruce seedlings using 22S, 23S-homobrassinolide. Embryogenic callus induction and growth of coffee, lettuce and potato was improved by the use of spirostane analogues of brassinosteroids in the culture medium as a cytokinin substitute or complement (Lu *et al*., 2003; Oh and Clouse, 1998; Nakajima *et al*., 1996; Nunez *et al*., 2004). Hu *et al*. (2000) suggested that 24-epiBrassinolide may promote cell division through Cyc D3, a D-type plant cyclin gene through which cytokinin activates cell division. They also showed that 24-epiBrassinolide can substitute cytokinin in culturing *Arabidopsis* callus and suspension cells. However, very few reports are available with respect to the effect of brassinosteroids in the micropropagation and tissue culture.

This study focused on the effect of 24-epiBrassinolide on somatic embryogenesis using the mature zygotic embryos of three genotypes of *P. wallichiana* A. B. Jacks. Our results demonstrated that 24-epiBrassinolide induces somatic embryogenesis in Himalayan blue pine and can be used as plant growth regulator. This is the first report of somatic embryogenesis induced by 24-epiBrassinolide in Himalayan blue pine.

MATERIALS AND METHODS

Plant Material

Pinus wallichiana A. B. Jacks seeds were collected from three open pollinated trees (PW145, PW21 and PW106) from the Arunachal Pradesh Forest Department, Itanagar, India. Seeds were washed with 1% (v/v) Citramide for 2 min and rinsed with sterilized distilled water three times. Seeds were further treated with sodium hypochlorite solution (4-5% available chlorine) for 2 min, rinsed 5 times with sterile distilled water and treated with 6% (v/v) hydrogen peroxide for 24 h. Immediately prior to excision of embryos, seeds were decontaminated sequentially with 0.1% (v/v) $HgCl_2$ for 2 min, immersed in 70% (v/v) ethanol for 3 min and finally rinsed thoroughly five times with sterile distilled water (Malabadi *et al*., 2002, 2003, 2005).

Culture Medium and Initiation of Embryogenic Tissue

Mature zygotic embryos (Fig. 1A) were cultured individually on half-strength inorganic salts MSG basal medium (Becwar *et al*., 1990) containing 2.0 g L^{-1} Gellan gum (Sigma), 90 mM maltose (Hi-media, Mumbai), 1.0 g L^{-1} L-glutamine, 1.0 g L^{-1} casein hydrosylate, 0.5 g L^{-1} meso-inositol, 0.2 g L^{-1} p-aminobenzoic acid and 0.1 g L^{-1} folic acid. The 24-epiBrassinolide was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada (www.cidtech-research.com/brass.html). The stock solutions of 24-epiBrassinolide were prepared in absolute ethanol. The medium was supplemented with a range of 24-epiBrassinolide (24-epiBl) concentrations (0.1, 0.5, 1, 2, 5, 10 and 15 μM) and 9.0 μM 2, 4-D. The cultures were initiated in 25×145 mm glass culture tubes (Borosil) with 15 mL medium and maintained in dark for 4-6 weeks at 25±3°C. Nutrient medium without 24-epiBrassinolide was used as a control. The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium was then sterilized by autoclaving at 121°C and 1.08 kg cm^{-2} for 15 min. L-glutamine, p-aminobenzoic acid and 24-epiBrassinolide were filter sterilized and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonal suspensor masses by morphological and cytological observations of callus. The cultures showing white mucilaginous embryogenic tissue were identified and subcultured on the initiation medium (Fig. 1B) for further three weeks for the better development of embryonal suspensor masses. The half-strength (inorganic salts) MSG medium supplemented with 9.0 μM 2, 4-D and 2.0 μM 24-epiBrassinolide was used as an initiation medium for this purpose.

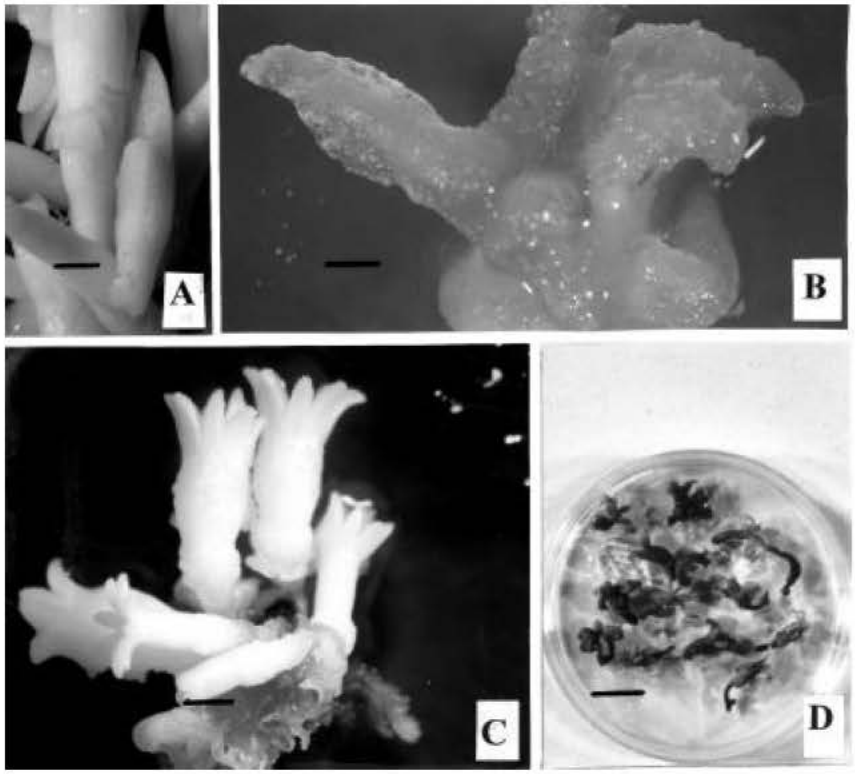

Fig. 1: Effect of 24-epiBr on somatic embryogenesis of *Pinus wallichiana*. A-group of mature zygotic embryos dissected from seed (10 = 8 mm). B-white mucilaginous embryogenic callus on initiation medium (I). (10 = 4 mm). C-development of advanced cotyledonary somatic embryos on maturation medium (10 = 10.97mm). D-somatic seedlings on germination medium

Maintanance of Embryogenic Tissue

The white mucilaginous embryogenic tissue developed on the above initiation medium (I) was subcultured on to maintenance MSG medium (II), containing 130 mM maltose, 4.0 g L^{-1} Gellan gum, 2 μM 2, 4-D and 0.5 μM 24-epiBrassinolide. On the maintenance medium, the embryogenic tissue containing embryonal suspensor masses was maintained for 3 weeks with two subcultures. All the cultures were maintained in dark.

Maturation of Somatic Embryos

After partial desiccation of 24 h (Malabadi and van Staden, 2003, 2005a-d; Malabadi *et al*., 2004; Malabadi and Nataraja, 2006a, b; Malabadi *et al*., 2006), the pieces of embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development (Fig. 1E). The half strength (inorganic salts) MSG medium supplemented with 180 mM Maltose, 60 μM ABA and 8.0 g L^{-1} Gellan gum (maturation medium) was used for this purpose (Malabadi *et al*., 2005). All the cultures were maintained in dark for 12 to 14 weeks.

Germination and Plantlet Recovery

After 12 to 14 weeks of maturation in presence of ABA and higher concentrations of maltose, advanced cotyledonary somatic embryos were picked from the cultures for germination. The

germination medium used was half strength (inorganic salts) MSG medium with 2.0 g L^{-1} Gellan gum. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in a growth room under a 16 h photoperiod (50 μmol m^{-2} s^{-1}) for hardening.

Statistical Analysis

In all the above experiments each culture tube received a single explant. Each replicate contained 50 cultures and one set of experiment was made up of 2 replicates (100 zygotic embryos were cultured for each genotype for one set of experiment). All the experiments were repeated 3 times (total 900 cultures for 3 independent experiments of three genotypes). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using a Duncan's multiple range test. All statistical analysis was performed at the 5% level using the SPSS statistical software package.

RESULTS AND DISCUSSION

In the present study, mature zygotic embryos cultured on half-strength MSG basal medium containing 9.0 μM 2, 4-D without 24-epiBr (control) and 24-epiBr at 5, 10 and 15 μM produced white, glossy non-embryogenic tissue in all three genotypes of *P. wallichiana*. Microscopic observation revealed loosely arranged elongated paranchymatous cells with globular and spherical cells. The cultures failed to produce embryonal suspensor masses and ultimately resulted in the browning of tissue and were discarded. In our preliminary screening of more than other ten genotypes of *P. wallichiana*, also resulted in the sudden browning of explants without callus formation (data not shown). On the other hand, mature zygotic embryos produced white mucilaginous embryogenic tissue on MSG containing 9.0 μM 2, 4-D and 24-epiBr at 0.5, 1.0 and 2.0 μM (Table 1). Mature zygotic embryos produced the highest percentage of embryogenic tissue on half strength MSG medium supplemented with 9.0 μM 2, 4-D and 2.0 μM 24-epiBr (Initiation medium) in all three genotypes tested (Fig. 1B and Table 1). However, the percentage of somatic embryogenesis was not similar in the three genotypes tested (Table 1 and 2). The highest percentage of somatic embryogenesis (91.5±3.0a) was recorded in PW106 genotype. On the other hand the lowest percentage of somatic embryogenesis (60.8±4.0b) was obtained in PW21 genotype. In case of PW145, (87.4±2.3a) of somatic embryogenesis was noticed (Table 1 and 2).

The white mucilaginous embryogenic tissue was subcultured on to maintenance medium for the further development of embryonal suspensor masses. The pro-embryos developed on the maintenance medium could not develop further; until they were transferred on a medium with maltose, ABA and

Table 1: The effect of various concentrations of 24-epiBrassilolide on the initiation of embryogenic cultures in three genotypes of *Pinus wallichiana* cultured on half-strength MSG basal medium containing 9.0 μM 2,4-D

	Initiation frequency (%)		
24-epiBrassinolide (μM)	PW145	PW21	PW106
Control	0	0	0
0.1	0	0	0
0.5	5.0±0.1b	9.0±0.3 b	14.0±1.2b
1.0	29.0±2.1b	31.2±2.8b	20.0±1.6b
2.0	87.4±2.3a	60.8±4.0 a	91.5±3.0a
5.0	0	0	0
10	0	0	0
15	0	0	0

Data scored after 6 weeks and represents the mean±SE of at least 3 different experiments; In each column the values with different letter(s) are significantly different ($p<0.05$); Control = MSG medium without 24-epiBrassinolide

Table 2: Somatic embryogenesis and seedling recovery in three genotypes of *Pinus wallichiana*

Genotype	Somatic embryogenesis (%)	Somatic embryos per gram fresh wt of embryogenic tissue	Seedlings per gram fresh wt of embryogenic tissue
PW145	87.4±2.3a	27.0±2.5b	22.0±2.6b
PW21	60.8±4.0a	22.0±1.8b	14.0±1.1b
PW106	91.5±3.0a	34.6±2.6b	26.0±1.3b

Data scored after 14 weeks and represent the means (±SE) followed by the same letter(s) in each column were not significantly different at $p<0.05$

Gellan gum respectively. The developed somatic embryos on maturation medium after 12 to 14 weeks in all the three genotypes tested (Fig. 1C and Table 2). The total number of somatic embryos recovered per gram fresh weight of embryogenic tissue and somatic seedlings (Table 2). After maturation, the advanced cotyledonary somatic embryos were picked for the germination (Fig. 1D). The half-strength MSG medium without growth regulators was used as a germination medium. After 6 weeks, the somatic seedlings were recovered (Fig. 1D). They were hardened in green house and ready for field transfer.

Brassinosteroids are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses (Gupta *et al*., 2004). In angiosperms species, brassinosteroids have been shown to have several effects, including stimulating cell division, ethylene production and adventitious tissue formation and increasing resistance to abiotic stress (Brosa, 1999). Although, little information is available for conifers, brassinosteroids have been isolated from conifers (Kim *et al*., 1990) and exogenous applications of brassinosteroids to pine seedlings and spruce cuttings have shown improved root growth, whole plant growth, or both (Rajasekaran and Blake, 1998; Ronsch *et al*., 1993). Pullman *et al*. (2003) reported that use of brassinolide at a concentration of 0.1 μM has improved the percentage of embryogenic cultures in loblolly pine, Douglas-fir (*Pseudotsuga menziesii*) and Norway spruce (*Picea abies*). They have also showed that brassinolide increased the weight of loblolly pine embryogenic tissue by 66% and stimulated initiation in the more recalcitrant families of loblolly pine and Douglas-fir, thus compensating somewhat for genotypic differences in initiation (Pullman *et al*., 2003). In the present study 24-epiBl at 0.5 and 1.0 μM yielded very lower percentage of somatic embryogenesis in three genotypes tested (Table 1). Two spirostane analogues of brassinosteroids (BB6 and MH5) were tested for callus induction and plant regeneration in lettuce. Results indicated that both BB6 and MH5 enhanced both callus formation and shoot regeneration from cotyledons in lettuce (Nunez *et al*., 2004). In case of rice seeds, the application of brassinosteroids reduced the impact of salt stress on growth, prevented photosynthesis pigment loss and increased nitrate reductase activity (Anuradha and Rao, 2003). Embryogenic callus induction and growth of coffee and potato was improved by the use of spirostane analogues of brassinosteroids in the culture medium as a cytokinin substitute or complement (Garcia, 2000; More *et al*., 2001). Frank-Duchenne *et al*. (1998) used 0.1 μM 24-epiBrassinolide to enhance the elongation of shoots grown through direct organogenesis on cotyledons and hypocotyls with a regeneration-recalcitrant cultivar of sweet pepper. Sasaki (2002) used brassinolide to increase adventitious shoot production on cauliflower hypocotyls segments. Wang *et al*. (1992) obtained embryogenic cotton cultures from seedling hypocotyls with the aid of 0.02 μM brassinolide. These results indicated ample evidence that brassinosteroids possess a broad spectrum of biological activities compared to the known plant hormones, including gibberellin, auxin and cytokinin- like activities (Brosa, 1999; Yopp *et al*., 1981).

In conclusion, we report here brassinolide-mediated induction of embryogenesis in *P. wallichiana*. Brassinolide promoted embryogenic tissue formation at 2.0 μM. Brassinolide shows much promise for use with conifer compared to other plant species. This is the first report of brassinolide- induced somatic embryogenesis in Himalayan blue pine.

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Abbreviations

ABA-abscisic acid, 24-epiBr-24-epiBrassinolide, MSG-Becwar *et al*., 1990 basal medium

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