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Induction of Somatic Embryogenesis in *Pinus caribaea*

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ABSTRACT

Brassinosteroids are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses including somatic embryogenesis in conifers. This study highlights the successful brassinolide-mediated stimulation of embryogenesis in all the five genotypes of *Pinus caribaea* tested. 24-epiBrassinolide at 2.0 µM with 9.0 µM 2, 4-dichlorophenoxy acetic acid enhanced the formation of embryogenic tissue from mature zygotic embryos on half-strength MSG basal medium. However, the frequency of somatic embryogenesis was not similar in all the five genotypes tested. The highest percentage of somatic embryogenesis (87.0 ± 1.8) was recorded in PC 05 genotype. On the other hand the lowest percentage of somatic embryogenesis (70.0 ± 1.7) was obtained in PC11 genotype. The developed somatic embryos on maturation medium after 12 to 14 weeks in all the five genotypes tested. Therefore, 24-epiBrassinolide can be used as growth regulator in conifer somatic embryogenesis for improving the initiation of embryogenic cultures of recalcitrant pines for the commercial forestry programmes particularly in Indian subcontinent.

Keywords: cell suspension, India, *Pinus caribaea*, somatic embryogenesis, tropical pine
Abbreviations: ABA, (±)-abscisic acid; **BA**, N^6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **24-epiBr**, 24-epiBrassinolide

INTRODUCTION

Conifers are an integral part of human life, and a vital component of biodiversity. Conifers in particular are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. The global demand for wood is not expected to decrease in the near future; in fact, the demand for several wood products is more likely to increase. To lower the pressure on existing forests, mainly rain and conifer forests, a global effort is very much needed to include trees in the modern era of plant breeding. Plantation forestry, with optimized and increased forest productivity, is likely to become the major source for wood products in the near future (Giri *et al*. 2004; Malabadi and Nataraja 2007c; Malabadi *et al.* 2008a, 2008b). Efficient and inexpensive large-scale clonal propagation of superior clones, are key elements for the successful reforestation and management of future commercial forests. To maintain and sustain forest vegetation, conventional approaches have been exploited in the past for propagation and improvement. However, such efforts are confronted with several inherent bottlenecks. Currently, most tree-improvement programs with biotechnological approach are based on the selection of superior clones from existing forests, and used for clonal propagation of the selected clones using tissue culture techniques. Plant tissue culture techniques *viz* somatic embryogenesis exploits the capacity for *in vitro* regeneration (Malabadi *et al.* 2009a, 2009a, 2009c). Somatic embryogenesis can be defined as the formation of an embryo from a cell other than a gamete or the product of gametic fusion (Konar and Nataraja 1965; Nataraja and Konar 1970). This approach appears to have several advantages over other *in vitro* propagation systems, including its potentially high multiplication rates, potential for scale up and delivery *via* bioreactor and synthetic seed technologies, and in fact that embryogenic cultures make suitable target tissue for gene transfer (Malabadi and Nataraja 2003; Malabadi and van Staden 2005b; Malabadi and Nataraja 2006, 2007c, 2007d; Malabadi *et al.* 2008a, 2008b, 2008c, 2008d, 2008e) Typically, conifer somatic embryogenesis proceeds through four steps; initiation, maintenance, maturation, germination, regeneration of plantlets, and cryopreservation of embryogenic tissue could be added as a final step when storage is needed (Malabadi and Nataraja 2006).

Of the coniferous trees, the genus *Pinus* is found to be most important, has the largest distribution, and is found in most diverse climates. *Pinus caribaea* Mor. (Caribbean pine) is one of the tropical pines that grow naturally at low altitudes. Its rapid growth and high-quality wood that is hard and resistant makes it a useful tree for pulp, carton, paper and veneer production. Consequently, it has become one of the most important *Pinaceae* for forestry use in tropical and subtropical areas (David *et al*. 1995). As an exotic species, *P. caribaea* has shown great promise in India for its fast growth and suitability for planting in low altitudes (Nadgauda *et al.* 1993). Somatic enbryogenesis was obtained from cultured immature embryos of *P. caribaea* Morelet var. *hondurensis* on a modified Hakman and von Arnold (1985) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), N^6-benzyladenine (BA) and/or kinetin. Embryogenic calli could be subcultured for more than 10 months and retain their embryogenic ability in *P. caribaea* (Laine and David 1990). Embryos bearing well differentiated cotyledons were recovered after transfer of calli to modified Hakman and von Arnold (1985) medium supplemented with abscisic acid and devoid of organic nitrogen. Rooted plantlets in *P. caribaea* were obtained after transfer to a hormone-free medium. Protoplasts were isolated from embryogenic cells suspensions of *P. caribaea*. Sustained divisions of protoplast-derived cells and regeneration of somatic pro-embryos were achieved using the medium designed for the culture of cotyledon protoplasts of *P. caribaea*, and then dilution with the cell suspension medium. The appearance and nutritional requirements of the established protoplast-derived cell suspension were similar to those of the original cell suspension. Cell aggregates were transferred to solid medium for callus development. Fully differentiated embryos with cotyledons and a hypocotyl

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were obtained 5 months after protoplast isolation in *P. caribaea* (Laine and David 1990). There are no reports of 24-epiBrassinolide (24-epiBr)-induced somatic embryogenesis in *P. caribaea*, and the existing protocols yield a very low percentage of somatic embryos and plantlets too (Laine and David 1990; Nadgauda *et al.* 1993).

Therefore, the aim of this study was to identify the role of 24-epiBr how different genotypes of *P. caribaea* responsive, and regulate the development of somatic embryos. The present investigation reports the role of 24-epiBr as a growth regulator for the induction of somatic embryos and the regeneration of plantlets using mature zygotic embryos as explants from five genotypes of *P. caribaea*.

MATERIALS AND METHODS

Plant material

Pinus caribaea seeds were collected from five trees (PC05, PC45, PC173, PC12, and PC11) from the Western Ghat Forests of Karnataka state, and from Panchgani (Maharashtra state), a town situated in the south western part of India. Seeds were washed with 1% (v/v) Citramide for 2 min, and rinsed with sterilized distilled water (SDW) three times. Seeds were further treated with sodium hypochlorite solution (4-5% available chlorine) for 2 min, rinsed 5 times with SDW and treated with 6% (v/v) hydrogen peroxide for 24 hr. 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 SDW (Malabadi *et al*. 2005a; Malabadi and Nataraja 2007e).

Culture medium and initiation of embryogenic tissue

Mature zygotic embryos were cultured individually on half-strength (inorganic salts) MSG basal medium (Becwar *et al*. 1990) containing 2.0 $g.l^{-1}$ Gellan gum (Sigma, St. Louis, USA), 90 mM maltose (Hi-media, Mumbai), 1.0 $g.l^{-1}$ L-glutamine, 1.0 $g.l^{-1}$ casein hydrosylate, 0.5 $g.l^{-1}$ *myo*-inositol, 0.2 $g.l^{-1}$ *p*-aminobenzoic acid and 0.1 $g.l^{-1}$ folic acid. 24-epiBr was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada (www.cidtech-research.com/brass.html). Stock solutions of 24-epiBr were prepared in absolute ethanol. The medium was supplemented with a range of 24-epiBr 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 mm × 145 mm glass culture tubes (Borosil) with 15 ml medium and maintained in the dark for 4-6 weeks at 25 ± 3°C. Media without 24-epiBr served as the control. The pH of the media was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. L-glutamine, *p*-aminobenzoic, and 24-epiBr were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm), 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 for a further 3 weeks for the improved development of embryonal suspensor masses. Half-strength (inorganic salts) MSG medium supplemented with 9.0 µM 2, 4-D and 2.0 µM 24-epiBr was used as an initiation medium for this purpose.

Maintenance 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-epiBr. On the maintenance medium, the embryogenic tissue containing embryonal suspensor masses was maintained for 3 weeks with two subcultures. All cultures were maintained in the dark.

Maturation of somatic embryos

For maturation, embryogenic tissue clumps of each of the 5 genotypes were incubated in the dark at room temperature (28 ± 2°C). The percentage somatic embryogenesis was calculated as responsive callus-based embryogenesis (expressed in terms of number of responsive regrowth of callus pieces/100 since a total of 100 pieces of calluses were subcultured). Therefore, 5 g of embryogenic tissue of each genotype was aseptically removed and chopped into 100 pieces under aseptic conditions by using a normal scalpel and blade and subcultured on maintenance medium for the growth of callus. The number of pieces re-callusing from 100 sub-cultured embryogenic tissues indicates the percentage somatic embryogenesis. This was estimated (i.e. the total number of somatic embryos, germinated embryos, and somatic seedlings produced/g fresh weight (FW) of tissue was calculated) before transferring the embryogenic tissue onto maturation medium. FW (1 g) of embryogenic tissue of each genotype was transferred to empty sterile Petri dishes (60 mm diameter) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 h to obtain the desired extent of desiccation. After desiccation, the partially desiccated embryogenic tissues (1 g-pieces ×5/Petri dish) of each genotype were transferred to maturation medium to induce cotyledonary embryo development. The half-strength (inorganic salts) MSG medium supplemented with 180 mM maltose, 120 µM abscisic acid (ABA; Sigma, ACS grade) and 10.0 $g.l^{-1}$ Gellan gum (maturation medium) was used for this purpose. All cultures were placed in the dark at 25 ± 2°C and maintained for 8-12 weeks (Malabadi and Nataraja 2005a, 2007e).

Germination and plantlet recovery

After 12 weeks of maturation in the presence of ABA and a higher concentration of maltose (60 g l^{-1} maltose), the cotyledonary somatic embryos were recovered from the cultures for germination. Before germination, cotyledonary somatic embryos of all 5 genotypes were cold pre-treated at 2°C and kept in the dark for 25 days. The germination medium consisted of half-strength MSG medium with 2 g l^{-1} Gellan gum (Malabadi and Nataraja 2005a, 2007e). In the first week of germination, cultures were kept in the dark then transferred to diffuse light (30 µmol m^{-2} s^{-1}) in the second week, and thereafter to a 16-hr photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for hardening. Somatic embryos were considered germinated as soon as radicals elongated while conversion to plantlets was based on the presence of epicotyls. After 4-6 weeks on germination medium, plantlets were transferred to vermiculite in a controlled growth room.

Statistical analysis

In above experiments, each replicate contained 50 cultures and one set of experiments consisted of two replicates (i.e. total of 100 cultures per experiment) for each genotype. All the experiments were repeated in triplicate. Data in **Tables 1** and **2** represent the average of three independent experiments. Data was arcsine transformed before being analyzed for significance using ANOVA ($p<0.05$) or evaluated for independence using the Chi-square test. Further, the differences in means were contrasted using Duncan's multiple range test (DMRT) (a=0.05) following ANOVA. All statistical analyses were performed using SPSS (Microsoft Windows ver. 13.0.1.1) 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 0.1 10 and 15 µM produced white, glossy non-embryogenic tissue in all five (PC05, PC45, PC173, PC12, and PC11) *P. caribaea* genotypes. The cultures failed to produce embryonal suspensor masses (ESMs) and ultimately resulted in the browning of tissue and were discarded. On the other hand, mature zygotic embryos produced white mucilaginous embryogenic tissue on MSG containing 9.0 µM 2,4-D and 24-

Table 1 The effect of various concentrations of 24-epibrassilolide on the initiation of embryogenic cultures in five genotypes of *Pinus caribaea* cultured on half-strength MSG basal medium containing 9.0 µM 2,4-D.

24-epibrassinolide (µM)	Embryogenic tissue initiation frequency (%)				
	PC05	PC173	PC45	PC12	PC11
Control	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.1	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.5	4.0 ± 0.1 b	0.0 ± 0.0 c	0.0 ± 0.0 c	8.0 ± 0.3 b	2.0 ± 0.1 b
1.0	11.0 ± 0.5 b	2.0 ± 0.1 b	10.0 ± 0.3 b	21.0 ± 0.7 b	11.0 ± 0.2 b
2.0	87.0 ± 2.1 a	57.0 ± 1.3 a	60.0 ± 1.8 a	80.0 ± 3.1 a	40.0 ± 2.6 a
5.0	3.0 ± 0.1 b	6.0 ± 0.2 b	2.0 ± 0.1 b	7.0 ± 0.3 b	2.0 ± 0.1 b
10	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
15	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

Data scored after 6 weeks and represents the mean ±SE of at least 3 different experiments. In each column the values with different letters are significantly different (P<0. 5) according to DMRT (Duncan's multiple range test).
Control = MSG medium without 24-epibrassinolide

Table 2 Somatic embryogenesis and seedling recovery in five genotypes of *Pinus caribaea*.

Genotype	Somatic embryogenesis* (%)	Somatic embryos per g fresh wt of embryogenic tissue	Seedlings per g fresh wt of embryogenic tissue
PC05	87.0 ± 1.8 a	30.0 ± 1.0 a	21.0 ± 0.9 a
PC173	75.0 ± 2.0 a	58.0 ± 3.0 a	45.0 ± 1.8 a
PC45	61.0 ± 1.9 a	40.0 ± 1.4 a	19.0 ± 1.2 a
PC12	83.1 ± 1.4 a	32.0 ± 1.6 a	11.0 ± 0.8 a
PC11	70.0 ± 1.7 a	47.0 ± 2.0 a	33.0 ± 2.6 a

Data scored after 14 weeks and represent the means (±SE) of at least 3 different experiments. Each values followed by the same letter in each column were not significantly different at P<0.5 according to DMRT (Duncan's multiple range test).
*(% of somatic embryogenesis) = 5 g of embryogenic tissue of each genotype was taken aseptically and chopped into 100 pieces and subcultured on the maintenance medium for the growth of callus. Out of 100 pieces, growth of the number of pieces was recorded and calculated in terms of percentage of SE).

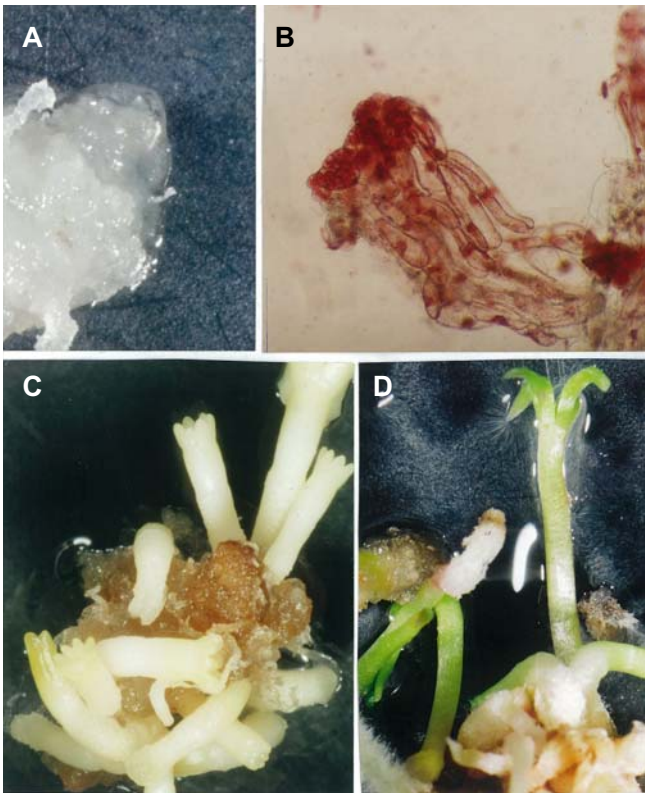

Fig. 1 Effect of 24-epiBr on somatic embryogenesis of *Pinus caribaea*. (**A**) White mucilaginous embryogenic callus on initiation medium (I) (10 mm = 4 mm). (**B**) Aceto carmine squash preparation of embryogenic tissue showing a well developed embryonal suspensor masses on maintenance medium (10 mm = 8 mm). (**C**) Development of advanced cotyledonary somatic embryos on maturation medium (10 mm = 10.97 mm). (**D**) Somatic embryos showing a positive sign of growth on germination medium (10 mm = 9.5 mm).

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 five (PC05, PC45, PC173, PC12, and PC11) genotypes tested (**Fig. 1A; Table 1**). However, the percentage of somatic embryogenesis was not similar in these genotypes (**Tables 1, 2**). Therefore, the potential for somatic embryogenesis is first of all determined at the level of the genotype. The highest percentage of somatic embryogenesis (87.0 ± 1.8) was recorded in PC 05. On the other hand, the lowest percentage of somatic embryogenesis (61.0 ± 1.9) was obtained in PC45. In PC11, 70.0 ± 1.7 somatic embryos was noted whereas PC12 and PC173 recorded 83.1 ± 1.4 and 75.0 ± 2.0, respectively (**Table 2**).

The white mucilaginous embryogenic tissue was subcultured onto maintenance medium for the further development of ESMs (**Fig. 1B**). The pro-embryos that developed on the maintenance medium could not develop further until they were transferred onto medium with maltose, ABA and Gellan gum. The somatic embryos developed on maturation medium after 12-14 weeks in all 5 genotypes tested (**Fig. 1C; Table 2**). The total number of somatic embryos recovered/g FW of embryogenic tissue and somatic seedlings is listed in **Table 2**. After maturation, the advanced cotyledonary somatic embryos were selected for germination (**Fig. 1D**). Half-strength MSG medium without plant growth regulators was used as a germination medium. After 6 weeks, somatic seedlings were recovered (**Fig. 1D**). They were hardened in the greenhouse and ready for field transfer. On the basis of this study we confirmed that embryogenic competence is expressed finally at the level of single cells. Embryogenically competent cells are those which are capable of differentiating into embryos if they receive differentiation inducers (Malabadi *et al*. 2009a). The signals inducing competence and triggering embryogenic development are not easy to separate. In spite of the continuously increasing group of conifer species where the conditions for somatic embryo induction have been established, there are a number of species still recalcitrant to form somatic embryos. Highly embryogenic and recalcitrant genotypes exist even within a given species. It has been emphasized, however, that in many instances, recalcitrance could be resolved by optimizing growth conditions of plants or by proper explant selection (Malabadi and Nataraja 2003; Malabadi and van Staden 2005b; Malabadi and Nataraja 2006, 2007c, 2007d; Aronen *et al*. 2008; Malabadi *et al.* 2008a, 2008b, 2008c, 2008d, 2008e, 2009a, 2009c; Park *et al.* 2009).

24-epiBr and other brassinolides are ubiquitous in plants and elicit a wide spectrum of physiological responses (Grove *et al*. 1979; Yopp *et al.* 1981; Mandava 1988; Sakurai and Fujioka 1993; Mayumi and Shibaoka 1995; Sasse

1997; Sakurai and Fujioka 1997; Fujioka *et al*. 1998; Altman 1999; Dhaubhadel *et al.* 1999; Fujioka 1999; Gupta *et al*. 2004; Malabadi and Nataraja 2007a, 2007b; Malabadi *et al*. 2008f, 2009b). In angiosperms, BRs have been shown to have several effects, including stimulating cell division, ethylene production, and adventitious tissue formation and increasing resistance to abiotic stress (Cluse *et al.* 1996; Clouse and Sasse 1998; Franck-Duchenne *et al.* 1998; Brosa 1999; Khripach *et al.* 2000). Mandava (1988) reported that brassinolide, a plant steroid lactone and the most active brassinosteroid (BR), and its analogues enhanced maturation and increased crop yield of several vegetables, including pepper. Since then, brassinolide has been regarded as a new plant growth regulator which is essential for normal plant growth and development (Franck-Duchenne *et al*. 1998).

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 (Ronsch *et al*. 1993; Rajasekaran and Blake 1998). Pullman *et al*. (2003) reported that the use of brassinolide at 0.1 µM 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). Embryogenic callus induction and growth of coffee and potato was improved by the use of spirostane analogues of BRs in the culture medium as a cytokinin substitute or complement (Garcia 2000; More *et al.* 2001). Two spirostane analogues of BRs (BB6 and MH5) were tested for callus induction and plant regeneration in lettuce. Both BB6 and MH5 enhanced callus formation and shoot regeneration from lettuce cotyledons (Nunez *et al*. 2004). 24-epiBr 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 in *Pinus wallichiana* (Malabadi and Nataraja 2007a). Embryogenic callus induction and growth of coffee, lettuce and potato was improved by the use of spirostane analogues of BRs in the culture medium as a cytokinin substitute or complement (Nakajima *et al*. 1996; Oh and Clouse 1998; Lu *et al*. 2003; Nunez *et al*. 2004). Successful initiation of embryogenic tissue in cotton (*Gossypium hirsutum*), organogenesis in sweet pepper (*Capsicum annuum* L. cvs. 'Jupiter' and 'Pimiento Perfection') and cauliflower (*Brassica oleracea* var. *botrytis* L.) was established using 24-epiBr (Wang *et al*. 1992; Franck-Duchenne *et al*. 1998; Sasaki 2002). When hypocotyl segments of cauliflower (*Brassica oleracea* var. *botrytis* L.) were cultured on MS medium containing 0.1 or 1 µM 24-epiBr in the light, a significant stimulation of adventitious shoot regeneration was observed (Sasaki 2002). Cytokinins (zeatin and iso-pentenylaminopurine) also promoted shoot regeneration in *B. oleracea* (Sasaki 2002). When 0.1 or 1 µM 24-epiBr was added together with these cytokinins, maximum regeneration was further improved (Sasaki 2002). Regeneration was much lower in the dark because of increased ethylene synthesis in the dark (Sasaki 2002). It was also noticed that when hypocotyl segments of cauliflower were cultured in the light on MS medium containing 24-epiBr at various concentrations, 0.1-10 µM 24-epiBr significantly promoted adventitious bud formation (Sasaki 2002). The highest percentage of regeneration occurred at 0.1 or 1 µM 24-epiBr in which 44% of explants formed buds. A maximum number of shoot buds per regenerating explant was achieved at 1 µM 24-epiBr. Sasaki (2002) also mentioned that zeatin also stimulated bud formation in Cauliflower. But when 24-epiBr was added with zeatin, regeneration was improved (91.7 vs. 42.3%) (Sasaki 2002). The interaction between cytokinin and BR suggests that BR makes more cells competent to respond to the organogenic signal of the cytokinin and that these cells became more sensitive to cytokinin (i.e., they required less cytokinins to achieve a response (Sasaki 2002).

Successful initiation of PLBs and *in vitro* regeneration was achieved using shoot tip (harvested from mother plants grown under greenhouse conditions) sections of *C. elegans* and 24-epiBr-supplemented Mitra *et al*. (1976) basal medium (Malabadi and Nataraja 2007b). The highest percentage of explants (91.0%) producing PLBs (24.0 ± 2.1) was recorded on 4.0 µM 24-epiBr and these PLBs or proliferating shoot buds formed the maximum number of healthy shoots (17.0 ± 1.23). Lower (0.5-1.0 µM) or higher (6.0-20.0 µM) concentrations of 24-epiBr resulted in the browning of explants and failed to produce PLBs. Initiation of PLBs or proliferation of shoot buds decreased with an increase in the concentration of 24-epiBr from 3.0 to 5.0 µM. All the newly formed PLBs survived and after nearly 12 weeks, small bud-like structures formed healthy shoots, which rooted when cultured on basal medium supplemented with 2.0 µM TRIA (Malabadi and Nataraja 2007b).

Using orchids as another model group of plants, the rapid clonal propagation of *Cymbidium bicolor* was achieved by induction of protocorm-like bodies (PLBs) using shoot tip thin cell layers (TCLs) when cultured on 24-epiBr-supplemented Mitra *et al*. (1976) basal medium. The highest percentage of explants (86.0%) producing PLBs (65.0 ± 3.9) was recorded when 3.0 µM 24-epiBr was used. All the newly formed PLBs survived and after nearly 12 weeks, small bud-like structures formed healthy shoots (Malabadi *et al*. 2008f). Recently in an another report, *Liparis elliptica* (Rees) Lindl., using transverse TCLs was established using 24-epiBr (Malabadi *et al.* 2009b). The initiation of PLBs and the regeneration of shoot buds from PLB TCLs relied significantly on the concentration of 24-epiBr-supplemented Mitra *et al*. basal medium. The highest percentage of PLB-TCL explants (93.0%) producing PLBs (71.0 ± 2.1) was recorded with 4.0 µM 24-epiBr in a period of 12 weeks.

In *Oryza sativa*, an increase in the soluble protein content was noticed following 3 µM 24-epiBr application and considerably alleviated oxidative damage that occurred under NaCl-stressed conditions and improved seedling growth in part under salt stress in sensitive 'IR-28 seedlings' (Ozdemir *et al*. 2004). Seedling growth of rice plants was improved by 3 µM 24-epiBr treatment under salt stress conditions. When seedlings treated with 3 µM 24-epiBr were subjected to 120 mM NaCl stress, the activities of superoxide dismutase, catalase and glutathione reductase did not show significant difference, whereas the activity of ascorbate peroxidase significantly increased (Ozdemir *et al*. 2004). *In vitro* regeneration of sweet pepper (*Capsicum annuum* L. cvs. 'Jupiter' and 'Pimiento Perfection') was performed via direct organogenesis (Franck-Duchenne *et al*. 1998). The resulting shoot-buds of these two cultivars were placed on media containing 0.1 µM 24-epiBr in the presence or absence of 9.1 µM zeatin plus 5.2 µM gibberellic acid for further stem elongation. Different responses to these treatments were recorded depending upon the protocols used and the genotypes tested. It appears that 24-epiBr does not always act directly on stem elongation but may be an elicitor and/or an enhancer of elongation in concert with endogenous and other exogenously added PGRs in sweet pepper. In various bioassays, 24-epiBr has been shown to be more active than, or synergistic with, auxins such as IAA or NAA (Brosa 1999). Oh and Clouse (1998) demonstrated that brassinolide increased the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Hu *et al*. (2000) suggested that 24-epiBr may promote cell division through Cyc D3, a D-type plant cyclin gene through which cytokinin activates cell division. In the same study, they also showed that 24-epiBr can substitute cytokinin in culturing *Arabidopsis* callus and suspension cells. Work with Chinese

cabbage protoplasts showed that 24-epiBr promoted cell division in the presence of 2,4-D and kinetin (Nakajima *et al*. 1996). However, very few reports are available with respect to the effect of brassinolide in micropropagation and tissue culture.

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