Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
an open access publisher
http://ansinet.com

Asian Journal of Plant Sciences 6 (2): 308-313, 2007
ISSN 1682-3974

Brassinosteroids Influences *in vitro* Regeneration Using Shoot Tip Sections of *Cymbidium elegans* Lindl

Ravindra B. Malabadi and K. Nataraja
Department of Botany, Division of Plant Biotechnology, Karnatak University,
Pavate Nagar, Dharwad-580003, Karnataka State, India

Abstract: This investigation highlights for the first time the application of 24-epibrassinolide in the micropropagation of orchids. Successful initiation of protocorm-like bodies and *in vitro* regeneration of *Cymbidium elegans* was achieved using shoot tip sections and 24-epibrassinolide supplemented Mitra *et al.* (1976) basal medium. The highest percentage of explants (91.0%) producing PLBs (24.0±2.1) was recorded on 4.0 μM 24-epibrassinolide supplemented basal medium. All the newly formed protocorm-like-bodies survived and after nearly 12 weeks, small bud-like structures formed healthy shoots. Shoots produced roots when cultured on basal medium supplemented with 2.0 μM triacontanol. The well-rooted shoots were transferred to pots containing charcoal chips, coconut husk and broken tiles (2:2:1) and 100% survival rate was achieved. Our results for the first time demonstrate that 24-epibrassinolide provided compelling evidence that they can be effectively used in the micropropagation of orchids.

Key words: Brassinosteroids, epiphytic orchid, micropropagation, triacontanol

INTRODUCTION

Brassinosteroids are widely distributed in plants and form a unique class of endogenous plant growth regulators with pleiotropic effects, which play an essential role in growth and developmental processes (Sasse, 1997; Clouse and Sasse, 1998). Since the first discovery of brassinosteroids from rape (*Brassica napus* L.) pollen by Grove *et al.* (1979) as a plant growth regulator with remarkable biological activity, 40 BRs and 4 BR conjugates have been characterized from 44 plant species including 37 angiosperms (9 monocots and 28 dicots), 5 gymnosperms, 1 algae and 1 pteridophyte (Fujioka, 1999). In several bioassays, they have been reported to affect cell elongation, division and differentiation of plant cells and splitting of internode (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). BRs have also been shown to enhance trachery element differentiation, act synergistically with cytokinins in the growth of cell cultures, stimulate hyperpolarisation, promote ethylene biosynthesis, control microtubule orientation, alter mechanical properties of cell walls and modulate environment stress signals as well as other developmental processes (Mayumi and Shibaoka, 1995; Dhaubhadel *et al.*, 1999).

Some workers have demonstrated their role in plant cell cultures. 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). Successful initiation of embryogenic tissue in conifers and cotton, organogenesis in sweet pepper and cauliflower was established using 24-epibrassinolide (Pullman *et al.*, 2003; Wang *et al.*, 1992; Franck-Duchenne *et al.*, 1998; Sasaki, 2002). Ronsch *et al.* (1993) reported an improvement in the rooting efficiency and survival of Norway spruce seedlings using 22S, 23S-homobrassinolide. In various bioassays, brassinolide 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-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. Work with Chinese cabbage protoplasts has shown that 24-epibrassinolide promoted cell division in presence of 2, 4-

Corresponding Author: Ravindra B. Malabadi, Division of Plant Biotechnology, Finnish Forest Research Institute (METLA), Punkaharju Research Unit, Finlandiantie 18, FIN-58450, Punkaharju, Finland

D and kinetin (Nakajima *et al*., 1996). However, very few reports are available with respect to the effect of brassinosteroids in the micropropagation and tissue culture.

Cymbidium elegans Lindl, an endangered orchid species is of grate horticultural value. This is an unusual species in which inflorescences carries a dense bunch of beautiful funnel-shaped buttercup yellow blossoms. It grows epiphytically and litophytically at elevations of 1500-2500 m (Kumar *et al*., 1994). Many attractive hybrids of Cymbidium orchid have become commercially important in cut flower and potted plant industries. Cymbidium was the first orchid to be propagated using shoot-tip culture (Wimber, 1963; Morel, 1964). Indiscriminate collection and severe habitat loss are the two potent factors responsible for its depletion in India (Kataki, 1977). Its large-scale multiplication in botanic gardens to meet trade demand has been recommended. Tissue culture techniques have been widely used for the *in vitro* mass multiplication of several commercially important orchids (Rao, 1977; Lakshmanan *et al*., 1995; Ichihashi, 1998; Kanjilal *et al*., 1999; Malabadi *et al*., 2004, 2005). *In vitro* regeneration of *Cymbidiums* has been reported by Wimber (1963), Morel (1964), Sagawa *et al*. (1966), Ueda and Torikata (1968), Steward and Mapes (1971), Wang (1988), Begum *et al*. (1994), Ichihashi (1997), Chang and Chang (1998), Phukan and Mao (1999), Nayak *et al*. (1997, 2002) and Huan *et al*. (2004). *In vitro* regeneration of *Cymbidium elegans* was achieved using axenic seeds germination (Sharma *et al*., 1991; Sharma and Tandon, 1990). In this study we report for the first time an efficient multiplication method for *Cymbidium elegans* through shoot tip thin section culture by using 24-epibrassinolide (24-epiBL) and higher percentage of rooting by the use of triacontanol (TRIA). Our results for the first time demonstrate that 24-epibrassinolide provided compelling evidence that they can be effectively used in the micropropagation of orchids.

MATERIALS AND METHODS

Twenty-five plants of *Cymbidium elegans* (Lindl.) collected from the Khasi and Jaintia Hills, Shillong, Meghalaya state, India were established in pots and grown under greenhouse conditions at Department of Botany, Karnatak University, Dharwad, India. Shoot tips (0.5-0.8 cm) harvested from mother plants were carefully washed in distilled water. They were surface decontaminated sequentially with 0.1% streptomycin (20 sec), 70% (v/v) ethanol (50 sec) and 0.1% (w/v) $HgCl_2$ (2 min) and thoroughly rinsed with sterilized double distilled water. Transverse- thin sections of 1-5 mm thick were cut from shoot tips and these sections were cultured on Mitra *et al*. (1976) basal medium with 3.0% sucrose (Hi media, Mumbai), 0.7% agar (Sigma), 0.5 g L^{-1} meso inositol, 1.0 g L^{-1} casein hydrosylate, 0.5 g L^{-1} L-glutamine, 250 mg L^{-1} peptone, 0.2 g L^{-1} p-aminobenzoic acid and 0.1 g L^{-1} biotin. The 24-epibrassinolide was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada. The medium was supplemented with a range of 24-epibrassinolide (24-epiBL) concentrations (0.5, 1, 2, 3, 4, 5, 6, 7 10, 15 and 20 μM) without any other growth hormones in 25×145 mm glass culture tubes (Borosil) containing 15 mL of the medium under cool white fluorescent light (100 μmol m^{-2} s^{-1}) at 25±3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with NaOH or HCl before agar was added. Media without 24-epibrassinolide is served as control. The media were then sterilized by autoclaving at 121°C at 1.04 kg cm^{-2} for 15 min. L-glutamine, biotin, p-aminobenzoic acid and 24-epibrassinolide (24-epiBL) were filter sterilized and added to the media after autoclaving when the medium had cooled to below 50°C.

The cultures were maintained for 6-10 weeks for the initiation of PLB's or proliferating shoot buds. The freshly initiated PLB's transferred to Mitra *et al*. (1976) basal medium containing 4.0 μM 24-epiBL. Healthy shoots with 2-3 leaves developed within 10-12 weeks. They were subcultured on the same medium for another 2 weeks for further shoot development. All experiments contained only 25 cultures for one replicate. Four replicates (100 cultures) were maintained for one set of experiment and experiments were repeated three times (100×3 = 300). 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 analyses were performed at the 5% level using the SPSS statistical software package.

The well-developed shoots were further transferred on fresh Mitra *et al*. (1976) basal medium supplemented with various concentrations of auxins (IAA, NAA, IBA) for rooting. All the shoot buds failed to produce rooting with IAA and IBA. Furthermore, poor rooting was observed with NAA. Therefore, the other growth regulators such as triacontanol (TRIA) were studied for the first time for the rooting efficiency in *C. elegans*. The well-developed shoot buds were transferred on fresh basal medium of Mitra *et al*. (1976) supplemented with various concentrations of TRIA (1, 2, 3, 4 and 5 μM) (Table 2). The shoots with well developed roots on 4.0 μM TRIA supplemented basal medium were washed thoroughly under running tap water and transplanted into 15 cm diameter pots in a potting mixture of charcoal chips, coconut husks and broken tiles (2:2:1). Three to four

plants were planted in each pot and the plants were watered daily and fertilized at weekly intervals with a foliar spray of a mixture of commercial DAP (Di Ammonium phosphate) and NPK (Nitrogen 20: phosphorous 10: Potassium 10) (Malabadi *et al*., 2004, 2005). Five months later, the rooted plants were individually transferred to 15 cm pots.

RESULTS AND DISCUSSION

This study demonstrated that 24-epiBL has a high potential to induce proliferating shoot buds or PLBs with callusing from thin shoot tip sections of *Cymbidium elegans*. The highest percentage of explants (91.0%) producing PLBs (24.0±2.1) was recorded on 4.0 μM 24-epiBL in a period of 14 weeks (Table 1) and (Fig. 1A). 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 concentrations (6.0-20.0 μM) of 24-epiBL resulted in the browning of explants and failed to produce PLBs (Table 1). Initiation of PLBs or proliferating shoot buds increases/decreases with increase in the concentrations of 24-epiBL from 2.0-5.0 μM (Table 1). However, the percentage of PLBs decreasing with increase in the concentrations of 24-epiBL from 4.0-5.0 μM. Explants cultured on basal medium (Mitra *et al*., 1976) supplemented with lower concentrations of 24-epiBL (3.0-4.0 μM) showed luxuriant growth of PLBs or proliferating shoot buds. Hence the effective range of 24-epiBL for the initiation of PLBs in *C. elegans* is 3.0-4.0 μM (Table 1). The thin shoot tip sections remained green and developed small bud-like structures when cultured on 4.0 μM of 24-epiBL supplemented basal medium of Mitra *et al*. (1976) within 9 weeks. They were further subcultured on the same medium and maintained for another 10-12 weeks. After nearly 12 weeks, small bud-like structures formed healthy shoots (Fig. 1A, B and C).

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). 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). Brassinosteroids are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses (Gupta *et al*., 2004). In this study, we have tested the influence of 24-epiBL alone without any combination with other plant growth regulator to test its role on *in vitro* regeneration of plantlets in orchids. Till today no other reports are available on the effect of brassinosteroids on *in vitro* regeneration of orchids. Embryogenic tissue were induced from longitudinally bisected segments of protocorm-like bodies of *Cymbidium* Twilight Moon Day Light, a hybrid orchid on

Table 1: Effect of various concentrations of 24-epiBL on the initiation of PLBs or proliferating shoot buds in *Cymbidium elegans*

24-epiBL (μM)	Responsive explants (%)	Total No. of PLBs or shoot buds per explant	Total No. of shoots per explant
Control	0	0	0
0.5	0	0	0
1.0	0	0	0
2.0	4.0±0.2c	1.0±0.0c	0
3.0	18.0±0.5b	10.0±0.9c	6.0±0.36c
4.0	91.0±4.7a	24.0±2.1b	17.0±1.23b
5.0	8.0±0.4c	3.0±0.1c	1.0±0.0c
6.0-20.0	0	0	0

Data scored after 14 weeks and represent the mean±SE of at least three different experiments. In each column, the values with different letter(s) are significantly different ($p<0.05$)

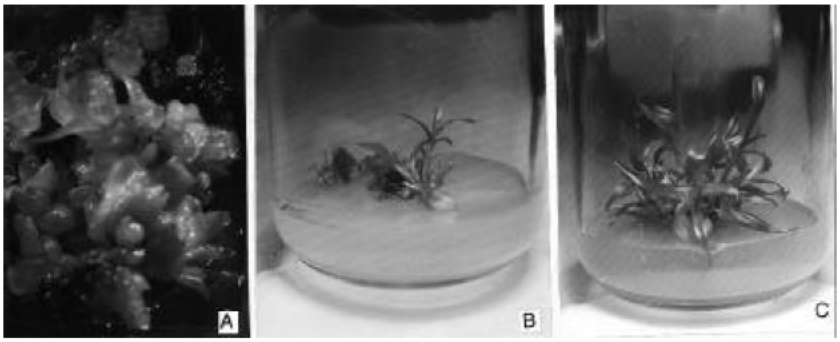

Fig. 1: *In vitro* multiplication of *C. elegans* using 24-epiBr. (A) Initiation of PLBs from thin sections of shoot tip on the basal medium of Mitra *et al*. (1976) supplemented with 4.0 μM 24-epibrassinolide. (B) Formation of healthy shoot from PLBs. (C) Healthy shoots with well-developed leaves ready for rooting

modified Vacin and Went medium (1949) supplemented with 2-4D in combination with 0.01 mg L^{-1} TDZ (Huan *et al*., 2004). Whereas, *in vitro* regeneration of *Cymbidium elegans* was achieved using 24-epiBL and survival rate of seedlings was 100%. The other advantages of using 24-epiBL were seedlings are very healthy and more resistant than rest of the regeneration protocols at least in *Cymbidium elegans*. In case of *Oryza sativa*, an increase in the soluble protein content was noticed by 24-epiBL 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). Our results are in conformity with the literature.

The shoots regenerated on 4.0 μM 24-epiBL supplemented basal medium were tested for rooting efficiency with different concentrations of auxins such as IAA, IBA and NAA. Shoots failed to produce roots at all the different concentrations of auxins (IAA, IBA). There was a poor rooting with different concentrations of NAA (data not shown) since this paper presents only optimum results. Therefore, the other growth regulators such as triacontanol (TRIA) were studied for the first time for the rooting efficiency in *C. elegans*. The highest percentage of rooting efficiency (95.0±6.0) was recorded at 2.0 μM of TRIA within a time period of 4 weeks (Table 2). On the other hand the lowest percentage of rooting (4.0±0.65) was observed at 4.0 μM of TRIA. Shoots failed to produce roots at higher concentrations (5.0 to 10.0 μM) of TRIA (Table 2). A sudden decrease in the rooting efficiency of shoots was noticed when the concentrations of TRIA was increased from 2.0 to 4.0 μM (Table 2). This also confirmed our previous results of higher rooting efficiency of an endangered orchid, *Dendrobium nobile* at a concentration of 2.0 μg L^{-1} TRIA supplemented with Mitra *et al.* (1976) basal medium (Malabadi *et al*., 2005). According to Tantos *et al.* (1999), successful rooting was observed at 2.0 μg L^{-1} TRIA in *M. officinalis*. Similarly Fraternale *et al.* (2003) reported that 2.0-5.0 μg L^{-1} TRIA was optimum for efficient rooting in *T. mastichina*. Lower concentrations of TRIA may be biologically effective because of the sensitivity of whole explants to extremely low doses of TRIA (Biernbaum *et al*., 1998; Malabadi *et al*., 2005). The well rooted shoots were washed thoroughly under running tap water and transplanted to 15 cm community pots in a potting mixture of charcoal chips, coconut husk and broken tiles (2:2:1) for hardening.

Table 2: Effect of different concentrations of TRIA on rooting of shoots regenerated at 4.0 μM 24-epiBL supplemented Mitra *et al.* (1976) basal medium

TRIA (μM)	Responsive explants (%)	Rooting (%)
Control	0	0
1	12.0±1.0c	32.0±2.9b
2	89.0±4.0a	95.0±7.3a
3	16.0±1.3c	19.0±1.3c
4	3.0±0.01d	4.0±0.65d
5-10	1.0±0.0d	0

Data scored after 4 weeks and represent the mean±SE of at least three different experiments. In each column, the values with different letter(s) are significantly different ($p<0.05$)

In conclusion this study reports for the first time the optimized parameters for the successful initiation of PLBs and regeneration of plantlets by using 24-epiBL from shoot tip sections and efficient rooting by the incorporation of TRIA in the basal medium in *Cymbidium elegans*. This protocol is very simple, significant for the large-scale propagation to meet commercial demands in a short period and conservation of this valuable orchid species.

ACKNOWLEDGMENT

We are grateful to the Head, Department of Botany, Karnatak University, Dharwad, India for providing necessary facilities for this work. RBM also wishes to thank lab mates for every help during this investigation. KSCST is warmly acknowledged for the financial support for this project.

REFERENCES

Altmann, T., 1999. Molecular physiology of brassinosteroids revealed by the analysis of mutants. Planta., 208: 1-8.

Anuradha, S. and S.R. Rao, 2003. Application of brassinosteroids to rice seeds (*Oryza sativa* L.) reduced the impact of salt stress on growth, prevented photosynthetic pigment loss and increased nitrate reductase activity. Plant Growth Regul., 40: 29-32.

Begum, A.A., M. Tamaki, S. Tahara and F. Kato, 1994. Somatic embryogenesis in *Cymbidium* through *in vitro* culture of inner tissue of protocorm-like bodies. Jap. Soc. Hortic. Sci., 63: 419-427.

Biernbaum, J.A., R.L. Houtz and S.K. Ries, 1998. Field studies with crops treated with colloidally dispersed triacontanol. J. Am. Soc. Hortic. Sci., 113: 679-684.

Brosa, D., 1999. Biological effects of Brassinosteroids. Cri. Rev. Biochem. Mol. Biol., 34: 339-358.

Chang, C. and W.C. Chang, 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* var. *misericors*. Plant Cell Rep., 17: 251-255.

Clouse, S.D., M. Langford and T.C. McMoris, 1996. A brassinosteroid-insensitive mutant in *Arabidopsis thaliana*. Proceedings of Plant Physiol. Plant Mol. Biol., 49: 427-451.

Clouse, S.D. and J.M. Sasse, 1998. Brassinosteroids: Essential regulators of plant growth and development. Ann. Rev. Plant Physiol. Plant Mol. Biol., 49: 427-451.

Dhaubhadel, S., S. Chaudhary, K.F. Dobinsen and P. Krishna, 1999. Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings. Plant Mol. Biol., 42: 333-342.

Franck-Duchenne, M., Y. Wang, S.B. Tahar and R.N. Beachy, 1998. *In vitro* stem elongation of sweet pepper in media containing 24-epi-brassinolide. Plant Cell Tiss. Org. Cult., 53: 79-84.

Fraternale, D., L. Giamperi, D. Ricci, M.B.L. Rocchi, L. Guidi, F. Epifano and M.C. Marcotullio, 2003. The effect of triacontanol on micropropagation and on secretory system of *Thymus mastichina*. Plant Cell Tiss. Org. Cult., 74: 87-97.

Fujioka, S., T. Noguchi, S. Takatsuto and S. Yoshida, 1998. Brassinosteroids in *Arabidopsis thaliana*. Phytochemistry, 48: 595-599.

Fujioka, S., 1999. Natural Occurrences of Brassinosteroids in the Plant Kingdom. In: Brassinosteroids; Steroidal Plant Hormones, Sakurai, A., T. Yokota and S.D. Clouse (Eds.), Springer-Verlag, Tokyo, pp: 21-45.

Garcia, D., 2000. Accion del analogo de brassinosteroides MH-5 y la kinetina en la formacion de biomasa en callos de *Coffea canephora* var. Robusta. Cultivos Tropicales, 21: 39-46.

Grove, M.D., G.F. Spencer, W.K. Rohwedder, N.B. Mandava, J.F. Worley, J.D. Warthen, G.L. Steffens, J.L. Flippen-Anderson and J.C. Cook, 1979. Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. Nature, 281: 216-217.

Gupta, D., R. Bhardwaj, P.K. Nagar and S. Kaur, 2004. Isolation and characterization of brassinosteroids from leaves of *Camellia sinensis* (L.) O. Kuntze. Plant Growth Regul., 43: 97-100.

Hu, Y.X., F. Bao and X.Y. Li, 2000. Promotive effect of brassinosteroids on cell division involves a distinct Cyc D3-induction pathway in *Arabidopsis*. Plant J., 24: 693-701.

Huan, L.T., T. Takamura and M. Tanaka, 2004. Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid. Plant Sci., 166: 1443-1449.

Ichihashi, S., 1997. Research on Micropropagation of *Cymbidium*, *Dendrobium* and *Phalaenopsis* in Japan. In: Orchid Biology: Reviews and Perspectives, Arditti, J. and A.M. Pridgeon (Eds.), Vol. VII. Dordrecth, The Netherlands: Kluwer Academic Publishers, pp: 285-316.

Ichihashi, S., 1998. Control of growth and development of protocorm-like bodies derived from callus by carbon sources. Plant Biotech., 15: 183-187.

Kanjilal, B., D.D. Sarkar, J. Mitra and K.B. Datta, 1999. Stem disc culture: Development of a rapid mass propagation method for *Dendrobium moschatum*, an endangered orchid. Curr. Sci., 77: 497-500.

Kataki, S.K., 1977. Endangered Orchids of Meghalaya State, India. Botanical Survey of India, Meghalaya State Forest Department, March Issue, pp: 85-87.

Khripach, V., V. Zhabinskii and A. deGroot, 2000. Brassinosteroids: A New Class of Plant Hormones. Academic Press, London.

Kumar, Y., J. Sharma and K.A. Joy, 1994. The orchids of north-east India-II: Towards a rainbow revolution. J. Orch. Soc. Ind., 8: 73-76.

Lakshmanan, P., C.L. Lee, C.S. Loh and C.J. Goh, 1995. *In vitro* Propagation of Commercial Orchids. An Assessment of Current Methodology and Development of a Novel Approach of thin Cross Section Culture. In: Plant Tissue Culture. Islam, A.S. (Ed.), New Delhi, India: Oxford IBH., pp: 42-49.

Lu, Z., M. Huang, D.P. Ge, Y.H. Yang, X.N. Cai, P. Qin and J.M. She, 2003. Effect of brassinolide on callus growth and regeneration in *Spartina patens* (Poaceae). Plant Cell Tiss. Org. Cult., 73: 87-89.

Malabadi, R.B., G.S. Mulgund and K. Nataraja, 2004. Efficient regeneration of *Vanda coerulea*, an endangered orchid using thidiazuron. Plant Cell Tiss. Org. Cult., 76: 289-293.

Malabadi, R.B., G.S. Mulgund and K. Nataraja, 2005. Micropropagation of *Dendrobium nobile* from shoot tip sections. J. Plant Physiol., 162: 473-478.

Mandava, N.B., 1988. Plant growth-promoting brassinosteroids. Ann. Rev. Plant Physiol. Plant Mol. Biol., 39: 23-52.

Mayumi, K. and H. Shibaoka, 1995. A possible double role for brassinolide in the reorientation of cortical microtubules in the epidermal cells of Azuki bean epicotyl. Plant Cell Physiol., 36: 173-181.

Mitra, G.C., R.N. Prasad and R.A. Choudhury, 1976. Inorganic salts and differentiation of protocorms in seed callus of orchid correlative changes in its free amino acid content. Indian J. Exp. Biol., 14: 350-351.

Morel, G.M., 1964. Tissue culture. A new means of clonal propagation of orchids. Am. Orch. Soc. Bull., 33: 473-478.

More, O., M. Hernandez, M. Nunez, A. Estevez and M.E. Gonzalez, 2001. Empleo de analogos de brassinosteroides en la formacion de callus embriogenicos en papa (*Solanum tuberosum* L.). Cultivos Tropicales, 224: 29-35.

Nakajima, N., A. Shida and S. Toyama, 1996. Effects of brassinosteroid on cell division and colony formation of Chinese cabbage mesophyll protoplasts. Jap. J. Crop. Sci., 65: 114-118.

Nayak, N.R., S. Sahoo, S. Patnaik and S.P. Rath, 1997. *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.), *Dendrobium nobile* and *Dendrobium moschatum* through thidiazuron-induced high frequency shoot proliferation. Sci. Hortic., 71: 243-250.

Nayak, N.R., S. Sahoo, S. Patnaik and S.P. Rath, 2002. Establishment of Thin Cross Section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) and *Dendrobium nobile*. Sci. Hortic., 94: 107-116.

Nunez, M., W.J. Siqueira, M. Hernandez, M.A.T. Zullo, C. Robaina and F. Coll, 2004. Effect of spirostane analogues of brassinosteroids on callus formation and plant regeneration in lettuce (*Lactuca sativa*). Plant Cell Tiss. Org. Cult., 78: 97-99.

Oh, M.H. and S.D. Clouse, 1998. Brassinolide affects the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Plant Cell Rep., 17: 921-924.

Ozdemir, F., B. Melike, T. Demiral and I. Turkan, 2004. Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, praline content and antioxidative system of rice (*Oryza sativa* L.) under salinity stress. Plant Growth Regul., 42: 203-211.

Phukan, Y.K. and A.A. Mao, 1999. A miniature orchid of northeast India. Orchid Rev., 107: 368-369.

Pullman, G.S., Y. Zhang and B.H. Phan, 2003. Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep., 22: 96-104.

Rao, A.N., 1977. Tissue Culture in Orchids Industry. In: Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture. Bajaj, Y.P.S. and J. Reinert (Eds.), Berlin: Springer, pp: 44-69.

Ronsch, H., G. Adam, J. Matschke and G. Schachler, 1993. Influence of (22S, 23S)-homobrassinolide on rooting capacity and survival of adult Norway spruce cuttings. Tree Physiol., 12: 71-80.

Sagawa, Y., T. Shoji and T. Shoji, 1966. Clonal propagation of *Cymbidiums* through shoot meristem culture. Am. Orch. Soc. Bull., 35: 118-122.

Sakurai, A. and S. Fujioka, 1993. The current status of physiology and biochemistry of brassinosteroids. Plant Growth Regul., 13: 147-159.

Sakurai, A. and S. Fujioka, 1997. Studies on biosynthesis of brassinosteroids. Biosc. Biotech. Biochem., 61: 757-762.

Sasaki, H., 2002. Brassinolide promotes an adventitious shoot regeneration from cauliflower hypocotyl segments. Plant Cell Tiss. Org. Cult., 71: 111-116.

Sasse, J.M., 1997. Recent progress in brassinosteroid research. Physiol. Plant., 100: 697-701.

Sharma, S.K. and P. Tandon, 1990. Asymbiotic germination and seedling growth of *Cymbidium elegans* Lindl. And *Coelogyne punctulata* Lindl as influenced by different carbon sources. J. Orch. Soc. Ind., 4: 149-159.

Sharma, S.K., P. Tandon and R.R. Mishra, 1991. Vitamins as related to axenic seed germination and seedling of *Cymbidium elegans* Lindl. and *Coelogyne punctulata* Lindl. J. Orch. Soc. Ind., 5: 25-28.

Steward, F.C. and M.O. Mapes, 1971. Morphogenesis in aseptic cell cultures of *Cymbidium*. Bot. Gaz., 132: 65-70.

Tantos, A., A. Meszaros and J. Kissimon, 1999. The effect of triacontanol on micropropagation of balm, *Melissa officinalis* L. Plant Cell Rep., 19: 88-91.

Ueda, H. and H. Torikata, 1968. Organogenesis in meristem culture of *Cymbidium*. I. Studies on the effects of growth substances added to culture media under continuous illumination. J. Jap. Soc. Hortic. Soc., 37: 240-248.

Vacin, E. and F.W. Went, 1949. Some pH changes in nutrient solutions. Bot. Gaz., 110: 605-613.

Wang, X., 1988. Tissue culture of *Cymbidium*: Plant and flower induction *in vitro*. Lindleyana., 3: 184-189.

Wang, W., X. Zhang and J. Liu, 1992. Effects of brassinolide on somatic embryogenesis of *Gossypium hirsutum*. Plant Physiol. Common., 28: 15-18.

Wimber, D.E., 1963. Clonal multiplication of *Cymbidium* through tissue culture of the shoot meristem. Am. Orch. Soc. Bull., 32: 105-107.