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In Vitro Shoot Regeneration by Culture of *Liparis elliptica* (Rees) Lindl. Shoot Tip-derived Transverse Thin Cell Layers Induced by 24-epi Brassinolide

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ABSTRACT

An efficient *in vitro* propagation protocol for *Liparis elliptica* (Rees) Lindl. using transverse thin cell layers (TCLs) was established. The initiation of protocorm-like bodies (PLBs) and the regeneration of shoot buds from PLB TCLs significantly relied on the concentration of 24-epi brassinolide (24-epiBL)-supplemented Mitra *et al*. basal medium. The highest percentage of PLB-TCL explants (93.0%) producing PLBs (71.0 ± 2.1) was recorded on 4.0 µM 24-epiBL in a period of 12 weeks. The cultures were maintained for 6-12 weeks for the initiation of PLBs or proliferating shoot buds. After nearly 12 weeks, small bud-like structures formed healthy shoots. The highest number of shoots with well developed roots was developed on 10.74 µM NAA-supplemented basal medium. This successful protocol will allow for the mass multiplication of *L. elliptica*, fulfilling the timely demand for clonal plantlets. This is the first ever report of *in vitro* culture for this epiphytic orchid.

Keywords: brassinosteroids, epiphytic orchid, micropropagation, Western Ghat forests
Abbreviations: 24-epiBL, 24-epibrassinolide; **IAA**, indole-3-acetic-acid; **IBA**, indole-3-butyric acid; **NAA**, 1-naphthalene acetic acid; **PLB**, protocorm-like body

INTRODUCTION

Current interest in propagation of commercially important and endangered orchid species has created a need to develop practical propagation methods. Orchids are commercially grown globally as cut flowers and pot plants. *Liparis elliptica* (Rees) Lindl. is one of the most important epiphytic orchids from the Western Ghat forests of Karnataka state, India known for its beautiful flowers. The ecology and economy of this region and of its people is greatly influenced by this orchid species to a large extent. The beautiful and intriguing flowers with bewildering colors, shape and sizes, persistence of blooms up to perfection and their ability to travel long distances uplifted them to 15th position of the top 50 (local newspaper finding) cut flowers in the Indian market. Most of the orchids are usually propagated sexually by seeds and asexually by division of offshoots. Besides low or no seed setting and germination, the seedling progenies are heterozygous, and do not warrant true-to-type plants of hybrid cultivars. *Liparis elliptica* is an epiphytic orchid, which is very difficult to propagate vegetatively. The characteristics of seedlings are not uniform, and propagation through tissue culture has been desired but not yet achieved. The source of this species, particularly in the Western Ghat forests of Karnataka, is experiencing a steady decline due to a lower rate of propagation in nature, over-exploitation and unfortunately, poachers. There are no reports of *in vitro* propagation methods available in the literature. Therefore, there is an urgent need to develop *in vitro* propagation protocols for the conservation of this orchid species. Conventional vegetative propagation is beset by a slow multiplication rate, and does not provide sufficient clones within a short timeframe. Therefore, it is essential to take immediate measures for the micropropagation of this orchid using *in vitro* culture techniques. Plant tissue culture methods have played an important role in the micropropagation of several commercially important orchids to meet the demands of a growing market throughout the world (Rao 1977; Lakshmanan *et al*. 1995; Ichihashi 1998; Kanjilal *et al*. 1999; Malabadi *et al*. 2004, 2005; Zhao *et al*. 2007; Malabadi and Nataraja 2007a, 2007b; Malabadi *et al*. 2008a, 2008b, 2008c). Transverse thin cell layers (TCLs) of plant tissues such as apical meristems, stem nodes, and protocorm-like bodies (PLBs) have been successfully used as explants for plant regeneration in a few orchids as well as other plant species (Begum *et al.* 1994; Nayak *et al*. 1997, 2002; Malabadi *et al.* 2004, 2005; Teixeira da Silva *et al.* 2006a, 2006b; Malabadi and Nataraja 2007a, 2007b; Zhao *et al.* 2007; Malabadi *et al.* 2008a, 2008b, 2008c). This culture system was first developed by Tran Thanh Van for programming different patterns of morphogenesis in *Nicotiana tobacum* (Tran Thanh Van 1973a, 1973b, 1980). TCLs allow for the isolation of specific cells or tissue layers, which, depending on the genetic state and epigenetic requirements and in conjunction with strictly controlled growth conditions (light, temperature, pH, PGRs, media additives and others) may lead to the *in vitro* induction of specific morphogenic programmes (Teixeira da Silva *et al*. 2006a, 2006b; Teixeira da Silva and Tanaka 2006; Teixeira da Silva *et al*. 2007a). The capacity of a TCL to enter a program depends upon a number of factors, including correct signal perception and transduction, capacity of the internal genetic machinery to respond and react to these signals and in the latter case, may depend on the physiological state and origin of the TCL (Teixeira da Silva *et al*. 2006, 2007a). This culture system was proved to be the best method than

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any other conventional *in vitro* culture methods with regard to the total output of plantlets for several ornamentals, including *Cymbidium* (Teixeira da Silva *et al*. 2005, 2006, 2007b, 2007c; Teixeira da Silva and Tanaka 2006). So in order to obtain rapid plant regeneration with a high frequency, TCL technology was exploited for the mass propagation of *L. elliptica*, and the influence of 24-epibrassinolide (24-epiBL) was evaluated during the plant regeneration of this orchid.

MATERIALS AND METHODS

Twenty five plants of *Liparis elliptica* (Rees) (Lindl.) collected from the Western Ghat Forests of Karnataka state, India were established in pots and grown under greenhouse conditions at the Department of Botany, Karnatak University, Dharwad, India. All the culture conditions and procedure for induction of *in vitro* plantlets of *L. elliptica* were adopted from our previous protocol of *C. elegans* (Malabadi and Nataraja 2007a), and *C. bicolar* (Malabadi *et al*. 2008b). Shoot tips of *L. elliptica* (0.5-0.8 cm) harvested from mother plants were carefully washed in double distilled water (DDW). They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, USA), and thoroughly rinsed with sterilized DDW. TCLs 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, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin (all medium constituents and reagents Sigma-Aldrich). 24-epiBL was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada. The medium was supplemented with a range of 24-epiBL concentrations (0.5, 1, 2, 3, 4, 5, 6, 7 10, 15 and 20 μM) without any other plant growth regulators in 25 mm × 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. Medium without 24-epiBL served as the 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-epiBL were filter sterilized (Whatman filter paper, pore size = 0.45 μm; diameter of paper = 25 mm) 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 PLBs or proliferating shoot buds. The freshly initiated individual PLBs were 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 30 cultures per replicate, with four replicates (120 cultures) per experimental treatment, and each treatment was repeated three times (120 × 3 = 360). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at the 5% level using the SPSS (Microsoft Windows ver. 13.0.1.1) statistical software package.

The well-developed shoots were further transferred to fresh Mitra *et al.* (1976) basal medium supplemented with various concentrations of auxins indole-3-acetic-acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), for rooting. All the shoot buds failed to produce roots with IAA (0.57, 8.56, 11.42, 14.27, 17.13, 19.98, 22.84 μM) and IBA (0.49, 2.45, 4.9, 7.35, 9.8, 12.25, 14.7, 19.6 μM), and rooting was observed with NAA (8.05, 10.74, 13.42 μM), whereas shoot buds failed to produce any rooting with higher concentrations of NAA tested (16.11, 18.79, 21.48, 24.16, 26.85 μM). Well-developed shoot buds were transferred to fresh Mitra *et al*. (1976) basal medium supplemented with various concentrations of NAA (8.05, 10.74, 13.42 μM), (**Table 2**). The shoots with highest percentage (84.0 ± 0.3) of roots on 10.74 μM NAA-supplemented basal medium were washed thoroughly under running tap water and transplanted into 15 cm diameter pots containing 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; Malabadi and Nataraja 2007a; Malabadi *et al*. 2008a, 2008b).

RESULTS AND DISCUSSION

Plant regeneration *via* PLBs formation using 24-epiBL from shoot tip TCL explants is a powerful method for *in vitro* propagation of *L. elliptica* for commercial applications. In the present study, 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**). The highest percentage of explants (93.0%) producing PLBs (71.0 ± 2.1) was recorded on 4.0 μM 24-epiBL in a period of 12 weeks (**Table 1; Fig. 1A**). These PLBs or proliferating shoot buds formed the maximum number of healthy shoots (59.0 ± 3.2). Initiation of PLBs increased or proliferating shoot buds decreased with an increase in the concentration of 24-epiBL from 2.0-5.0 μM (**Table 1**). However, the percentage of PLBs decreased as the concentration of 24-epiBL increased from 4.0 to 5.0 μM. Explants cultured on basal medium (Mitra *et al*. 1976) supplemented with lower concentrations of 24-epiBL (2.0-4.0 μM) showed prolific growth of PLBs or shoot buds. Hence the effective range of 24-epiBL for the initiation of PLBs in *L. elliptica* is 2.0-4.0 μM (**Table 1**). The TCLs remained green and developed small bud-like structures when cultured on 4.0 μM of 24-epiBL-supplemented basal medium within 9 weeks. These were further subcultured on the same medium and maintained for another 6-9 weeks. After nearly 12 weeks, small bud-like structures formed healthy shoots (**Figs. 1A-C**). tTCL explants cultured on 24-epiBL-free medium (control) remained green for 2 weeks and gradually turned brown and died later without forming shoots or PLBs. In another similar study, successful initiation of PLBs and *in vitro* regeneration of *C. elegans* was achieved using shoot tip tTCL sections in the presence of 24-epiBL on Mitra *et al*. (1976)

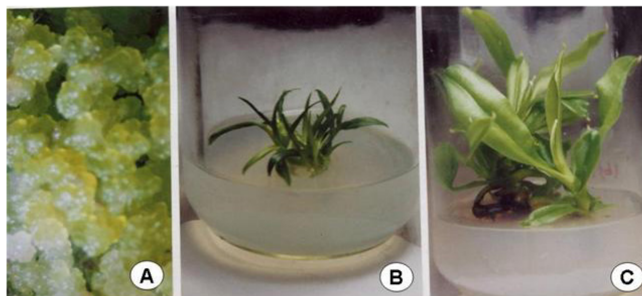

Fig. 1 *In vitro* multiplication of *L. elliptica* using 24-epiBL. (**A**) Initiation of PLBs from thin sections of shoot tips on Mitra *et al*. (1976) basal medium supplemented with 4.0 μM 24-epiBL. (**B**) Formation of healthy shoots with well developed leaves from PLBs ready for rooting. (**C**) Well developed hardened plants ready for transfer to field conditions.

Table 1 Effect of various concentrations of 24-epiBL on the initiation of PLBs or proliferating shoot buds in *L. elliptica*

24-epiBL (μM)	Responsive explants (%)	Total № of PLBs or shoot buds per explant	Total № of shoots per explant
Control	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
0.5	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c
1.0	0.0 ± 0.0c	0.0 ± 0.0 c	0.0 ± 0.0 c
2.0	23.0 ± 1.0 b	11.0 ± 0.2 b	6.0 ± 1.3 b
3.0	31.0 ± 2.2 b	15.0 ± 2.1 b	10.0 ± 0.5 b
4.0	93.0 ± 3.4 a	71.0 ± 2.1 a	59.0 ± 3.2 a
5.0	12.0 ± 0.4 b	2.0 ± 0.1 b	0.0 ± 0.0 c
6.0-20.0	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

Data scored after 12 weeks and represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different (P<0.05) according to DMRT (Duncan's multiple range test).

basal medium, with the highest percentage of explants (91.0%) producing PLBs (24.0 ± 2.1) on 4.0 μM 24-epiBL; all the newly formed PLBs survived and after nearly 12 weeks, small bud-like structures formed healthy shoots (Malabadi and Nataraja 2007a). Therefore, the results of the present study are similar to the findings with *C. elegans* and *C. bicolar* (Malabadi and Nataraja 2007a; Malabadi *et al.* 2008a).

24-epiBL 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). 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). 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). In our study, we tested the influence of 24-epiBL alone without any combination with other PGRs to test its role on *in vitro* regeneration of this epiphytic orchid, *L. elliptica*. This is the third report for orchids. 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).

The first was in *C. elegans* in which 24-epiBL and the survival rate of seedlings was 100% (Malabadi and Nataraja 2007a). Successful initiation of PLBs and *in vitro* regeneration was achieved using shoot tip (harvested from mother plants grown under greenhouse conditions) sections and 24-epiBL-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-epiBL 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-epiBL 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-epiBL from 3.0 to 5.0 μM. In a second report, the rapid clonal propagation of *Cymbidium bicolor* was achieved by induction of PLBs using shoot tip TCLs (similar to this study) when cultured on 24-epiBL-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-epiBL was used. All the newly formed PLBs survived and after nearly 12 weeks, small bud-like structures formed healthy shoots (Malabadi *et al.* 2008a). In various bioassays, 24-epiBL has been shown to be more active than, or synergistic with, auxins such as IAA or NAA (Brosa 1999).

In *Oryza sativa*, an increase in the soluble protein content was noticed following 3 μM 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). Seedling growth of rice plants was improved by 3 μM 24-epiBL treatment under salt stress conditions. When seedlings treated with 3 μM 24-epibL 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). 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-epiBL (Wang *et al.* 1992; Franck-Duchenne *et al.* 1998; Sasaki 2002). 24-epiBL 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 2007c). Oh and Clouse (1998) demonstrated that brassinolide increased the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. However, very few reports are available with respect to the effect of brassinolide in micropropagation and tissue culture.

Cytokinins (zeatin and iso-pentenylaminopurine) also promoted shoot regeneration in *B. oleracea* (Sasaki 2002). When 0.1 or 1 μM 24-epiBL was added together with these cytokinins, maximum regeneration was further improved (Sasaki 2002). When hypocotyl segments of cauliflower (*Brassica oleracea* var. *botrytis* L.) were cultured on MS medium containing 0.1 or 1 μM 24-epiBL in the light, a significant stimulation of adventitious shoot regeneration was observed (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-epiBL at various concentrations, 0.1-10 μM 24-epiBL significantly promoted adventitious bud formation (Sasaki 2002). The highest percentage of regeneration occurred at 0.1 or 1 μM 24-epiBL in which 44% of explants formed buds. A maximum number of shoot buds per regenerating explant was achieved at 1 μM 24-epiBL. Sasaki (2002) also mentioned that zeatin also stimulated bud formation in Cauliflower. But when 24-epiBL 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). Hu *et al.* (2000) suggested that 24-epiBL 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-epiBL can substitute cytokinin in culturing *Arabidopsis* callus and suspension cells. Work with Chinese cabbage protoplasts showed that 24-epiBL promoted cell division in the presence of 2,4-D and kinetin (Nakajima *et al.* 1996).

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-epiBL 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-epiBL 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. Shoots regenerated on 4.0 μM 24-epiBL-sup-

Table 2 Effect of different concentrations of auxin (IAA, IBA, NAA) on rooting of shoots regenerated with 4.0 µM 24-epiBL treatment.

IAA (µM)	Rooting (%)	IBA (µM)	Rooting (%)	NAA (µM)	Rooting (%)
Control*	0.0 ± 0.0 c	control	0.0 ± 0.0 c	control	0.0 ± 0.0 c
0.28	0.0 ± 0.0 c	0.24	0.0 ± 0.0 c	0.26	0.0 ± 0.0 c
0.57	0.0 ± 0.0 c	0.49	0.0 ± 0.0 c	0.53	0.0 ± 0.0 c
2.85	0.0 ± 0.0 c	2.45	0.0 ± 0.0 c	2.68	0.0 ± 0.0 c
5.71	0.0 ± 0.0 c	4.90	0.0 ± 0.0 c	5.37	0.0 ± 0.0 c
8.56	0.0 ± 0.0 c	7.35	0.0 ± 0.0 c	8.05	11.0 ± 0.6 b
11.42	0.0 ± 0.0 c	9.8	0.0 ± 0.0 c	**10.74**	**84.0 ± 0.3 a**
14.27	0.0 ± 0.0 c	12.25	0.0 ± 0.0 c	13.42	5.0 ± 0.1 b
17.13	0.0 ± 0.0 c	14.7	0.0 ± 0.0 c	16.11	0.0 ± 0.0 c
19.98	0.0 ± 0.0 c	17.15	0.0 ± 0.0 c	18.79	0.0 ± 0.0 c
22.84	0.0 ± 0.0 c	19.6	0.0 ± 0.0 c	21.48	0.0 ± 0.0 c
25.69	0.0 ± 0.0 c	22.05	0.0 ± 0.0 c	24.16	0.0 ± 0.0 c
28.55	0.0 ± 0.0 c	24.5	0.0 ± 0.0 c	26.85	0.0 ± 0.0 c

*Control= Basal medium without IAA, IBA and NAA

Data scored after 4 weeks and represent the mean ± SE of at least three different experiments. In each column, the values with different letters are significantly different (P<0.05) according to DMRT (Duncan's multiple range test).

plemented 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 concentrations of IAA and IBA. On other hand shoots or proliferating shoot buds showed rooting on Mitra *et al*. (1976) basal medium supplemented with various concentrations of NAA (8.05, 10.74, 13.42 µM) (**Table 2**). The highest number of shoots with well developed roots was developed on 10.74 µM NAA-supplemented basal medium. Finally, the well-rooted shoots that regenerated on 4.0 µM 24-epiBL-supplemented basal medium 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. All the plants were normal and showed healthy growth with an 89% survival rate.

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