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Shoot Tip Transverse Thin Cell Layers and 24-Epibrassinolide in the Micropropagation of *Cymbidium bicolor* Lindl.

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ABSTRACT

The rapid clonal propagation of *Cymbidium bicolor* was achieved by induction of protocorm-like-bodies (PLBs) using transverse thin cell layers of shoot tips when cultured on 24-epibrassinolide (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. Shoots produced roots when cultured on the same 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. This is only the second report of the use of 24-epiBL, a plant steroid lactone, in the micropropagation of orchids.

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

INTRODUCTION

Cymbidium bicolor Lindl is one of the most important orchids from the Western Ghat forests of Karnataka state, India known for its beautiful flowers. It has thick succulent leaves, and spends its life as an epiphyte, perched high on suitable trees in full sunlight. The source of this species particulary in the Western Ghat forests of Karnataka is experiencing a steady decline due to a lower rate of propagation in nature and over exploitation. Conventional vegetative propagation is beset with slow multiplication rate, does not provide sufficient clones within 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; Malabadi and Nataraja 2007a, 2007b). In orchids plantlets are usually regenerated through protocorm-like bodies (PLBs) or through direct shoot organogenesis. *In vitro* regeneration of *Cymbidium* has been reported by Wimber (1963), Morel (1964), Sagawa *et al*. (1966), Ueda and Torikata (1968), Steward and Mapes (1971), Wang (1988), Sharma and Tandon (1990), Sharma *et al.* (1991), Begum *et al.* (1994), Ichihashi (1997), Chang and Chang (1998), Phukan and Mao (1999), Nayak *et al*. (1997, 2002), Huan *et al*. (2004), Teixeira da Silva *et al*. (2006) and Malabadi and Nataraja (2007a). Transverse thin-cell layers of plant tissues such as apical meristems, stem nodes, and 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*. 2002; Malabadi *et al.* 2004, 2005; Teixeira da Silva *et al*. 2006; Malabadi and Nataraja 2007a, 2007b; Zhao *et al.* 2007). This culture system was first developed by Tran Thanh Van for progrmming different patterns of morphogenesis in *Nicotiana tobacum* (Tran Thanh Van 1973a, 1973b, 1980). TCL systems allow for the isolation of specific cell or tissue layers, which depending on the genetic state and epigenetic requirements and in conjuction with strictly controlled growth conidtions (light, temperature, pH, PGRs, media additives and others) may lead to the *in vitro* induction of specific morphogenic progrms (Teixeira da Silva *et al*. 2006, 2007). 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, 2007). This culture system was proved to be the best method than any other conventional *in vitro* culture methods with regard to the total output of plantlets. So in order to obtain rapid plant regeneration with a high frequency, TCL technology was exploited for the mass propagation of *C. bicolor* and the influence of 24-epibrassinolide (24-epiBL) and higher percentage of rooting by the use of triacontanol (TRIA) were evaluated during the plant regeneration of this orchid.

MATERIALS AND METHODS

Ten plants of *Cymbidium bicolor* (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 *Cymbidium bicolor* were adopted from our previous protocol of *C. elegans* (Malabadi and Nataraja 2007a). Shoot tips of *C. bicolor* (0.5-0.8 cm) harvested from mother plants were carefully washed in double distilled water. 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), and thoroughly rinsed with sterilized double distilled water. Transverse thin sections of 1-

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5 mm thick were cut from shoot tips and these sections were cultured on Mitra *et al.* (1976) basal medium with 3.0% sucrose (Sigma), 0.7% agar (Sigma), 0.5 gl^{-1} *myo*-inositol (Sigma), 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine (Sigma), 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin (Sigma). 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 growth hormones 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. Media 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 3.0 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 25 cultures per replicate, with four replicates (100 cultures) per experimental treatment, and each treatment was 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 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 (IAA, NAA, IBA) 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 poor rooting was observed with NAA (0.53, 2.68, 5.37 µM), whereas shoot buds failed to produce any rooting with higher concentrations of NAA tested (8.05, 10.74, 13.42, 16.11 µM). Therefore, other growth regulators such as TRIA were studied for rooting efficiency in *C. bicolor.* Well-developed shoot buds were transferred to fresh Mitra *et al*. (1976) basal medium supplemented with various concentrations of TRIA (1, 2, 3, 4 and 5 µM) (**Table 2**). The shoots with well developed roots on 2.0 µM TRIA-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).

RESULTS AND DISCUSSION

The success of plant regeneration via PLBs formation using 24-epiBL from TCL explants opens another efficient way to mass propagate *C. bicolor*. This novel tissue culture method is unique and may be superior to other *in vitro* culture methods. In the present study the highest percentage of explants (86.0%) producing PLBs (65.0 ± 3.9) was recorded on 3.0 µM 24-epiBL in a period of 14 weeks (**Table 1; Fig. 1A**). These PLBs or proliferating shoot buds formed the maximum number of healthy shoots (54.0 ± 3.1). Lower (0.5 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 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 4.0-5.0 µM. Explants cultured on basal medium (Mitra *et al*. 1976) supplemented with lower concentrations of 24-epiBL (2.0-3.0 µM) showed prolific growth of PLBs or shoot buds. Hence the effective range of 24-epiBL for the initiation of PLBs in *C. bicolor* is 2.0-3.0 µM (**Table 1**). The thin shoot tip sections remained green and developed small bud-like structures when cultured on 3.0 µM of 24-epiBL-supplemented basal medium within 9 weeks. These were further subcultured on the same medium and maintained for another 10-12 weeks. After nearly 14 weeks, small bud-like structures formed healthy shoots (**Fig. 1A, 1B**). 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. 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) 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.*

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

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	12.0 ± 0.3 b	8.0 ± 0.2 b	3.0 ± 0.1 b
2.0	38.0 ± 3.5 b	21.0 ± 0.0 b	16.0 ± 2.3 b
3.0	86.0 ± 5.2 a	65.0 ± 3.9 a	54.0 ± 3.1 a
4.0	10.0 ± 0.4 b	6.0 ± 0.1 b	5.0 ± 0.2 b
5.0	2.0 ± 0.1 b	3.0 ± 0.1 b	2.0 ± 0.1 b
6.0-20.0	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c

Data scored after 14 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).

Fig. 1 ***In vitro*** **multiplication of** ***C. bicolor*** **using 24-epiBr.** (**A**) Initiation of PLBs from thin sections of shoot tips on Mitra *et al*. (1976) basal medium supplemented with 3.0 µM 24-epiBL. (**B**) Formation of healthy shoots with well developed leaves from PLBs ready for rooting.

elegans (Malabadi and Nataraja 2007a).

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).

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 plant growth regulators to test its role on *in vitro* regeneration of orchids. This is only the second report in orchids.

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. 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 2007a). Embryogenic tissue were induced from longitudinally bisected segments of *Cymbidium* Twilight Moon 'Day Light' PLBs, a hybrid orchid, on modified Vacin and Went medium (1949) supplemented with 2,4-D in combination with 0.01 mg l^{-1} TDZ (Huan *et al*. 2004).

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).

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). 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). 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).

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. 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). In various bioassays, 24-epiBL 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-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). However, very few reports are available with respect to the effect of brassinolide in micropropagation and tissue culture. Shoots regenerated on 3.0 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 concentrations of all three auxins. Therefore, TRIA (Malabadi *et al.* 2005b) was studied for its rooting efficiency in *C. bicolor.* TRIA, a long 30-carbon primary alcohol, is a naturally occurring plant growth promoter (Ries *et al*. 1977; Ries and Houtz 1983; Ries and Wert 1988). TRIA is a component of the epicuti-

Table 2 Effect of different concentrations of TRIA on rooting of shoots regenerated at 3.0 µM 24-epiBL-supplemented Mitra *et al*. (1976) basal medium.

TRIA (µM)	Responsive explants (%)	Rooting (%)
Control	0.0 ± 0.0 c	0.0 ± 0.0 c
1	22.0 ± 1.5 b	29.0 ± 1.9 b
2	91.0 ± 3.6 a	93.0 ± 3.0 a
3	26.0 ± 2.0 b	17.0 ± 1.0 b
4	13.0 ± 0.6 b	8.0 ± 0.3 b
5-10	3.0 ± 0.0 b	0.0 ± 0.0 c

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).

cular waxes of alfalfa and many other plants (Chibnall *et al*. 1933). The plant growth stimulating property of TRIA was demonstrated for the first time by Crosby and Vlitos (1959) and later by and Stoutemyer and Cook (1987). The rooting efficiency of somatic seedlings of *Pinus kesiya* as well as *Pinus roxburghii* was improved by the use of TRIA induced somatic embryogenesis (Malabadi *et al.* 2005b; Malabadi and Nataraja 2007c).

The highest percentage of rooting efficiency (93.0 ± 3.0) was recorded with 2.0 µM TRIA within 4 weeks (**Table 2**) but the lowest percentage of rooting (8.0 ± 0.3) was observed at 4.0 µM 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 3.0 to 4.0 µM (**Table 2**).

TRIA was also studied for the first time for the rooting efficiency in *Cymbidium elegans* (Malabadi and Nataraja 2007a). The highest percentage of rooting efficiency (95.0 ± 6.0) was recorded at 2.0 µM TRIA within 4 weeks while the lowest percentage (4.0 ± 0.65) was observed at 4.0 µM TRIA. Shoots failed to produce roots at higher concentrations (5.0 to 10.0 µM).

This also confirmed our previous results in which higher rooting efficiency of an endangered orchid, *Dendrobium nobile* was possible on Mitra *et al.* (1976) basal medium supplemented with 2.0 µg l^{-1} TRIA (Malabadi *et al*. 2005a). The highest rooting efficiency (95.0 ± 6.0%) was also recorded with 2.0 µM of TRIA within 4 weeks in *C. elegans* (Malabadi and Nataraja 2007a). According to Tantos *et al.* (1999), successful rooting was observed at 2.0 µg l^{-1} TRIA in *Melissa officinalis*. Similarly Fraternale *et al*. (2003) reported that 2.0-5.0 µg l^{-1} TRIA was optimum for efficient rooting in *Thymus 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). Recently, TRIA was show to positively influence somatic embryogenesis and rooting of *Pinus roxburghii* and *Pinus kesiya* somatic seedlings (Malabadi *et al*. 2005b; Malabadi and Nataraja 2007c). Mature zygotic embryos produced white, mucilaginous embryogenic callus when cultured on full strength LM basal medium supplemented with 90 mM maltose, 2.0 gl^{-1} Gellan gum, 9.0 µM 2, 4-D and 7 µgl^{-1} TRIA. On subculture of such embryogenic callus onto maintenance medium containing 2.0 µM 2, 4-D and 2.0 µgl^{-1} TRIA pro-embryos were induced. The highest percentage of somatic embryogenesis (83.5%) was recorded in PR810 genotype. On the other hand embryogenic cultures were initiated and established for the first time in 3 different genotypes of *P. kesiya* using mature zygotic embryos and TRIA (Malabadi *et al.* 2005b). Mature zygotic embryos produced white mucilaginous embryogenic callus when cultured on half strength MSG basal medium supplemented with 90 mM maltose, 2.0 gl^{-1} Gellan gum, 9.0 µM 2,4-D and 10 µgl^{-1} TRIA (Malabadi *et al.* 2005). On sub culture of such embryogenic callus on the maintenance medium (II) containing 2.0 µM 2,4-D and 2.0 µgl^{-1} TRIA induced cleavage polyembryogenesis with proembryos. The percentage somatic embryogenesis was not similar in all the three genotypes (Malabadi *et al.* 2005b). The highest percentage of shoot regeneration of *Costus speciosus* was achieved using thin rhizome sections and TRIA. Initiation of shoot buds was observed when rhizome thin sections were cultured on B5 basal medium supplemented with 5 µ$g1^{-1}$ TRIA. Shoots with two to three leaves produced roots when cultured on B5 basal medium supplemented with 2 µ$g1^{-1}$ TRIA. Results of this study showed that TRIA can be used as an effective growth regulator in the micropropagation and conservation of *C. speciosus* (Malabadi *et al.* 2005c).

Finally, the well-rooted shoots that regenerated on 3.0 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 80% survival rate.

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