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TDZ induced *in vitro* propagation of an epiphytic orchid *Xenikophyton smeeanum* (Reichb. f.)

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An efficient shoot regeneration of *Xenikophyton smeeanum* (Reichb. f.). was achieved using shoot tip sections and thidiazuron (TDZ). Protocorm-like bodies (PLB's) or proliferating shoot buds was observed when thin cell layers of shoot tip sections were cultured on Mitra *et al.* (1976) basal medium supplemented with 11.35µM thidiazuron. Shoots produced roots when cultured on Mitra *et al* (1976) basal medium supplemented with 8.56 µM IAA. This simple protocol will be useful for large-scale propagation of native epiphytic orchid *Xenikophyton smeeanum.*

Key words: Conservation, micropropagation, Karnataka, native orchids, Western Ghat forests

Abbreviations: IAA-indole-3-acetic acid; PLB's-protocorm-like bodies; TDZ-thidiazuron (N-phenyl-N-1,2,3-thidiazuron-5'-ylurea);IBA-indolebutyric acid; NAA-napthaleneacetic acid.

Conservation and *in vitro* propagation of native orchid species is one of the biggest challenges to meet commercial demands of native orchids. The demand for native orchids is also growing day by day. *Xenikophyton smeeanum* (Reichb. f.) is one of the important epiphytic native orchid species of Western Ghat forests of Karnataka. Plant tissue culture methods played an important role in the micropropagation of several commercially important orchids to meet the demands of growing market throughout the world (Wimber 1963; Morel 1964; Rao 1977; Wang 1988; Sharma and Tandon 1990; Sharma *et al*. 1991; Lakshmanan *et al*. 1995; Ichihashi 1997, 1998; Chang and Chang 1998; Kanjilal *et al*. 1999; Malabadi *et al*. 2004, 2005; Malabadi and Nataraja 2007a, 2007b; Nayak *et al*. 1997, 2002; Huan *et al*. 2004; Teixeira da Silva *et al*. 2006; Malabadi *et al*. 2008a, 2008b, 2009a, 2009b, 2011). 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; Malabadi *et al*. 2008a). The objective of this study is to develop an *in vitro* propagation method using tTCL technology (Tran Thanh Van 1973a, 1973b, 1980) in *Xenikophyton smeeanum* (Reichb. f.). Recently we have also reported

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for the first time a successful *in vitro* seed germination of *Xenikophyton smeeanum* (Reichb. f.) using smoke saturated water as the natural growth promoter (Malabadi *et al.* 2011). This could help in the conservation of this native orchid, and also TDZ *in vitro* multiplication method can be used for the large scale production to meet the growing demands of native orchids.

Materials and methods

Seven plants of *Xenikophyton smeeanum* (Reichb. f.), 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, Karnataka state India. Shoot tips of *Xenikophyton smeeanum* (Reichb. f.) (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 1-5 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). The medium was supplemented with a range of TDZ concentrations (0. 04, 0.22, 0.45, 2.27, 4.54, 9.08, 11.35, 13.62, 18.16, 22.71, 27.24, 31.78, 36.32, 40.86 and 45.41 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 TDZ 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 TDZ 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 11.35µM µM TDZ. 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 20 cultures per replicate, with four replicates (80 cultures) per experimental treatment, and each treatment was repeated three times (80 × 3 = 240). 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 were cultured on Mitra *et al.* (1976) basal medium supplemented with IAA (0.57, 8.56, 11.42, 14.27, 17.13, 19.98, 22.84 µM), IBA (0.49, 2.45, 4.9, 7.35, 9.8, 12.25, 14.7, 19.6 µM) and NAA (0.53, 2.68, 5.37, 8.05, 10.74, 13.42, 16.11 µM) for the rooting efficiency (**Table 2**). The shoots with well developed roots on 8.56 µM IAA supplemented basal medium were washed thoroughly under running tap water and transplanted into 15 cm diameter plastic pots containing a potting mixture of charcoal chips, coconut husks and broken tiles (2: 2: 1) (Figure-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). 60% of plants were survived.

Results and Discussion

In the present study, lower concentrations (0.04, 0.22, 0.45 µM) of TDZ was not effective on the initiation of PLB's or proliferating shoot buds (Table-1). All the explants (tTCL of shoot tip) remained green for 2 weeks, eventually turned brown, and finally dead. Incorporation of higher concentrations (22.71, 27.24, 31.78, 36.32, 40.86, 45.41 µM) of TDZ leads to the browning of explants, and ultimately resulted in the death of explants (Table-1). Few explants remained green for 3 weeks and failed to induce any organogenesis. All the explants cultured on Mitra *et al*. (1976) basal medium without TDZ (control) failed to induce PLB's. On the other hand low initiation of PLB's or proliferating shoot buds was noticed with incorporation of TDZ at 4.54, 11.35, and 13.62 µM in the Mitra *et al*. (1976) basal medium. The highest number of responsive explants (60.0±4.1b) showing maximum number of PLB's or proliferating shoot buds was observed at 11.35µM µM of TDZ (Table-1) (Figure-1A). Therefore, 11.35µM µM of TDZ is the optimum concentration for inducing PLB's or proliferating shoot buds in *Xenikophyton smeeanum* (Reichb. f.) (Table-1; Figure-1A). The tTCL of shoot tip explants remained green and developed small bud-like structures when cultured on 11.35µM µM of TDZ supplemented Mitra *et al.* (1976) basal medium after two weeks (Figure-1A). The small bud like structures were maintained for 4 weeks and then further subcultured on the same fresh medium for another 4 weeks. After 8 weeks, small bud-like structures formed healthy shoots with 2-3 leaves. The shoots regenerated on 11.35µM µM of TDZ supplemented basal medium were tested for rooting efficiency with different concentrations of auxins such as IAA, IBA and NAA (Table-2). The shoots failed to produce roots with lower concentrations of IAA (from 0.28 to 5.71 µM). Rooting efficiency was satisfactory by increasing the concentration of IAA from 8.56 µM to 19.98 µM (Table-2).

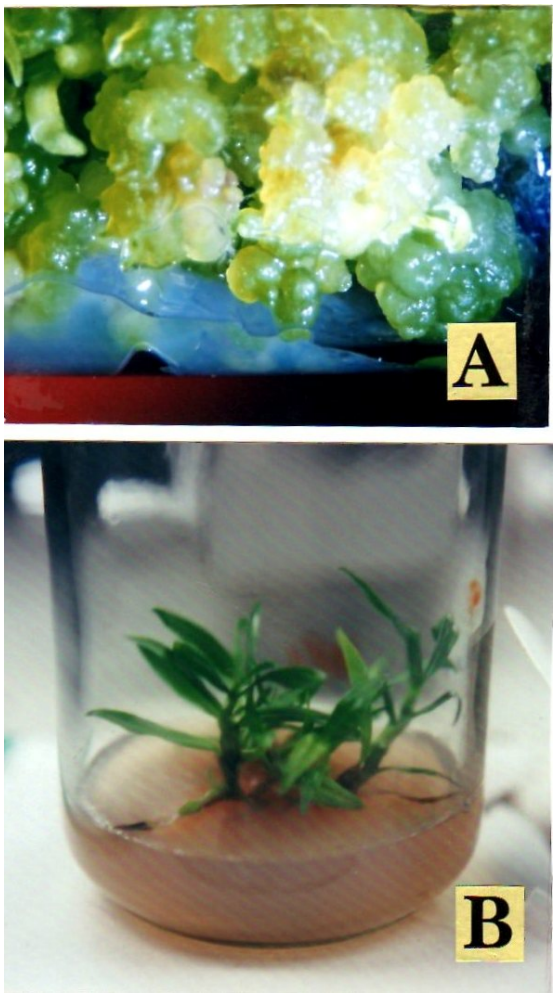

Figure 1: TDZ induced *in vitro* multiplication of *Xenikophyton smeeanum* (Reichb. f.). A) Initiation of protocorm-like bodies or proliferating shoot buds (60%) in tTCL of shoot tip after 3-4 weeks of culture on Mitra *et al.* (1976) basal medium supplemented with 11.35µM TDZ. B) Healthy shoots formed rooting (70%) on Mitra *et al.* (1976) basal medium supplemented with 8.56 µM IAA.

Highest percentage of rooting (70%) was with 8.56 µM IAA supplemented basal medium (Table-2) (Figure-1B). The shoots cultured on basal medium supplemented with various concentrations of IBA (0.24-7.35 µM) and NAA (0.26-8.05 µM) failed to produce roots (Table-2).

Table 1: Effect of different concentrations of TDZ on the initiation of protocorm-like bodies (PLBs) or proliferating shoot buds in *Xenikophyton smeeanum* (Reichb. f.).

TDZ (µM)	Responsive explants (%)	Number of PLBs or shoot buds per explant	Number of shoot buds produced per explant
Control*	0.0±0.0c	0.0±0.0c	0.0±0.0c
0.04	0.0±0.0c	0.0±0.0c	0.0±0.0c
0.22	0.0±0.0c	0.0±0.0c	0.0±0.0c
0.45	0.0±0.0c	0.0±0.0c	0.0±0.0c
2.27	1 .0±0.1b	0.0±0.0c	0.0±0.0c
4.54	5.0±0.8b	1.0±0.1b	0.0±0.0c
9.08	9.0±0.2b	3.0±0.6b	1.0±0.1b
11.35	60.0±4.1a	11.0±0.8b	4.0±2.1b
13.62	11.0±0.2b	4.0±0.3b	2.0±0.1b
18.16	1.0±0.1b	0.0±0.0c	0.0±0.0c
22.71	0.0±0.0c	0.0±0.0c	0.0±0.0c
27.24	0.0±0.0c	0.0±0.0c	0.0±0.0c
31.78	0.0±0.0c	0.0±0.0c	0.0±0.0c
36.32	0.0±0.0c	0.0±0.0c	0.0±0.0c
40.86	0.0±0.0c	0.0±0.0c	0.0±0.0c
45.41	0.0±0.0c	0.0±0.0c	0.0±0.0c

*Control=Basal medium without TDZ. 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 ($P<0.05$)

The potentiality of TDZ to stimulate shoot formation is very common in dicotyledonous plants as *Pisum sativum* (Massimo *et al.* 1996), *Cajanus cajan* (Eapen *et al.* 1998), *Arachis hypogaea* (Kanyand *et al.* 1994) as well as in monocotylednous plants viz. *Dendrocalamus strictus* Nees (Singh *et al.* 2001). In orchids, an efficient shoot regeneration of *Vanda coerulea* was achieved using thin shoot tip sections and TDZ (Malabadi *et al.* 2004a). Protocorm-like-bodies (PLBs) or proliferating shoot buds was observed when thin shoot tip sections were cultured on Vacin and Wents (VW) basal medium supplemented with 11.35 µM TDZ. The highest percentage of protocorm-like-bodies (95%) survived and ultimately produced healthy shoots with 2-3 leaves when subjected to a 4 week TDZ treatment. A culture period longer than 8 weeks with TDZ resulted in the formation of fascinated or distorted shoots. Shoots produced roots when cultured on half strength VW (Vacin and Went medium) supplemented with 11.42 µM IAA in *Vanda coerulea* (Malabadi *et al.* 2004a). A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. *Chiengmai* Pink was developed using TDZ (Chung *et al.* 2007). 5-25% of leaf tip segments of *in vitro* grown

plants *Dendrobium* cv. *Chiengmai* Pink directly formed somatic embryos on half strength MS medium supplemented with 0.3, 1 and 3.0mg\l TDZ(Chung *et al.* 2007). TDZ is effective in induction of in vitro morphogenesis in shoot regeneration and multiplication (Earnst 1994; Chen and Piluek 1995; Nayak *et al.* 1997; Chen and Chang 2000) and direct somatic embryogenesis (Chen et al. 1999; Chen and Chang 2000) of several orchids (*Phalaenopsis*, *Doritaenopsis*, *Cymbidium aloifolium*, *Dendrobium aphyllum*, *Dendrobium moschatum*, *Oncidium*). Moreover, TDZ combined with 2,4-D are required for callus induction in *Cymbidium ensifolium* var. misericors. (Chang and Chang 1998), *Oncidium* (Chen and Chang 2000a, 2000b), *Phalaenopsis* (Chen *et al.* 2000) and *Paphiopedilum* (Lin *et al.* 2000). However, Huang et al. (2001) reported that TDZ inhibits shoot proliferation and rooting in *Paphiopedilum*. In addition, Chen *et al.* (2002) reported that the best treatment of 4.54 µM TDZ induced only 20% of leaf explants to form shoots in *Paphiopedilum*. Very recently Hong and coworkers (2008) reported induction of totipotent callus on half strength MS medium supplemented with 22.60 µM 2,4-D and 4.54 µM TDZ in darkness in a maudiae type slipper orchid, *Paphiopedilum* Alma Gavaert (Hong *et al.* 2008).

Table 2: Effect of different concentrations of auxin (IAA, IBA, NAA) on rooting of *Xenikophyton smeeanum* (Reichb. f.). shoots regenerated with 11.35µM TDZ treatment.

IAA (µM)	Rooting (%)	IBA (µM)	Rooting (%)	NAA (µM)	Rooting (%)
Control*	0.0±0.0c	control	0.0±0.0c	control	0.0±0.0c
0.28	0.0±0.0c	0.24	0.0±0.0c	0.26	0.0±0.0c
0.57	0.0±0.0c	0.49	0.0±0.0c	0.53	0.0±0.0c
2.85	0.0±0.0c	2.45	0.0±0.0c	2.68	0.0±0.0c
5.71	0.0±0.0c	4.90	0.0±0.0c	5.37	0.0±0.0c
8.56	70.0±3.2a	7.35	0.0±0.0c	8.05	0.0±0.0c
11.42	16.0±0.2b	9.8	2.0±0.1b	10.74	2.0±0.1b
14.27	0.0±0.0c	12.25	1.0±0.1b	13.42	4.0±0.1b
17.13	0.0±0.0c	14.7	0.0±0.0c	16.11	0.0±0.0c
19.98	0.0±0.0c	17.15	0.0±0.0c	18.79	0.0±0.0c
22.84	0.0±0.0c	19.6	0.0±0.0c	21.48	0.0±0.0c
25.69	0.0±0.0c	22.05	0.0±0.0c	24.16	0.0±0.0c
28.55	0.0±0.0c	24.5	0.0±0.0c	26.85	0.0±0.0c

*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 ($P<0.05$)

The callus was proliferated more and maintained without any morphogenesis on the same medium with a 2 month interval. When callus was transferred on half strength MS medium supplemented with 26.85 µM NAA, an average of 4.7 protocorm-like bodies (PLBs)\shoot buds formed from each explant after 120 days of culture in *Paphiopedilum* Alma Gavaert (Hong *et al.* 2008). Nayak *et al.* (1997) developed a 4.0 µM TDZ-induced high frequency shoot proliferation in *Dendrobium moschatum* , *Cymbidium aloifolium*, and *Dendrobium apyllum.* Stem nodal explants of *Paphiopedilum philippinense* hybrids (hybrid PH59 and PH60) directly formed shoots

when cultured on modified-half strength MS basal medium supplemented with a combination of 4.52 µM 2,4-D and 0.45 µM TDZ (Chen et al. 2002). On hormone free basal medium, the percentages of explants with shoots were 33.3% and 0% and the shoot numbers per explant were 1 and 0 in hybrid PH59 and hybrid PH60 respectively. In hybrid PH59, 4.52 µM 2,4-D plus 0.45 µM TDZ induced a higher percentage of explants with shoots and shoot number per explant than did the hormone-free treatment. In hybrid PH60, although 4.52 µM 2, 4-D and 0.45 µM TDZ promoted shoot formation, the highest shoot number was found with 4.52 µM 2, 4-D alone (Chen *et al*. 2002). Thidiazuron (TDZ) is a substituted phenyl urea with cytokinin like activity (Mok *et al.* 1982) and therefore, stimulates rapid shoot differentiation. Thidiazuron (TDZ) aids in rapid plant regeneration of a number of plant species through organogenesis (Malik and Saxena 1992).

Singh *et al.* (2001) also reported TDZ induced shoot multiplication in bamboo (*Dendrocalamus strictus*) and maximum number of shoots (14.8 ± 1.0) were obtained from shoot explants cultured in 2.27 µM TDZ supplemented half strength MS basal medium. Treatment of soybean callus with TDZ, stimulated cytokinin accumulation (Thomas and Katterman 1986). In *Costus speciosus*, rhizome thin sections cultured on B_5 basal medium without TDZ (control) or with low concentrations 0.45 and 4.54 µM TDZ completely failed to produce shoot buds. Higher concentrations of TDZ particularly 36.32, 40.86 and 45.41 µM resulted in the browning of explants which finally necrosed (Malabadi *et al.* 2004b). On the other hand initiation of shoot buds was observed in the range 11.35 µM -27.34 µM TDZ with highest percentage of rhizome thin sections (92%) producing shoot buds (12 ± 2.01) at 18.16 µM TDZ (Malabadi *et al.* 2004b). Eighty seven percentage of these shoot buds elongated and gives rise to shoots. On this medium the explants remained green for 3 weeks and developed small bud like structures from the central as well as peripheral region during this period. After 2 weeks, the shoot buds showed further elongation and formed 2 to 3 leaves. Sharp decrease in the number of shoot buds formation was also noticed when the concentration of TDZ was increased from 18.16 to 31.78 µM (Malabadi *et al*. 2004b). Least and poor growth of shoot buds (1.8 ± 0.02) was noticed at 31.78 µM TDZ in *Costus speciosus* (Malabadi *et al.* 2004b). Wilhelm (1999) reported successful micropropagation of juvenile Sycamore maple (*Acer pseudoplatanus*) *via* adventitious shoot formation by the use of 0.04 µM TDZ. In case of pea cvs Sugar Ann and Patriot, an average of 20 shoots formed on MS basal medium supplemented with 0.5 or 1.0 µM TDZ (Massimo *et al.* 1996). TDZ either alone (4.54 or 9.08 µM) or in combination with IAA (5.71 µM) on MS supplemented medium induced high frequency of shoot regeneration from primary leaf segments of 3 pigeonpea (*Cajanus cajan* L.) cultivars (Eapen *et al.* 1998). These investigations are in conformity with the present findings.

A reliable protocol *via* PLB's or proliferating shoot buds formation for *Xenikophyton smeeanum* (Reichb. f.) orchid's propagation was achieved. The described protocol for micropropagation of *Xenikophyton smeeanum* (Reichb. f.) with TDZ is simple, efficient and can be applied for the conservation of this native orchid.

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