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In vitro propagation and genetic stability assessment of an endangered terrestrial jewel orchid *Anoectochilus elatus* Lindl.

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Anoectochilus elatus Lindl. is an endangered terrestrial jewel orchid habituated in the Eastern and the Western Ghats of Tamil Nadu, India. It is endangered due to lack of suitable pollinators for ovary fertilization and low fruit development. Moreover, issues such as its germinationwhich depends up on thesymbiotic association with mycorrhizal fungus with their tiny embryosand indiscriminate collection cause scarcity of this species in nature. Hence, in the present study we have developed an efficient and reproducible micropropagation protocol for eco-restoration programmes for this species. The explants axillary bud and shoot tip responded positively in Murashige and Skoog medium (MS) supplemented with different concentrations of thidiazuron (TDZ) alone and combination with cytokinins, auxins, complex extracts and antioxidants at various concentrations. Among the different experimental trails, MS medium supplemented with 1.5 mg/L TDZ and 50 mg/L peptone proved beneficial for producing multiple shoots. A maximum of 17.2 shoots/explant were derived from axillary bud followed by shoot tip (14 shoots/explant) explants. *In vitro* rooting of micro shoots was observed on MS medium supplemented with 3 g/L activated charcoal (AC). Genetic stability of *in vitro* raised plantlets was analysed through Inter Simple Sequence Repeats (ISSRs) molecular markers. Band patterns revealed 2.38% polymorphism and 97.61% monomorphism with genomic uniformity that of the mother plant. The regenerated plantlets were successfully acclimatized in a greenhouse environment with 68.42% survival rate. Finally, acclimatized plants were shifted to the Vattakanal Conservation Trust, Pambarpuram, Kodaikanal, Tamil Nadu for further maintenance and establishment under natural conditions.

Keywords: Activated charcoal, Antioxidants, Conservation, Eastern Ghats, Genomic uniformity, Micropropagation, Molecular markers, Thidiazuron (TDZ), Western Ghats

Orchidaceae, one of the largest families of flowering plants, comprises 30000-35000 species belonging to 850 genera and the taxa are terrestrial, epiphytes, lithophytes and saprophytes[1,2]. Orchid plants have become commercially important due to their attractive flowers and are marketed as both cut flowers and potted plants. Some orchid species play an important role as traditional system of medicine in many countries to cure various diseases; because orchid phytomolecules are rich in alkaloids, flavonoids, glycosides and other phytochemicals[3,4].

The genus *Anoectochilus* is an important perennial medicinal herb of the family Orchidaceae. It belongs to a group of terrestrial orchids commonly known as 'Jewel Orchids' due to their attractive foliage[5]. *Anoectochilus* has 30-40 species distributed in Sri Lanka, Malaysia, India, Japan, New Caledonia, Southern China, Australia, Hawaii and South Pacific Islands[5,6]. Several species of this genus such as *A. roxburghii*, *A. formosanus* and *A. koshunensis* have been used as folk medicine in China and Taiwan[7]. *Anoectochilus* is conventionally propagated through seed; however, the germination rate is very low, plants tend to grow slowly and cultivation period is long. Additionally, the occurrence of diseases such as root and stem rots caused by *Fusarium oxysporum* and *Pythium ultimum*, bacterial soft rot disease, pest infection by red spider, mites and scale insects often result in the continuous usage of pesticides and fungicides during cultivation may lead to fragmentation of their population[8]. Of late, this orchid genus is under threat of extinction due to excessive collection from natural resources to meet the requirements of the pharmaceuticals[9].

In India, *Anoectochilus brevilabris*, *A. narasimhanii*, *A. nicobaricus*, *A. roxburghii* and *A. tetrapterus* are endemic[10]. *Anoectochilus elatus* Lindl. is an endangered terrestrial orchid distributed in the Eastern and the Western Ghats of Tamil Nadu and Kerala[11]. It was earlier considered as endemic but distributed

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in other regions also, particularly in Sri Lanka[12]. It is a monopodial perennial medicinal herb with rhizomatousstem, pinkish white flowers, unique ornamental leaves that appear as dark green covers with golden and silver colour veins[13] (Fig. 1 A-D). It is found in the slopes of evergreen forest under shadow regions at an altitude of 1300-2100 m. Several factors affect their natural regeneration process, especially lacking of suitable pollinators for ovary fertilization, low fruit development and absence of suitable mycorrhizal fungus for seed germination. Finally, these obstacles cause scarcity of this species in nature[11,13,14].

Plant tissue culture facilitates a fast method for production of a large number of uniform plantlets in a short time irrespective of season and serves as alternative sources of seeds materials. *In vitro* preservation of germplasm is also as safe method in protecting plant species by reducing the risk of natural vagaries[15]. Repeated large scale multiplication process may affect recovery of true-to-type regenerants. Chromosomal rearrangements, gene amplification, gene mutation and retrotransposon are activated in the *in vitro* regenerated plants due to the stress given by the synthetic plant growth regulators (PGRs) and culture conditions which result in somaclonal variations among the sub-clones[16]. Genetically, stable true-to-type of regenerates is required for successful germplasm conservation and commercial utilizations. Currently, the genetic stability between *in vivo* and *in vitro* plants were accessed using molecular markers and become desirable technique for recognizing genetic similarity or dissimilarity of *in vitro* propagated plants[17].

Our earlier reports described an initial effort of plant regeneration through *in vivo* explants of *A. elatus* using individual cytokinins. Among the various concentrations of cytokinins tried, thidiazuron (TDZ) induced maximum number of shoots, although *in vitro* regeneration efficiency was considerably very low[14]. Hence, in the present study we attempted to improve and establish mass multiplication of *Anoectochilus elatus* through axillary bud and shoot tip explants using different concentrations of TDZ alone and combination with different PGRs, complex extracts and antioxidants. The genetic stability of *in vitro* raised plants were also analysed through Inter Simple Sequence Repeats (ISSRs) molecular markers.

Materials and Methods

In vitro propagation

Explant source

The juvenile axillary bud (≥1 cm) and shoot tip (≥0.5 cm) explants were excised from 6 month old *in vitro* raised plants of *A. elatus*[14].

Culture medium and condition

The juvenile axillary bud and shoot tip explants were cultured on Murashige and Skoog medium (MS)[18] supplemented with thidiazuron (TDZ) alone at different concentrations (0.1-2.5 mg/L). For enhancing shoot multiplication, TDZ was combined with different PGRs, including the cytokinins *viz*., N^6-benzyladenine (BA), kinetin (KN), N^6-(2-isopentenyl) adenine (2iP), zeatin (ZEA) and auxins *viz*., 2,4-dichloro-phenoxy-acetic acid (2,4-D), α-naphthalene-acetic acid (NAA) and indole-3-butyric acid (IBA) at concentrations ranging from 0.01-3.0 mg/L. In addition to the PGRs mentioned above, various complex extracts such as peptone (PEP), yeast extract (YE), malt extract (ME), beef extract (BE) at 50-200 mg/L, coconut water, coconut milk at 5-20% and antioxidants such as ascorbic acid (AA), glutamic acid (Glu), citric acid (CA), trisodium citrate (STC), activated charcoal (AC) and polyvinylpyrrolidone (PVP) at 50-200 mg/L were added to the medium for multiple shoot formation. Healthy elongated shoots (≥2.5 cm) separated from multiple shoot clumps and cultured on MS medium supplemented with IBA, indole-3-acetic acid (IAA) at 0.1-5.0 mg/L, AC and PVP at different concentrations (0.5-3.0 g/L) for rooting. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH or HCl before adding 0.8 % Agar-Agar as a solidifying agent and autoclaving at 121°C for 15 min. All the cultures were maintained under 16-h photoperiod with cool white fluorescent tubes (Philips TL-D Super 80, Gurgaon, India; 40 μM m^{-2} s^{-1}) at a constant temperature of 23±2°C.

Acclimatization

Rooted shoots were removed from the rooting medium and washed thoroughly in distilled water to remove any adhering medium. The plantlets were successfully acclimatized based on the previous report[11]. The plantlets were frequently irrigated with MS liquid solution (without carbon source and agar) every three days once for 4 weeks. Well acclimatized plants were finally transported to Vattakanal Conservation Trust, Pambarpuram, Kodaikanal, Tamil Nadu for re-establishment in nature.

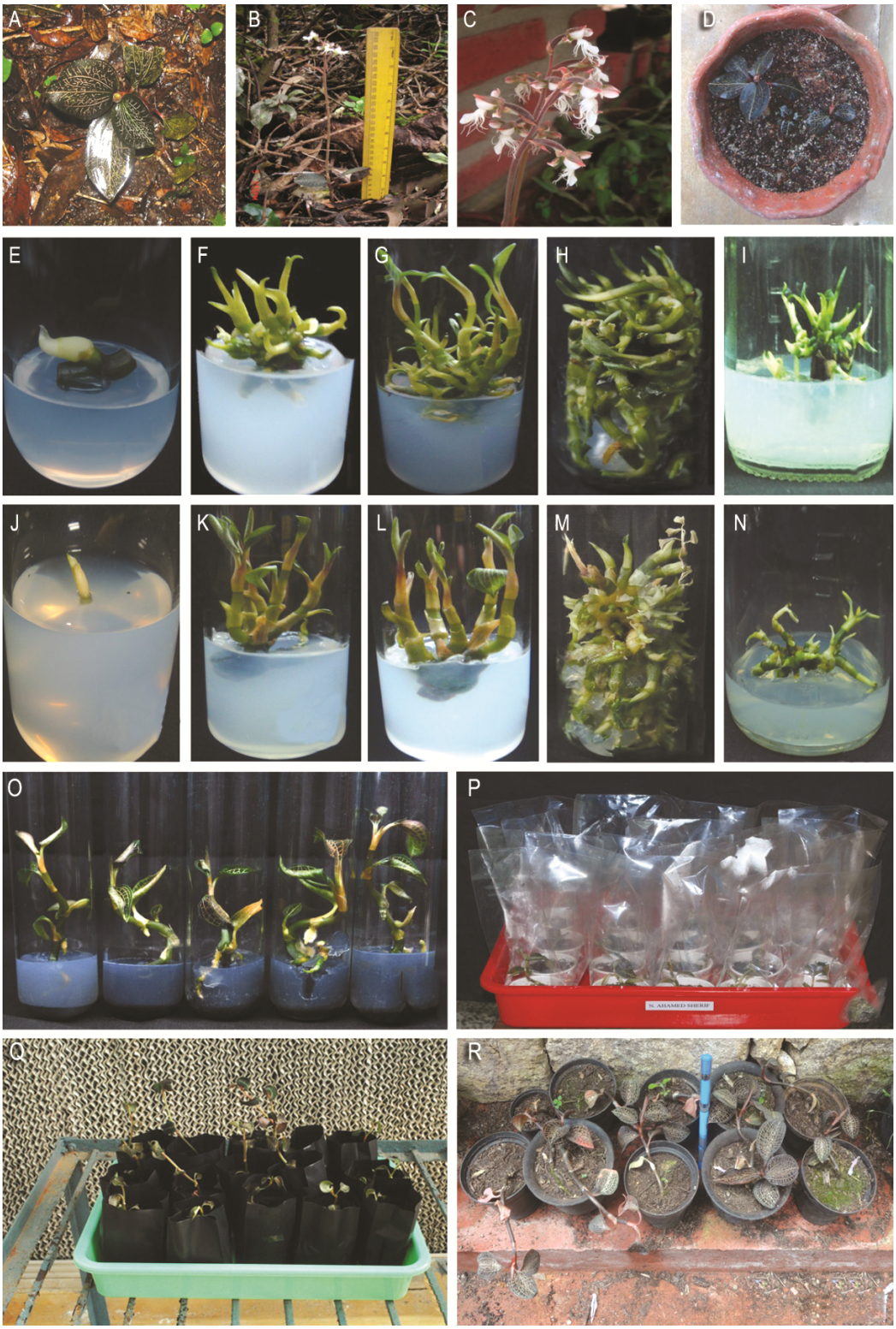

Fig. 1—Micropropagation of *Anoectochilus elatus* Lindley. All Scale bars= 1cm. (A) Habit; (B) Habit with infloresence; (C) Infloresence with flowers; (D) Wild plants maintained at green house;(E to I) shoot development from axillary bud explant; (J to N) shoot development from shoot tip explant; (O) *In vitro* rooting; (P) Hardening of regenerted plantlets covered with transparent polythene bags; (Q) Well acclimatized plants in greenhouse conditions; and (R) *In vitro* raised well acclimatized plants maintained at Vattakanal Conservation Trust, Kodaikanal, Tamil Nadu.

Data collection and statistical analysis

All the experiments were conducted as a completely randomized design. Each experiment included with seven replicates and experiments were performed three times. Data on shoot bud induction, shoot multiplication, shoot frequency percentage, number of shoot, mean shoot length (cm) were recorded every week for 4 to 12 weeks and the number of roots and average root length (cm) were recorded for 6 weeks. The data were analyzed through analysis of variance (One-wayANOVA) and significant differences between treatments were determined based on Duncan's multiple range test at $P<0.05$ level using SPSS-PASW statistical program version 18.0.0 (SPSS, Chicago, IL, USA).

Genetic stability analysis

Genomic deoxyribonucleic acid (*DNA*) *isolation*

Ten numbers of *in vitro* raised well acclimatized plants were randomly selected along with single *in vivo* mother plant and their genomic DNA was isolated from the fresh leaves using Cetyltrimethylammonium bromide (CTAB) method[19]. The purified DNA was subjected to genetic stability studies with molecular marker.

Inter Simple Sequence Repeats (*ISSRs*) *primers*

Six ISSRs primers were obtained from Xcelris Genomics & Labs Ltd, Ahmedabad, India. Each being 17-18 nucleotides in length were selected for further analysis.

Polymerase chain reaction (*PCR*)

The reactions were carried out in a total volume of 20 µL, comprising 10 µL Taq premix, 4 µL of molecular grade water, 3 µL primer and 3 µL of template DNA. The PCR amplification was performed with 96-well Thermal Cycler Eppendorf Pros, Hamburg, Germany under the following condition programmed for 40 cycles of 0.5 min at 94°C for denaturation, 0.5 min at 52°C for annealing, 0.5 min at 72°C for extension and then final extension with 7 min at 72°C. Amplified products were separated by electrophoresis on 1.2 % agarose gel buffered with 1x TAE and 3 µL ethidium bromide. Fragment sizes were estimated based on 1 kb DNA ladder (Thermo Scientific, USA) as size markers. To check the absence of contamination, DNA was replaced by water and performed experiment alongside of the every PCR amplification. DNA fragments were identified by image analysis software on gel documentation system (AlphaImager® EP Gel Documentation System).

Results

Effect of TDZ on shoot bud induction

The axillary bud and shoot tip explants were cultured on MS medium supplemented with TDZ at different concentrations (0.1-2.5 mg/L). After 4 weeks of culture, 1.5 mg/L TDZ induced maximum 4.2 shoots per explant from axillary bud (70% shoot bud initiation) followed by shoot tip explants (2.7 shoots per explant with 60% shoot bud initiation; Table 1).

Effect of TDZ with cytokinins

The respective explants were cultured on MS medium contains 1.5 mg/L TDZ supplemented with different cytokinins namely, BA, KN, 2iP and ZEA at different concentrations for shoot multiplication. After 12 weeks of culture, among the different concentrations and treatments, maximum 10.8 shoots per axillary bud (100% shoot bud proliferation) was observed in 1.5 mg/L TDZ combined with 0.01 mg/L

Table 1 — Effect of different concentrations of TDZ supplemented to MS medium on *in vitro* shoot establishment from axillary bud and shoot tips explants of *A. elatus*. (Data scored after 4 weeks of culture)

TDZ (mg/L)	Axillary bud			Shoot tip		
	Mean number of shoot/ explant	Mean shoot length/explant (cm)	Shoot bud induction (%)	Mean number of shoot/ explant	Mean shoot length/explant (cm)	Shoot bud induction (%)
Control MS medium	1.00±0.13^{c}	0.7±0.11^{c}	100±0.00^{a}	1.1±0.14^{a}	0.8± 0.16^{a}	100±0.00^{a}
0.1	2.3±0.14^{bc}	1.0±0.16^{a}	90±0.57^{a}	1.3±0.11^{b}	0.6±0.60^{a}	95±0.43^{a}
0.5	2.1±0.15^{bc}	0.4±0.32^{b}	70±1.23^{b}	2.1±0.23^{ab}	0.4±0.12^{ab}	70±1.33^{b}
1.0	2.8±0.23^{bc}	0.4±0.33^{b}	60±0.78^{d}	2.0±0.18^{ab}	0.2±0.10^{b}	43±1.23^{e}
1.5	4.2±0.14^{a}	0.6±0.42^{b}	70±1.52^{bc}	2.7±0.23^{a}	0.2±0.10^{b}	60^{b}±0.67^{c}
2.0	3.2±0.18^{ab}	0.3±0.34^{b}	50±1.33^{e}	2.1±0.13^{ab}	0.3±0.12^{b}	55±0.57^{c}
2.5	2.8±0.23^{bc}	0.5±0.12^{ab}	60±1.73^{d}	1.8±0.12^{b}	0.4±0.14^{ab}	55±0.57^{c}

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

BA (Fig. 1E & F; Table 2). Whereas, 4.6 shoots per shoot tip (90.5% shoot bud proliferation) was observed in 1.5 mg/L TDZ combined with 0.01 mg/L KN (Fig. 1J & K; Table 2).

Effect of TDZ with auxins

The MS medium contains 1.5 mg/L TDZ along with different types of auxins namely, 2,4-D, NAA and IBA at different concentrations are used for shoot multiplication. After 12 weeks of culture, maximum 10 shoots per axillary bud (100% shoot bud proliferation) followed by 4.2 shoots per shoot tip (85.7% shoot bud induction) was observed on MS medium supplemented with 1.5 mg/L TDZ and 0.01 mg/L NAA (Fig. 1G & L; Table 3).

Effect of TDZ with complex extracts and antioxidants

The MS medium supplemented with different complex extracts namely, YE, ME, BE, PEP, CW and CM at various concentrations used for synergistic effect along with 1.5 mg/L TDZ. After 12 weeks of culture, maximum 17.2 shoots per axillary bud followed by 14 shoots per shoot tip explant was observed on MS medium supplemented with 1.5 mg/L TDZ and 50 mg/L peptone (Fig. 1H & M; Table 4). Apart from these treatments, influence of some antioxidants such as AA, Glu, CA, STC, AC and PVP on multiple shoot formation was also examined. After 12 weeks of culture, maximum 10.8 shoots per axillary bud followed by 9.0 shoots per explant shoot tip was observed on MS medium supplemented with 1.5 mg/L TDZ and 50 mg/L AA (Fig. 1I & N; Table 5).

***In vitro* rooting and acclimatization**

In vitro raised shoots (≥4 cm) were transferred to MS medium supplemented with different concentrations of IAA, IBA, AC and PVP. After 6 weeks of culture, among the various treatments, 3.0 g/L AC induced maximum 3.8 number of roots with 3.1 cm

Table 2 — Effect of TDZ with cytokinins supplemented to MS medium on *in vitro* shoot multiplication from axillary bud and shoot tip explants of *A. elatus*. (Data scored after 12 weeks of culture)

Cytokinins (mg/L)				Axillary bud		Shoot tip	
BA	KN	2ip	Zea	Mean number of shoots/explant	Shoot bud proliferation (%)	Mean number of shoots/explant	Shoot bud proliferation (%)
0.01				10.8±1.01^{a}	100±0.00^{a}	3.0±0.13^{c}	80.9±1.22^{c}
0.1				6.2±1.59^{bc}	85.7±0.30^{bc}	2.6±0.40^{cd}	76.2±1.83^{cd}
0.5				6.0±1.26^{bc}	76.1±0.90^{cd}	2.2±0.48^{de}	71.4±1.63^{cd}
1.0				8.0±1.58^{ab}	85.7±0.28^{bc}	2.2±0.20^{de}	85.7±1.28^{bc}
2.0				7.0±1.41b	66.7±1.30^{e}	3.2±0.73^{bc}	85.7±1.33^{bc}
3.0				4.8±1.06^{e}	76.1±0.88^{cd}	3.4±0.92^{bc}	80.9±1.13^{c}
	0.01			5.6±0.87^{c}	76.2±0.77^{cd}	4.6±0.24^{a}	90.5±1.56^{ab}
	0.1			5.6±1.16^{cd}	61.9±1.10^{ef}	2.4±0.24^{de}	80.9±1.13^{c}
	0.5			5.6±0.50^{cd}	85.7±0.40^{bc}	2.0±0.00^{e}	71.4±1.47^{cd}
	1.0			5.4±0.87^{cd}	80.9±1.13^{cd}	3.8±1.06^{ab}	90.5±0.53^{ab}
	2.0			4.8±0.37^{de}	100±0.00^{a}	2.8±0.58^{cd}	90.5±0.48^{ab}
	3.0			4.6±1.16^{de}	76.2±0.78^{cd}	2.8±0.37^{cd}	94.7±0.33^{ab}
		0.01		6.6±1.02^{bc}	100±0.00^{a}	3.6±0.40^{b}	85.7±1.40^{bc}
		0.1		4.4±0.40^{e}	100±0.00^{a}	2.2±0.20^{de}	76.2±1.85^{cd}
		0.5		5.0±0.77^{d}	100±0.00^{a}	2.0±0.12^{e}	95.2±0.87^{a}
		1.0		4.0±0.54^{ef}	100±0.00^{a}	2.4±0.24^{d}	90.5±0.49^{ab}
		2.0		3.4±0.24^{ef}	100±0.00^{a}	2.0±0.14^{e}	80.9±1.10^{c}
		3.0		3.0±0.31^{f}	85.7±1.30^{bc}	2.4±0.24^{de}	90.5±0.43^{ab}
			0.01	2.7±0.11^{g}	61.9±1.10^{e}	2.3±0.14^{de}	73.9±1.15^{cd}
			0.1	3.3±0.20^{ef}	68.2±1.82^{ef}	2.7±0.14^{cd}	70.8±1.42^{d}
			0.5	3.2±0.14^{ef}	70.2±0.78^{de}	3.0±0.12^{cd}	89.5±1.42^{b}
			1.0	6.2±0.20^{bc}	70.8±1.23^{de}	3.8±0.43^{ab}	90.9±0.47^{ab}
			2.0	5.3±0.20^{cd}	83.7±1.33^{bc}	3.5±0.34^{bc}	68.8±1.13^{de}
			3.0	4.2±0.17^{de}	80.8±1.26^{cd}	2.3±0.43^{de}	73.4±1.60^{cd}

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

Table 3 — Effect of TDZ with auxins supplemented to MS medium on *in vitro* shoot multiplication from axillary bud and shoot tip explants of *A. elatus*. (Data scored after 12 weeks of culture)

Auxins (mg/L)			Axillary bud		Shoot tip	
2,4-D	NAA	IBA	Mean number of shoots/explant	Shoot bud proliferation (%)	Mean number of shoots/explant	Shoot bud proliferation (%)
0.01			5.6±0.87^{ab}	76.2±0.88^{cd}	3.2±0.20^{ab}	85.7±0.38^{bc}
0.1			3.8±0.37^{bc}	85.7±0.34^{bc}	2.2±0.20^{bc}	80.8±1.33^{cd}
0.5			3.4±0.74^{bc}	66.7±1.33^{d}	2.2±0.20^{bc}	95.2±0.43^{ab}
1.0			2.8±0.37^{d}	85.7±0.42^{bc}	2.0±0.00^{c}	80.9±1.13^{cd}
2.0			2.4±0.24^{de}	52.4±1.70^{ef}	2.0±0.00^{c}	90.5±0.47^{b}
3.0			2.6±0.60^{de}	95.2±0.83^{ab}	2.0±0.00^{c}	85.7±0.35^{bc}
	0.01		10.0±0.44^{a}	100±0.00^{a}	4.2±0.96^{a}	85.7±0.36^{bc}
	0.1		6.0±0.44^{ab}	100±0.00^{ab}	2.4±0.40^{bc}	66.7±1.38^{e}
	0.5		5.8±0.66^{ab}	85.7±0.36^{bc}	2.6±0.40^{bc}	76.2±0.78^{d}
	1.0		5.8±1.01^{ab}	85.7±0.33^{bc}	3.4±0.97^{ab}	71.4±1.53^{de}
	2.0		4.8±0.73^{b}	76.2±0.80^{c}	2.0±0.00^{c}	76.2±0.77^{de}
	3.0		5.2±0.96^{ab}	71.4±1.62^{de}	3.6±1.36^{ab}	80.9±1.13^{c}
		0.01	5.0±1.04^{ab}	85.7±0.32^{bc}	3.0±0.00^{b}	71.4±0.66^{de}
		0.1	2.8±0.58^{de}	100±0.00^{ab}	2.6±0.24^{bc}	95.4±0.53^{a}
		0.5	2.6±0.24^{de}	52.4±1.63^{ef}	2.0±0.00^{c}	80.5±1.43^{cd}
		1.0	3.4±0.97^{bc}	76.2±1.78^{cd}	2.0±0.00^{c}	61.9±1.23^{f}
		2.0	2.6±0.40^{de}	57.1±1.83^{e}	2.0±0.00^{c}	71.4±1.56^{de}
		3.0	4.2±0.37^{bc}	90.5±0.44^{b}	2.0±0.00^{c}	76.2±0.83^{de}

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

Table 4 — Effect of TDZ with complex extracts supplemented to MS medium on *in vitro* shoot multiplication from axillary bud and shoot tip explantsof *A. elatus*. (Data scored after 12 weeks of culture)

Complex extracts (mg/L)						Axillary bud	Shoot tip
YE	ME	BE	PEP	CW%	CM%	Mean number of shoots/explant	
50						14.2±0.67^{ab}	9.8±0.67^{bc}
100						8.4±0.87^{bc}	7.6±0.43^{cd}
200						7.2±0.73^{cd}	6.2±0.11^{de}
	50					14.8±0.67^{ab}	9.8±0.15^{b}
	100					9.6±0.50^{bc}	6.4±0.92^{de}
	200					8.6±0.22^{bc}	7.0±0.81^{cd}
		50				8.0±0.70^{bc}	5.0±0.48^{ef}
		100				5.6±0.81^{de}	3.4±0.50^{fg}
		200				4.2±0.58^{de}	4.4±0.69^{ef}
			50			17.2±0.28^{a}	14.0±0.49^{a}
			100			9.6±0.77^{bc}	11.6±0.66^{ab}
			200			8.2±0.86^{bc}	7.6±0.32^{c}
				5		10.4±0.77^{b}	5.8±0.28^{e}
				10		8.2±0.66^{bc}	4.2±0.39^{ef}
				20		7.0±0.70^{cd}	3.8±0.39^{f}
					5	7.6±0.92^{c}	4.6±0.27^{ef}
					10	6.8±0.67^{cd}	2.8±0.60^{g}
					20	5.6±0.29^{d}	3.0±0.70^{fg}

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

Table 5 — Effect of TDZ with complex extracts supplemented to MS medium on *in vitro* shoot multiplication from axillary bud and shoot tip explantsof *A. elatus*. (Data scored after 12 weeks of culture)

Antioxidants (mg/L)						Axillary bud	Shoot tip
AA	Glu	CA	STC	AC	PVP	Mean number of shoots/explant	
50						10.8±0.86^{a}	9.0±0.70^{a}
100						7.6±0.67^{bc}	4.0±0.44^{cd}
200						4.2±0.86^{cd}	3.6±0.20^{cd}
	50					9.0±0.70^{ab}	8.8±0.57^{ab}
	100					6.8±0.58^{bc}	7.8±0.66^{ab}
	200					4.8±0.58^{d}	6.6±0.50^{b}
		50				9.0±0.70^{b}	5.6±0.27^{c}
		100				6.0±0.70^{c}	6.2±0.24^{bc}
		200				5.6±0.81^{cd}	5.8±0.69^{bc}
			50			9.6±0.92^{ab}	8.6±0.27^{ab}
			100			7.8±0.96^{bc}	7.4±0.81^{ab}
			200			5.6±0.87^{cd}	5.0±0.14^{cd}
				50		3.6±0.92^{de}	2.6±0.67^{d}
				100		1.6±0.40^{ef}	1.8±0.58^{e}
				200		1.8±0.73^{ef}	1.4±0.50^{e}
					50	2.0±0.89^{e}	0.8±0.37^{ef}
					100	0.4±0.24^{fg}	0.8±0.48^{ef}
					200	0.6±0.40^{f}	1.0±0.31^{e}

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

root length and 98.5% of rooting response was observed in axillary bud derived shoots followed by 3.0 number of roots with 2.2 cm root length and 93.1% of rooting response in shoot tip derived shoots (Fig. 1O; Table 6). Rooted shoots were acclimatized in paper cups covered with polythene bags and maintained at 24±2°C and 80–85% humidity (Fig. 1P). After 2 months, the bags were removed and the humidity was gradually reduced to 60-70%. Plantlets were successfully established in the greenhouse with a 68.42% survival rate (Fig. 1Q). After successful adaptation in green house conditions the plants were shifted to Vattakanal Conservation Trust for further maintenance and conservation (Fig. 1R).

Genetic stability analysis

In the present study, DNA fingerprinting profiles for *in vivo* and 10 *in vitro* raised plants were generated and the ISSRs profile of the entire sample is represented in Fig. 2 A-F. A total of 6 ISSRs primers were used to amplify each genotype of *in vivo* and *in vitro* raised plants. All DNA samples of 11 individuals produced distinct reproducible amplification with six screened ISSRs primers. The size of the amplified

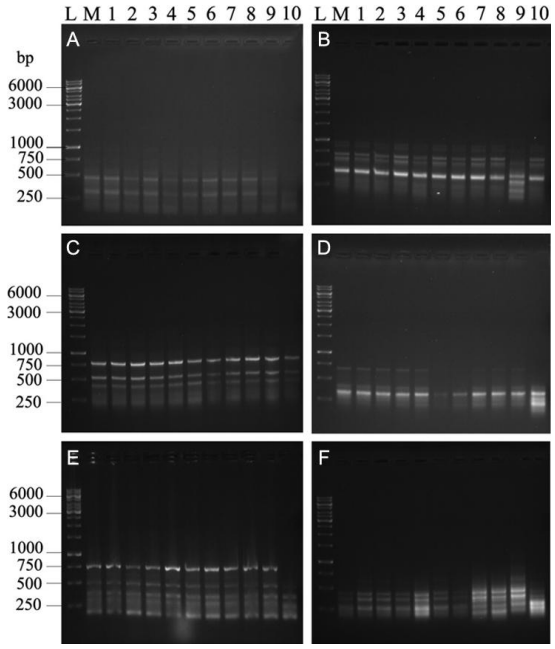

Fig. 2 — Amplified products obtained with ISSRs primers. (A) UBC-808; (B) UBC-827; (C) UBC-835; (D) UBC-36; (E) UBC-841; and (F) UBC-842. [LaneL is 1kb molecular weight marker, lane M, represents mother plant (*Anoectochilus elatus*) and lanes 1-10 represent selected sub-clones (Tissue culture raised plants)].

Table 6 — Root inductions of shoots derived from axillary bud and shoot tip explants of *A. elatus* on MS medium supplemented with auxins, activated charcoal and polyvinylpyrrolidone (Data scored after 6 weeks of culture)

Growth regulators (mg/L)	Axillary bud			Shoot tip		
	Mean number of roots/explant	Mean root length/explant	Root induction (%)	Mean number of roots/explant	Mean root length/explant	Root induction (%)
Control MS medium	1.0±0.10^{de}	0.8±0.25^{cd}	10.5±0.48^{h}	1.1±0.10^{bc}	0.3±0.08^{bc}	33.9±1.27^{e}
IAA						
1.0	1.4±0.67^{cd}	0.7±0.45^{cd}	80.9±1.23^{b}	1.5±0.50^{bc}	0.8±0.23^{bc}	57.1±1.88^{d}
2.0	1.3±0.25^{cd}	1.1±0.17^{bc}	76.2±1.88^{c}	1.3±0.94^{bc}	0.3±0.19^{bc}	71.4±1.67^{cd}
3.0	1.0±0.57^{de}	0.3±0.17^{cd}	61.9±1.14^{cd}	0.8±0.47^{cd}	0.4±0.22^{bc}	73.1±1.90^{cd}
4.0	2.5±0.28^{ab}	0.7±0.15^{cd}	100±0.00^{a}	1.3±0.75^{bc}	0.5±0.30^{bc}	76.2±1.73^{cd}
5.0	2.8±0.28^{ab}	0.7±0.14^{cd}	100±0.00^{a}	2.5±0.64^{ab}	1.0±0.12^{b}	100±0.00^{a}
IBA						
1.0	0.8±0.37^{de}	0.6±0.24^{cd}	42.9±1.32^{fg}	0.8±0.58^{cd}	0.2±0.12^{bc}	57.1±1.72^{d}
2.0	1.4±0.87^{cd}	0.1±0.08^{d}	57.1±1.87^{ef}	1.0±0.63^{cd}	0.3±0.19^{bc}	76.2±1.80^{cd}
3.0	2.0±0.40^{c}	0.6±0.18^{cd}	61.9±1.13^{cd}	1.0±0.44^{c}	0.3±0.21^{bc}	71.4±1.47^{cd}
4.0	2.3±0.62^{ab}	1.0±0.23^{c}	100±0.00^{a}	2.0±0.70^{b}	0.7±0.30^{bc}	80.9±1.33^{bc}
5.0	2.8±0.20^{ab}	1.2±0.25^{bc}	100±0.00^{a}	2.8±0.57^{ab}	0.7±0.34^{bc}	100±0.00^{a}
AC						
0.5	2.7±0.46^{ab}	2.2±0.14^{ab}	80.3±0.73^{bc}	1.6±0.23^{bc}	1.8±0.21^{ab}	83.5±1.23^{bc}
1.0	2.5±0.28^{ab}	2.7±0.23^{ab}	98.3±0.27^{ab}	2.5±0.28^{ab}	1.5±0.14^{ab}	78.3±1.47^{c}
2.0	3.0±0.12^{ab}	2.8±0.57^{ab}	86.4±0.73^{bc}	2.1±0.20^{ab}	2.0±0.33^{ab}	86.6±1.22^{b}
3.0	3.8±0.43^{a}	3.1±0.58^{a}	98.5±0.47^{ab}	3.0±0.37^{a}	2.2±0.38^{a}	93.1±0.89^{ab}
PVP						
0.5	1.2±0.45^{cd}	1.2±0.21^{bc}	58.1±1.87^{e}	1.2±0.30^{bc}	0.9±0.21^{bc}	45.3±1.67^{de}
1.0	1.2±0.18^{cd}	1.8±0.79^{b}	45.6±1.36^{f}	1.1±0.11^{bc}	0.4±0.31^{bc}	33.9±1.59^{e}
2.0	1.0±0.15^{d}	0.8±0.13^{cd}	38.2±1.67^{fg}	0.0±0.00	0.0±0.00	00.0±0.00
3.0	1.0±0.20^{de}	0.8±0.31^{cd}	20.2±1.88^{g}	0.0±0.00	0.0±0.00	00.0±0.00

Table 7 — List of primers, their sequences and size of the amplified fragments generated by Inter Simple Sequence Repeats (ISSRs) markers for DNA fingerprinting of micropropagated plants of *A. elatus*.

Sequence (5'-3') (Primer code)	Nucleotide fragment size (bp)	Total bands amplified	Average bands/ individual	Mono-morphic band	Poly-morphic band	Mono-morphism (%)	Poly-morphism (%)
AGAGAGAGAGAGAGAGC (UBC808)	150-750	53	4.8	50	3	94.33	5.66
ACACACACACACACACG (UBC827)	200-750	55	4.9	53	1	98.14	1.85
AGAGAGAGAGAGAGAGYC (UBC835)	200-750	66	6	66	0	100	0
AGAGAGAGAGAGAGAGYG (UBC36)	200-900	87	7.9	86	1	98.85	1.14
GAGAGAGAGAGAGAGAYC (UBC841)	100-900	88	8	88	0	100	0
GAGAGAGAGAGAGAGAYG (UBC842)	200-900	71	6.5	66	5	92.95	7.04
Total		420	38.1	410	10	97.61	2.38

[Data represented the Mean±SE of three repeated experiment. Means followed by the same letter in a column were not different by Duncan's multiple range test ($P \leq 0.05$)]

products ranged from 150-900 bp. A total of 420 bands were amplified with six primers. In this, 410 were monomorphic band and 10 were polymorphic. Polymorphic bands varied from 1-5 in numbers. The percentage of monomorphism is 97.61% and polymorphism is 2.38% (Table 7).

Discussion

Effect of TDZ on shoot bud induction

Thidiazuron (TDZ) is a phenylureal compound and emerged as a multidimensional plant growth regulator[20]. TDZ reveals a unique dual property of auxins, cytokinins and plays an important role in shooting and rooting[21]. The culture of axillary bud and shoot tip explants on MS medium containing 1.5 mg/L TDZ effectively induced *in vitro* shoot regeneration. While increasing and decreasing of TDZ concentrations on medium reduction in the frequency and number of shoots formation was observed. Studies on *Vanda coerulea*[22] and *Prunus dulcis*[23], showed increased and decreased shoot development based on TDZ concentration in the medium. In the present study, TDZ showed least effect on shoot bud induction from shoot tip compared to axillary bud explants. It reveals the fact that role of TDZ on the shoot bud induction was explant dependent. TDZ is known to persuade cytokinin like effect in several orchid species and provided greatest effects on different explants[24,25].

Effect of TDZ with cytokinins and auxins

The TDZ with additional PGRs induced high frequency of regeneration in several orchid species and their hybrids like, *Cattleya* hybrid[26], *Cymbidium giganteum*[27], *Vanilla planifolia*[24] and *Malaxis densiflora*[28]. To test the efficacy of TDZ along with cytokinins and auxins, the explants were cultured on MS medium containing 1.5 mg/L TDZ along with different cytokinins like BA, KN, 2iP, ZEA and auxins like 2,4-D, NAA and IBA. Axillary bud and shoot tip showed shoot bud multiplication at all concentrations of PGRs. The MS medium supplemented with TDZ and BA was more effective for axillary buds, KN for shoot tip explants. The number of shoots were increased when, 1.5 mg/L TDZ combined with the auxin NAA. In contrast, TDZ in combination with BA, KN and NAA was found to be the best for shoot proliferation. This shows that the type and combination of PGRs play a crucial role for shoot multiplication. The effective role of TDZ along with BA, KN and NAA in bud breakage and shoot proliferation has been well documented. Similar observations have also been made in *A. formosanus*[5], *A. regalis* and *A. sikkimensis*[29].

Effect of TDZ with complex extracts and antioxidants

Among the different types of complex extracts and antioxidants tested with TDZ, PEP and AA were found to be the most suitable for multiple shoot formation in both explants. Peptone is a simple nitrogen source routinely used in plant tissue culture medium and easily assimilated by plants[30]. In some circumstances, PEP is used as elicitor in *in vitro* propagation and embryo production in orchid species[31,32]. The present study indicates TDZ

supplemented with low amount of PEP stimulating multiple shoot formation. It shows that these explants required small amount of additional nitrogen containing compounds to stimulate the growth and multiple shoot formation in both explants of *A. elatus*. Other complex extracts also promoting multiple shoots but they are differed from PEP. A perusal of literatures revealed, TDZ combined with PEP beneficial for early plant development and shoot multiplication in many orchid species[33,34].

Antioxidants generally function as electron donors and avoiding quick maturity in plants helping to induce multiple shoot formation[35]. TDZ incorporated with AA increased the shoot formation in both explants followed by STC. AA proved to be obligatory for inducing shoot proliferation in *Eulophia ochreata*[36] and *Hoya wightii* ssp. palniensis[37]. STC at minimum concentration present in medium producing multiple shoots in callus culture of the same species[11]. Other antioxidants, such as CA and Glu also play a significant role in the multiple shoot formation but they are significantly differed from AA and STC. When, TDZ combined with AC and PVP did not augment multiple shoot formation.

In vitro rooting and acclimatization

Among the different concentrations of auxins, AC and PVP, AC alone promoted root formation in both types of explants. Auxins either alone or combinations (data not shown) and PVP did not enhance root formation in this species. AC promotes or inhibits *in vitro* morphogenetic characters depending on the species. It is an essential component in rooting medium for inducing dark environment and releasing undefined compounds in the medium[38]. Plastic bag cultivation method is used to grow and protect the plants from water loss, diseases and mite infections[11]. In this method, totally 50 plants were hardened and the process was completed within six months. Out of which, 68% of plants were survived successfully, remaining 32% of the plants were eliminated due to the effect of soil born fungus and varied temperature level in the environmental conditions. The similar method has been previously used in the same species and other species of *Anoectochilus*[14,39]. The well acclimatized plants were successfully maintained in Vattakanal Conservation Trust for reintroduction of this species in Kodaikanal, Western Ghats of Tamil Nadu.

Genetic stability analysis

Pre-existing bud culture technique especially *in vitro* micropropagation system is a favourable method to derive genotypically stable plants[17]. ISSRs primers were used to analyse the genetic stability of *in vitro* regenerated plants. It is a simple and fast method to target the non-coding regions of the template DNA[40]. The assessment of genetic stability were successfully analysed in *A. elatus* with the help of ISSRs primers and the percentage of polymorphism between *in vivo* and *in vitro* plant was 2.38%. It reveals that micropropagated plants were 97.61% similar that of the mother plant based on ISSRs band patterns. The present study suggests that tissue culture raised plants from axillary bud and shoot tip explants of *A. elatus* have the same genotype which is similar to the mother plant. Genetic stability analysis observed in many tissue culture raised orchid species, such as *Habenaria edgeworthii*[41], *Dendrobium bellatulum*[42], *Phalaenopsis gigantea*[43] shows similarity to that of the mother plant. These results suggest that genotypically stable true-to-type plants may be utilized for industrial oriented commercial application and germplasm conservation.

Conclusion

In conclusion, we improved *in vitro* regeneration efficacy from axillary bud and shoot tip explants of *Anoectochilus elatus* on MS medium supplemented with TDZ as potent growth regulator and combined with different types of cytokinins, auxins, complex extracts and antioxidants. *In vitro* regeneration of *A. elatus* was achieved using MS medium supplemented with 1.5 mg/L TDZ and 50 mg/L PEP. For shoot multiplication, axillary bud showed better response and produced more number of shoots as compared to the shoot tip explants. Optimal concentration of TDZ along with suitable complex extract at low concentration could increase multiple shoot formation. Genetic stability analysis using ISSRs primers revealed that *in vitro* raised plants were genetically stable and micropropagated plants were true-to-type to the mother plant. This standardized protocol may be helpful for large-scale propagation and also for conservation of this jewel orchid species.

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