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Full Length Research Paper

Regeneration of plantlets from nodal and shoot tip explants of *Anoectochilus elatus* Lindley, an endangered terrestrial orchid

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Anoectochilus elatus **Lindley is an endangered terrestrial orchid. A procedure for the regeneration of complete plantlets of *A. elatus* Lindley through node and shoot tip explants resulted directly in shoots when cultured on a full strength Murashige and Skoog (1962) medium supplemented with cytokinins at different concentrations. An average number of shoots per explant is 3 in shoot tip and 4 in node, respectively. The best shoot proliferation was observed in 3.0 mg/l 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) and the mean shoot length in 3.5 mg/l 6-furfurylaminopurine (KIN) (node) and 0.01 mg/l (shoot tip). Hundred percent rooting was achieved with the regenerated shoots in the same medium with the addition of 0.3 g/l activated charcoal (AC). Plantlets with well developed leaves and roots were transplanted to pots filled with a mixture of coconut coir, activated charcoal, commercial fertilizers (3:1:1) and acclimatized before been transferred to the greenhouse.**

Key words: *Anoectochilus elatus*, activated charcoal, regeneration, rooting.

INTRODUCTION

Orchidaceae is amongst the most diverse family of the flowering plants consisting of 35,000 species under 880 genera (Chugh et al., 2009) occurring widely in the humid tropical forests of India, Sri Lanka, South Asia, South and Central America and Mexico. Orchids belong to the highest level of organization and are distinguished by the complicated biology of reproduction with long developmental cycle (Vakhrameeva et al., 1991). The genus *Anoectochilus* consists of approximately 40 species belonging to a group of terrestrial orchids commonly known as Jewel orchids due to their attractive foliage, distributed throughout Southeast Asia, New Caledonia and Hawaii (Ket et al., 2004). *Anoectochilus elatus* Lindley is an endangered orchid endemic to Tamil Nadu and Kerala. It is a terrestrial herb found in the forest floors rich in humus in the evergreen forest at an altitude of 4265.091 ft. *A. elatus* prefers a lot of air and light but grows away from sunshine. It was listed endangered in the recent Conservation Assessment and Management Plan (CAMP) Workshop Report for Endemic Orchids of the Western Ghats (Kumar et al., 2001). IUCN (2001) categorizes a species as endangered (EN) when it meets any of the criteria A-E. The extent of occurrence of this species (IUCN Criteria B1) is about 10 to 5000 sq. km and the area of occupancy is 10 to 500 sq. km (IUCN Criteria B2). The number of subpopulation range from 10 to 50 which shows highly fragmented nature. Mature individuals in all the population are estimated to be less than 250 (IUCN Criteria D). There is continuing decline in the number of location or subpopulation as a consequence of decrease in its habitat (more than 20% in the last 10 to 20 years and more than 20% decline is predicted in the next 10 years); fragmented populations with lesser mature individuals leading to demographic

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Abbreviations: MS, Murashige and Skoog; **BA,** 6-benzyl adenine; **TDZ,** 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; **KIN,** 6-furfurylaminopurine; **AC,** activated charcoal.

instability; human interference. Based on the above data, *A. elatus* has been listed as endangered. *A. elatus* being endemic to the Tamil Nadu and Kerala is one of the few orchids or Western Ghats having an extended distribution in Eastern Ghats. A long felt need for the conservation of this species is to undertake proper survey and analyze its genetic relationship of species (Kumar et al., 2001).

Several species have been used in Chinese folk medicine and used for hypertension, lung and liver diseases (Liang et al., 1990; Kan, 1986; Zheng et al., 1996). This genus is conventionally propagated by seed; however, the germination rate is very low and nowadays this orchid is under threat of extinction due to over collection from natural resources (Belitsky and Bersenev, 1999). Plant tissue culture technology has been successfully used for the commercial production of pathogen-free plants (Debergh and Maene, 1981) and to conserve the germplasm of rare and endangered species (Fay, 1992). This paper describes the propagation of *A. elatus* by using nodal and shoot tip explants by means of micropropagation.

MATERIALS AND METHODS

The plants of *A. elatus* were collected from Pambar Shola in the Western Ghats of Tamil Nadu, India and maintained in community pots in the glass house of Bharathidasan University, Tiruchirappalli under controlled conditions (temperature 78.8 ± 35.60°F and RH 70%). Explants were collected from these plants after stabilization (Figure 1a).

Surface sterilization

Shoot tip (1 to 3 mm in length) and nodal parts (0.20 to 0.28 in length) were washed under running tap water for 45 min and surface sterilized with 70% ethanol for 30 s, then rinsed 5 times with sterile distilled water and treated with 5% sodium hypochlorite for 5 min, then again rinsed with sterile distilled water. Finally, the explants were treated with 0.1% $HgCl_2$ for 2 min and then washed thoroughly in sterile distilled water.

Influence of cytokinins

After surface sterilization, the explants were inoculated on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations (0.01to 3.5 mg/l) of cytokinins (BA, TDZ and KIN) individually for shoot bud proliferation. The medium was supplemented with 3% (w/v) sucrose and solidifying agent 0.7% agar (Bacterial grade, Himedia, India).

Rooting and transplantation of the regenerated plantlet

The individual regenerated shoots were transferred to rooting medium consisting of MS medium and activated charcoal (50 mg/l to 2.5 g/l). The rooted healthy plantlets were washed off adhering agar in sterile distilled water and were transferred to paper cups (0.98 in diameter) containing coconut coir, activated charcoal, commercial fertilizer in the ratio of 3:1:1 under controlled growth chamber conditions of 78.8 ± 35.60°F, 16 h photoperiod, 80 to 85% relative humidity and 60 µmol m^{-2} s^{-1} light intensity. The potted plants were irrigated with MS basal salt solutions every 4 days for 3 weeks and the plants were then transferred to glass house.

Culture conditions

The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl after addition of the growth regulators, prior to the addition of 0.7% agar (Himedia, India). The medium was autoclaved at 249.80°F for 30 min. All the cultures were maintained in sterilized culture room at 78.80 ± 35.60°F, under 16/8 h light regime provided by cool white fluorescent light (60 µmol m^{-2} s^{-1} light intensity) and with 55 to 60% relative humidity.

Data collection and analysis

Experiments were set up in completely randomized design and repeated three times. Each treatment had seven replications for shoot proliferation and eight replications for rooting. Observations on the number of shoots, shoot height, root and root length were recorded after 4 months of culture. Data were subjected to Duncan's multiple range tests (DMRT) (Duncan, 1955).

RESULTS AND DISCUSSION

In general, the type, concentration and combination of cytokinins will play an important role during *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). MS medium is widely used in tissue culture technology for the growth of orchid seedlings. Nodal and shoot tip explants of *A. elatus* cultured on MS medium without phytohormones supplement developed 1 to 3 shoots within 4 months of culture (Table 1). In this medium supplemented with BA, TDZ and KIN, shoot bud differentiations occurred within 20 days of culture and were free of any intervening callus or protocorm like body formation. Among cytokinins, 3.0 mg/l TDZ developed an average of 2.86 to 3.71 shoots per explants and highest mean shoot length was observed in 3.5 mg/l KIN (node) and 0.01 mg/l (shoot tip), respectively (Figure 1b, c and Table 1). KIN is the most common cytokinins, it does not affect the growth of nodal explants; 3.5 mg/l KIN developed excellent growth as compared to the other two cytokinins. However, increasing the concentrations of KIN produced lower proliferation rates and diminutive growth in shoot tip explants of *A. elatus*. Level of cytokinin, which is too high in shoot tip cultures results to small shoots which typically fail to elongate. TDZ is widely used for micropropagation of several plants including many orchids because of its incredible ability to induce organogenesis (Ernst, 1994; Nayak et al., 1997). Shoots developed on a TDZ containing medium had slow growth and morphological abnormalities as well as small leaves as compared to KIN. This may be due to the physiological condition of the explants or TDZ failure with its high cytokinin activity (Huetteman and Preece, 1993). Addition of BA had slight effect on proliferation rate as compared to the control. Different concentrations of AC were used (without phytohormones) with the aim of

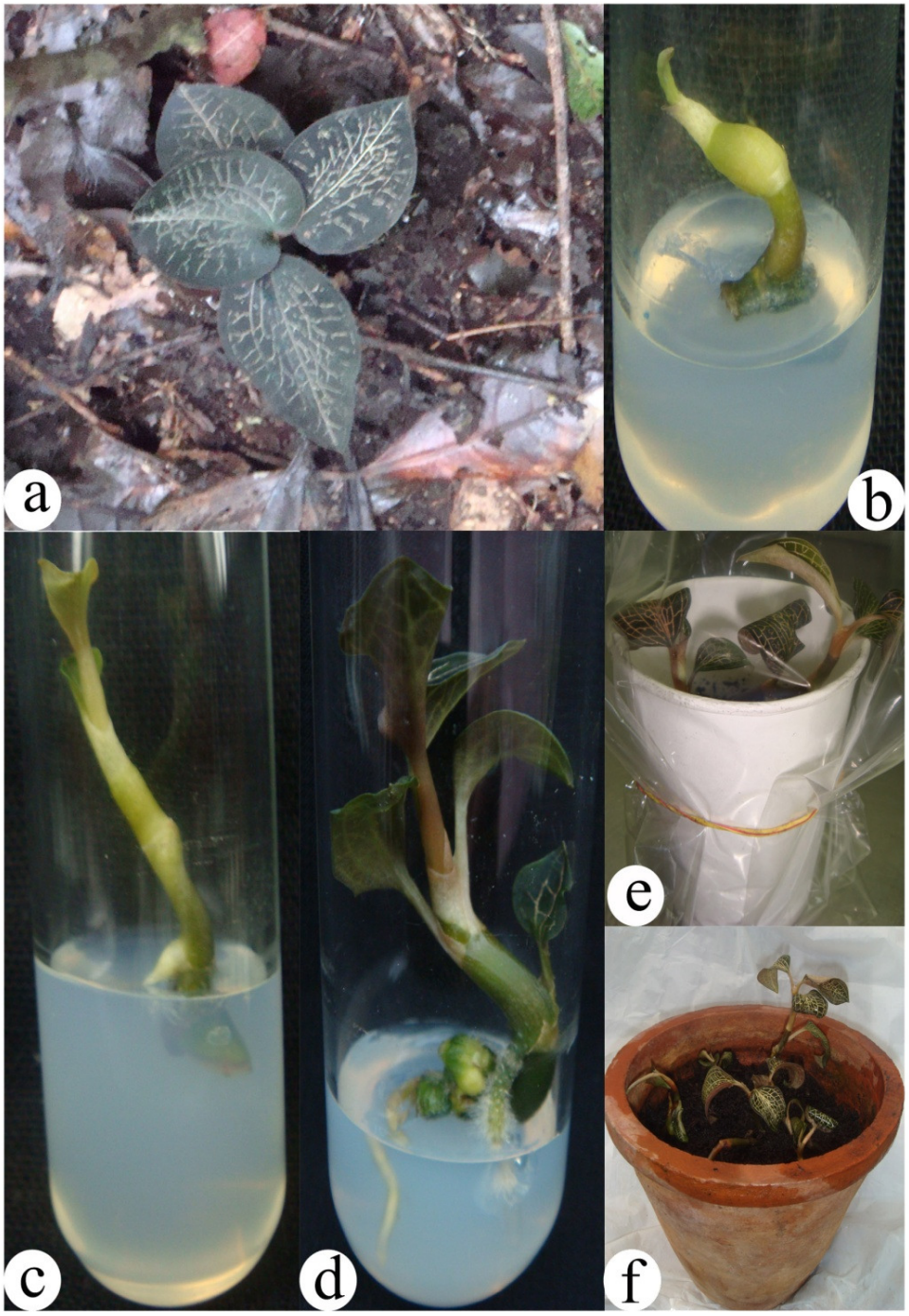

Figure 1. Micropropagation of *A. elatus* Lindley. a) Habitant; b and c) Shoot proliferation on MS + BA 2.5 mg/l; d) Rooting on MS + AC 0.3 g/l; e) Pre-hardening under culture room condition; f) Hardened plants in green house.

stimulating *in vitro* root development. Full strength MS medium supplemented with 0.3 g/l AC gave the maximum rooting response with an average of 4.25 ± 0.49 roots per microshoot and the mean root length of 0.87 ± 0.14. Young roots are very sensitive, white in color surrounded by root hair; rooted plants showed vigorous growth and produced more expanded leaves than the control (Table 2).

The positive effect of AC may be attributed to establishment of darkened environment, adsorption of inhibitory substances and the release of growth promoting substances or adsorption by AC (Pan and Staden, 1998). The tissue culture raised plants are heterotrophic in their mode of nutrition as they grow on medium rich in

Table 1. Effect of BA, KIN and TDZ on *in vitro* shoot proliferation of *A.elatus* after four months of culture.

Cytokinins concentration (mg/l)	Number of Shoots/explant		Shoot length/Explant	
	Shoot tip (Mean±SE)	Node (Mean±SE)	Shoot tip (Mean±SE)	Node (Mean±SE)
Control	1.20±0.33^{ab}	2.23±0.55^{ab}	1.59±0.35^{bcde}	2.25±0.44^{bc}
BA				
0.01	0.86±0.26^{d}	1.86±0.88^{ab}	1.88±0.43^{abcde}	2.28±0.62^{bc}
0.05	1.71±0.29^{abcd}	1.00±0.00^{ab}	2.93±0.38^{bc}	3.38±0.36^{abc}
0.1	2.14±0.51^{abcd}	1.14±0.14^{ab}	2.18±0.20^{abcde}	3.18±0.39^{abc}
0.5	1.71±0.29^{abcd}	1.14±0.14^{ab}	2.59±0.29^{abcd}	2.95±0.40^{abc}
1.0	2.71±1.06^{ab}	1.43±0.20^{ab}	2.19±0.16^{abcde}	2.64±0.37^{bc}
1.5	2.14±0.34^{abcd}	1.86±0.26^{ab}	2.11±0.23^{abcde}	2.46±0.34^{bc}
2.0	1.86±0.14^{abcd}	1.71±0.42^{ab}	2.27±0.23^{abcde}	3.35±0.34^{abc}
2.5	1.14±0.26^{bcd}	1.43±0.29^{ab}	2.59±0.19^{abcd}	3.49±0.65^{abc}
3.0	2.00±0.72^{abcd}	2.00±0.57^{ab}	1.78±0.46^{abcde}	3.43±0.30^{abc}
3.5	1.86±0.34^{abcd}	2.00±0.43^{ab}	2.72±0.42^{abc}	2.64±0.46^{bc}
TDZ				
0.01	1.71±0.42^{abcd}	2.14±0.40^{ab}	1.83±0.16^{abcde}	2.80±0.22^{abc}
0.05	2.57±0.37^{abc}	2.43±0.75^{ab}	1.65±0.38^{bcde}	2.07±0.20^{c}
0.1	2.88±0.61^{abcd}	1.86±0.14^{ab}	1.02±0.09^{e}	2.16±0.27^{bc}
0.5	1.67±0.21^{abcd}	1.14±0.26^{ab}	1.40±0.28^{cde}	1.88±0.33^{c}
1.0	2.43±0.81^{abcd}	1.14±0.26^{ab}	1.37±0.24^{de}	2.39±0.28^{bc}
1.5	1.29±0.18^{abcd}	1.71±0.52^{ab}	1.32±0.24^{de}	2.45±0.30^{bc}
2.0	2.14±0.34^{abcd}	1.29±0.29^{ab}	1.61±0.34^{bcde}	2.54±0.26^{bc}
2.5	2.14±0.55^{abcd}	1.14±0.14^{ab}	1.93±0.19^{abcde}	2.50±0.34^{bc}
3.0	2.86±0.73^{a}	3.71±0.97^{a}	2.41±0.28^{abcd}	1.83±0.46^{c}
3.5	2.57±0.29^{abc}	2.29±0.75^{ab}	2.05±0.41^{abcde}	2.84±0.30^{abc}
KIN				
0.01	2.00±0.76^{abcd}	1.43±0.43^{ab}	3.01±0.54^{a}	2.10±0.46^{c}
0.05	0.86±0.26^{d}	1.14±0.67^{ab}	2.17±0.93^{abcde}	3.41±1.01^{abc}
0.1	1.14±0.14^{bcd}	1.00±0.00^{ab}	2.26±0.26^{abcde}	2.75±0.27^{bc}
0.5	1.57±0.61^{abcd}	1.14±0.14^{ab}	1.88±0.32^{abcde}	2.23±0.48^{bc}
1.0	1.43±0.29^{abcd}	1.00±0.00^{ab}	2.31±0.33^{abcde}	2.85±0.12^{abc}
1.5	1.14±0.14^{bcd}	1.00±0.00^{ab}	2.01±0.16^{abcde}	2.38±0.38^{bc}
2.0	1.14±0.14^{bcd}	1.00±0.00^{ab}	2.31±0.16^{abcde}	2.24±0.38^{bc}
2.5	1.29±0.47^{abcd}	1.00±0.00^{ab}	2.33±0.17^{abcde}	1.85±0.40^{c}
3.0	1.57±0.37^{abcd}	1.86±0.94^{ab}	2.30±0.70^{abcde}	3.85±0.80^{ab}
3.5	1.00±0.38^{cd}	1.00±0.30^{ab}	2.63±0.70^{abcd}	4.43±1.17^{a}

[1]Means with different letters within columns are significantly different according to Duncan's multiple range tests at 0.05% level. [2]All the cultures were maintained in sterilized culture room at 78.80 ± 35.60°F, under 16/8 h light regime provided by cool white fluorescent light (60 µmol m^{-2} s^{-1} light intensity). BA, 6-Benzyl adenine; TDZ, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; KIN, 6-furfurylaminopurine.

minerals and sugar and thus, cannot withstand the environmental conditions without proper hardening and acclimatization. For hardening, the *in vitro* healthy plantlets were transferred to autoclaved paper cups containing coconut coir, AC, and commercial fertilizers (3:1:1). These plantlets were irrigated with half-strength MS medium without organics and maintained in culture room for 4 to 5 weeks (Figure 1e). This was followed by transfer to growth chamber for three weeks at relative humidity of 80 to 90% and 68 ± 75.2°F temperatures.

Table 2. Effect of activated charcoal concentrations on growth of *A.elatus* cultured on MS medium for four months.

Activated charcoal (g/l)	Number of day taken to induce root	Number of roots/microshoot (Mean±SE)	Average root length (cm) (Mean±SE)	Rooting (%)
Control	12-15	2.13±0.52^{c}	1.60±0.33^{abc}	87.50
0.05	12-15	1.88±0.79^{c}	1.15±0.45^{bc}	87.50
0.10	13-16	2.12±0.52^{c}	1.20±0.30^{bc}	50.00
0.20	12-16	2.88±0.23^{ab}	2.01±0.26^{ab}	100.00
0.30	12-15	4.25±0.49^{a}	2.21±0.35^{a}	100.00
0.40	10-15	4.00±0.87^{a}	2.20±0.28^{a}	100.00
0.50	12-15	3.00±0.63^{ab}	1.43±0.35^{abc}	100.00
1.00	10-15	3.50±0.46^{ab}	1.23±0.18^{bc}	100.00
1.50	13-19	3.25±0.25^{ab}	0.95±0.15^{c}	100.00
2.00	16-26	3.00±0.50^{ab}	1.11±0.11^{bc}	100.00
2.50	16-24	2.00±0.26^{c}	0.98±0.20^{c}	100.00

[1]Means with different letters within columns are significantly different according to Duncan's multiple range tests at 0.05% level. [2]All the cultures were maintained in sterilized culture room at 78.80 ± 35.60°F, under 16/8 h light regime provided by cool white fluorescent light (60 µmol m^{-2} s^{-1} light intensity).

Plants were then shifted to community pots containing the same mixture and maintained in the green house where a survival rate of over 80% was recorded (Figure 1f). They are easy to grow, preferring high humidity, low temperature, moist and well drained soil. For proper growth and development, maintenance of continued optimum temperature of 75.2 to 82.4°F and high humidity of 80 to 85% is essential for this species. This successful protocol is suitable for large scale propagation as well as *ex-situ* conservation of this endangered terrestrial orchid.

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