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In-vitro Regeneration of Endemic Medicinal Plant *Anodendron paniculatum* from Nodal Segment Explants

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A B S T R A C T

An effective protocol was developed for the regeneration of rare endemic medicinal plant, *Anodendron paniculatum* L. from nodal explants. The highest number of shoots (7 ± 0.05), maximum shoot length (62 ± 0.76 mm) and the highest response of shoot induction (68%) were recorded on MS medium supplemented with 3.0 mg/L BAP. Rooting was achieved with quarter strength of MS medium added with 1.0% IBA and 0.75% sucrose. The highest number of roots (8.75 ± 0.25) with 4.1 cm length was achieved. The plantlets regenerated were successfully established in earthen pots containing sterile sand and vermiculate (1:1) and grown in green house. This simple protocol can be used in large scale multiplication of this rare endemic plant.

Key words: *Anodendron paniculatum,* Regeneration, Plant growth regulators, Nodal explants, Endemic

Tissue culture technology is used to produce high quality seedlings instead of the traditionally used cuttings. It has a high fecundity, producing thousands of propagules unlike conventional techniques. Micro propagation is an effective means for rapid multiplication of endangered species in which conventional methods have limitations. Established aseptic cultures and development of an efficient protocol for regeneration and multiplication of plants are required for developing *in-vitro* strategies for conservation. Micro propagation not only ensures the supply of quality planting material on regular basis but storage of germplasm in the form of *in-vitro* cultures has been an additional advantage (Arya *et al*. 2006). *Anodendron* belongs to the subfamily Apocynoideae, family Apocynaceae (Takhtajan 2009). Anodendron genus consists of 17 species (Middleton 2007). It is naturally distributed from India, Japan, South China, to Vanuatu. According to Flora of China Editorial Committee (Ma and Clemants 2006), *Anodendron* consists of 16 species covering a large area of India, Malaysia, Sri Lanka and Vietnam. In China, there are five species of Anodendron namely *Anodendron benthamianum*, *A. howii*, *A. punctatum*, *A. affine*, and *A. formicinum*. This species does not have a local name, so there is no local knowledge regarding its potential (Widodo 2015). In Andhra Pradesh, India, *A. paniculatum* included in the list of medicinal plants with the International Union for Conservation of Nature (IUCN) status as endangered or threatened species (Ved *et al*. 2002).

The present study describes the invitro regeneration of shoot multiplication callus culture from nodal segments and effect of plant growth regulators on shoot regeneration and rooting of nodal segments from *Anodendron paniculatum*. Studies regarding regeneration of the plant by organogenesis from tissue cultures of *A. paniculatum* plant have not been reported so far. In this study, the protocol was optimized using nodal segments for regeneration of the plant.

MATERIALS AND METHODS

Plant materials

Nodal explants of 1-2 cm size containing a single node were collected from the plants growing in the Botanical garden of the University. The explants were washed thoroughly under running water for 10 min and then surface sterilized with 0.1% $HgCl_2$ for 1 min, after three successive

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washings with sterile distilled water. After sterilization once again the segments were washed repeatedly for 2-3 times in sterile distilled water, and transferred into culture tubes (25×150 mm) with 15 ml MS medium supplemented with hormones BAP, TDZ and KIN in different concentrations (1,2,3,4 and 5) for shoot inductions and auxins IBA and NAA for root induction. The culture tubes were incubated at room temperature 25±4°C under the fluorescent light with intensity from 2000-3000 lux. The final pH was adjusted to 5.8 prior to autoclaving and the cultures were incubated at 25°+5°C with 8 hours of photoperiod.

Media preparation

MS basal medium was prepared with different plant growth regulator combinations and the pH was adjusted to 5.7. Murashige and Skoog (1962) medium and stock solutions of micro salts, vitamins (w/v) and plant growth regulators (PGRs) were prepared in sterile distilled water. Vitamins and plant growth regulators like BAP, IAA, NAA and IBA were pipette out; sucrose was weighed, dissolved and the media is made up to the desired volume. The pH of the medium was adjusted to the suitable range (5.6±0.2) with 1N NaOH or 1N HCL before adding 0.8% agar and dissolving it by heating at 80°C in a water bath. Interaction between the *in-vitro* raised plantlets with the gelling agent in culture medium is a dynamic process and the changes in gel consistency affect the regeneration of plants or tissues. Traditionally, 0.8% agar is added to the culture medium to increase its viscosity. The required media were dispensed in culture vessels (15-20 ml medium in 25×150 mm culture tubes and 50-60 ml medium in 250 ml culture flasks) and closed tightly with non-absorbent cotton plugs. The medium was sterilized by autoclaving at 1.06 kg/cm pressure at 121°C for 15 min. Cultures were incubated at 24±2°C under cool white florescent light (with quantum flux density of 40 µ mol/m/s) with 16-8h regime photoperiod.

Shoot and root induction

Nodal explants were cultured in MS medium supplemented with different concentrations of (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) BAP, KiN and TDZ at each concentration 10 explants were cultured in triplicates and the data on percentage shoot regeneration, shoot number per explants and shoot length were recorded after 6 weeks of culture. For root induction, regenerated micro shoots of 4-5 cm length were excised and transferred to rooting medium comprising of MS basal medium, half and quarter strength MS with auxins- IAA or IBA (0.05, 0.10 mg, 1.5 mg/L) and along with three different concentrations (3%, 1.5% and 0.75%) of sucrose. the number of roots and length were recorded after 6 weeks of culturing.

Hardening

The plantlets after root induction were transferred to small paper cups containing the medium of sterile sand and vermiculate (1:1). These cups were covered with transparent polythene bags to maintain humidity and irrigated with ¼ strength MS salt solution without (vitamins) foe 2 weeks followed by tap water. After successful hardening process in lab, the plantlets were transferred to earthen pots kept at room temperature and finally kept in the green house.

Statistical analysis

The mean values were analyzed with SPSS (Software, version 10.0). Statistical significance were considered when p was less than 0.05

RESULTS AND DISCUSSION

The regeneration of shoots and roots from nodal explants was carried out using various concentrations of growth regulators in MS medium.

a) Effect of plant growth regulators on shooting response of nodal segments

In this study, the nodal segments were cultured by using different plant growth regulators individually in order to find out the most efficient PGR to propagate the *A. paniculatum*. The individual PGRs applied to the MS medium to initiate shoots were cytokinins like BAP, Kin and TDZ at different concentrations i.e. 1.0-5.0 mg/L and the results were recorded and tabulated (Table 1).

Table 1 Effect of plant growth regulators on shooting response of nodal segments

Plant growth Regulator conc.	% of explants responded	Number of shoots	Shoot length (mm)
	MS + BAP		
1.0 mg/L	18±0.91	1±0.02	14±0.21
2.0 mg/L	49±1.32	3±0.13	25±0.37
3.0 mg/L	68±1.90*	7±0.05*	62±0.76*
4.0 mg/L	40±1.02	4±0.10	39±0.20
5.0 mg/L	27±0.33	1±0.03	27±0.45
	MS + KIN		
1.0 mg/L	09±0.10	1±0.05	11±0.09
2.0 mg/L	18±0.19	2±0.09	17±0.15
3.0 mg/L	35±0.23	4±0.51	21±0.31
4.0 mg/L	24±0.08	1±0.25	29±0.28
5.0 mg/L	19±0.31	1±0.29	18±0.06
	MS + TDZ		
1.0 mg/L	05±0.14	1±0.00	5±0.09
2.0 mg/L	09±0.10	1±0.00	7±0.13
3.0 mg/L	0±0.0	0±0.00	0±0.00
4.0 mg/L	0±0.0	0±0.00	0±0.00
5.0 mg/L	0±0.0	0±0.00	0±0.00

Means followed by *in column are significantly different at p≤0.05

At each concentration, 10 explants were cultured in triplicates. Growth started from 3rd week, and the results were tabulated from 6th week. From the study it was evident that, the application of BAP to the medium gave best results in terms of explants response, number of multiple shoots and shoot length, when compared to other plant growth regulators like kinetin and TDZ at all concentrations. BAP at concentration of 3 mg/L showed maximum growth in cotyledonary node with 68% explants response, followed by 4 mg/L, 2 mg/L, 5 mg/L and 1 mg/L with 49%, 40%, 27%,

and 18%, respectively. Where as in terms of number of shoots and shoot length with 3 mg/L BAP, outnumbered other concentrations with 7 multiple shoots and with an average length of 62 mm, when compared to other PGRs like Kin and TDZ in this study (Fig 1). Growth increased from 1 mg/L to 3mg/L of BAP, after that the growth decreased with increase in BAP concentration above 3 mg/L, i.e. at 4 mg/L and 5mg/L. Kin at concentration of 4 mg/L showed better results within kinetin concentrations, with 35% growth response. Whereas TDZ was found to be inefficient, at lower concentrations i.e. at 1 and 2 mg/L which showed mild response with 5% and 9% growth response respectively. Plant regeneration from shoot explants of *Amsonia orientation* is achieved on MS supplemented with 1mg/1 IBA and 0.5 mg/L Kinetin (Oz *et al*. 2008). The superiority of BAP over other cytokinins on shoot induction has been reported for *Catheranthus roseus* (Mahammed *et al*. 2011). BAP at 0.5 mg/L concentration induced more shoots (9.2 + 0.37 / explant) with 3.4 cm length was reported in *Catheranthus roseus* nodal explants (Mohammed *et al*. 2011). However, in the present study, maximum of 7 roots with length of 62 mm were recorded when BAP was used as a concentration of 3 mg/L.

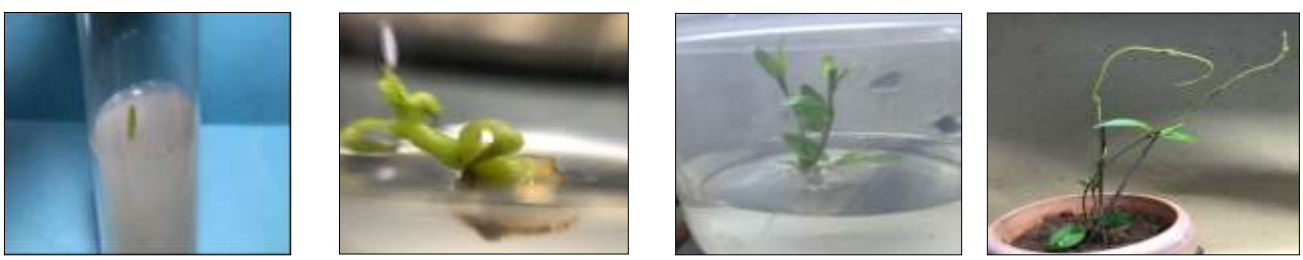
b) In-vitro rooting from the micro-cuttings of Anodendron paniculatum

To optimize the rooting response, two different auxins, IBA and IAA were tested at different concentrations. The rooting was further optimized at different concentrations of sucrose and 3 different strengths of MS medium. Quarter strength of MS medium with IBA (1.0 mg/L) was found most effective for induction of roots. In this optimized medium maximum number of roots (8.75 ± 0.25) with 4.1 + 0.18 cm length was observed. IAA in all the three different strengths of MS could not induce roots properly as IBA (Table 2).

Table 2 *In-vitro* rooting from the micro-cuttings of *Anodendron paniculatum*

(RP) PGR	PGR conc. + % (w/v) of Sucrose + Strength of MS media	No. of roots	Root length (cm)
IBA	0.5 IBA + 3% + Full strength of MS	0±0.0	0±0.0
	0.5 IBA + 1.5% + 1/2 strength of MS	0±0.0	0±0.0
	0.5 IBA + 0.75% + 1/4 strength of MS	0±0.0	0±0.0
	1.0 IBA + 3% + Full strength of MS	2.4±0.18	2.3±0.13
	1.0 IBA + 1.5% + 1/2 strength of MS	4.3±0.11	3.1±0.14
	1.0 IBA + 0.75% + 1/4 strength of MS	8.75±0.25*	4.1±0.18*
	1.5 IBA + 3% + Full strength of MS	1.47±0.41	2.1±0.20
	1.5 IBA + 1.5% + 1/2 strength of MS	1.66±0.09	2.5±0.21
	1.5 IBA + 0.75% + 1/4 strength of MS	2.11±0.21	3.1±0.10
IAA	0.5 IAA + 3% + Full strength of MS	0±0.0	0±0.0
	0.5 IAA + 1.5% + 1/2 strength of MS	0±0.0	0±0.0
	0.5 IAA + 0.75% + 1/4 strength of MS	0±0.0	0±0.0
	1.0 IAA + 3% + Full strength of MS	2.10±0.31	1.8±0.08
	1.0 IAA + 1.5% + 1/2 strength of MS	3.97±0.68	2.5±0.12
	1.0 IAA + 0.75% + 1/4 strength of MS	5.08±0.24*	3.1±0.10*
	1.5 IAA + 3% + Full strength of MS	2.23±0.06	1.8±0.17
	1.5 IAA + 1.5% + 1/2 strength of MS	2.11±0.55	1.9±0.11
	1.5 IAA + 0.75% + 1/4 strength of MS	3.86±0.67	2.6±0.28

Means followed by *in column are significantly different at p≤0.05



A: Nodal explant B: Multiple shoots C: *In-vitro* rooting D: Plantlets with leaves, stem of *A. paniculatum* in earthen pots

Fig 1 *In-vitro* regeneration and establishment of *Anodendron paniculatum*

Best rooting response were obtained with half strength of MS medium containing IBA (0.10 mg/L) where 30 healthy roots were produced with 6.87 cm root length were reported in *Catheranthus roseus* (Mahammed *et al*. 2011). Whereas maximum rooting with half strength of MS medium containing IBA (1.0 mg/L) produced 45.27 roots while it increased upto 72.02 roots/explants when IBA was mixed with NAA at 2.5 mg/L and 0.5mg/L concentrations respectively in *Catheranthus roseus* was also reported (Singh *et al*. 2011). Finally, the plantlets with Shoots and well-developed roots were transferred to small plastic cups containing sterile sand vermiculate (1:1). After successful

hardening, 92% survival rate of the plantlets was recorded in earthen pots kept in Green house. Similar reports of IBA induced rooting system was also reported in other member of Apocynaceae but not on *Anodendron* species. IBA induction rooting was reported in *Hemidesmus indicus* (Sreekumar *et al*. 2000), *Holostemmaada-kodien schult* (Martin 2002), *C. noorjahaniae* (Chavan *et al*. 2014).

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