INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY

RESEARCH ARTICLE

In-vitro Regeneration of An Endangered Medicinal Plant *Anodendron paniculatum* (Apocynaceae)

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OPEN ACCESS

Received: 29-06-2024

Accepted: 17-07-2024

Published: 26-07-2024

Citation: Reddy GS, Bhagirath G, Md Mustafa (2024) *In-vitro* Regeneration of An Endangered Medicinal Plant *Anodendron paniculatum* (Apocynaceae) . Indian Journal of Science and Technology 17(28): 2945-2953. https://doi.org/10.17485/IJST/v17i28.2121

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Funding: None

Competing Interests: None

Published By Indian Society for Education and Environment (iSee)

ISSN
Print: 0974-6846
Electronic: 0974-5645

Abstract

Objectives: The present study aims to establish a standardized and efficient regeneration protocol for *Anodendron paniculatum* A. DC. (Apocynaceae) by optimizing the effect of various plant growth regulators as it is categorized globally as an endangered medicinal plant. **Methods**: MS medium fortified with 3% sucrose (w/v) and different concentrations of cytokinins 6-benzylamino purine (BAP), kinetin, thidiazuron either alone or in combination with auxins, indole 3-acetic acid (IAA), naphthalene acetic acid (NAA) and GA_3. For the in vitro root induction, individual and healthy shoots (3-4 cm in length) were excised in the 7^{th} week and were cultured onto basal and half-strength MS medium supplemented with various concentrations of auxins and incubated in the culture room. **Findings**: MS medium supplemented with thidiazuron (2.5 μM) in combination with indole 3-acetic acid (2.5 μM) and GA_3 (1.25 μM) was most effective, providing multiple shoot regeneration for 79.33% of cotyledonary leaf explants associated with a high number of shoots per explant (20.00 ± 1.15) and length of shoot (2.66 ± 0.08 cm). Whereas, the frequency of response, mean number of shoots, and respective lengths were varied with different concentrations of 6-benzylamino purine when used in combination with IAA or NAA and BAP (5.0 μM) along with IAA (2.5 μM) at the frequency of 47.66%. Whereas, the addition of GA_3 (1.25 μM) in combination with BAP (5.0 μM) and IAA (2.5 μM) has shown a maximum number of multiple shoot formation/explant (12.33) with length of shoot (1.53 ± 0.08 cm). MS medium supplemented with 10 μM 6-benzyl amino purine and 2.5 μM naphthalene acetic acid recorded the highest response (53.66%) with more number of the shoot (9.66) and length of the shoot (1.83 ± 0.03 cm) per explant. The maximum percentage of response (84.33%) was recorded at $\frac{1}{2}$ strength MS medium with 5.0 μM NAA, whereas the highest number of roots per shoot (6.66 ± 0.33) was induced at MS + 5.0 μM NAA. The highest length of roots (3.66 cm) was recorded at basal MS medium fortified with 5.0 μM NAA and 7.5 μM NAA. **Novelty**: The standardized protocol method is of first of its kind, a quick and suitable approach for the high quantity production of *Anodendron paniculatum* with appropriate concentrations of plant growth regulators that meet the

commercial demand of endangered medicinal plants.
Keywords: *Anodendron paniculatum*; Endangered plant; Plant growth regulators; Regeneration; Protocol standardization

1 Introduction

Medicinal plants play a vital role in various ancient traditional medicinal systems like Ayurveda, Siddha, and Unani in India and emerging in other developing countries of Asia, both in preventive and curative treatments, even in the era of modern Western medicine[(1)]. The development of modern medicine in and around the 1990s, along with the introduction of modern drugs produced by pharma companies, has dealt a strong blow to traditional medicine due to the scarcity of raw materials. The high cost of modern drugs, unavailability in remote areas, and side effects of certain drugs have all led to a swing back to traditional medicine in recent years. The importance and value of traditional and indigenous herbal medicine were the subject of a campaign of the World Health Organization (WHO) which in turn in the 1970s, led to an appeal to all the nations to do their utmost to bring back the use of known and tested medicinal plants and derivatives into primary health care in rural areas and as alternatives when modern medicine was not available[(2)]. Some plant species such as *Anodendron paniculatum*, *Chamaesyce hirta, Ficus racemosa, Litsea glutinosa, Nyctanthes arbor-tristis, Ziziphus rugosa* are very important to heal bone fracture in Telangana. Among these, the local people in the area hugely depend on *Anodendron paniculatum* to cure the bone fracture which is being caused to depleting its occurrence due to its over exploitation for use. To overcome this issue, the present technique is adopted for the mass production of it in a short period.

Anodendron paniculatum is a woody climber of Apocynaceae (dogbane) family in undisturbed marshy and rocky areas, which bark and roots can be used to heal bone fractures and control vomiting and cough. It is an invasive in Tahiti from Sri Lanka. The genus *Anodendron* consists ca. 17 species and is distributed in natural forests of India, Japan, and South China. According to Flora of China Editorial Committee *Anodendron* consists of 19 plant taxa spread all over India, namely, *Anodendron benthamianum, A. formicinum, A. howii, A. paniculatum, A. punctatum, A. affine*, etc.

Plant tissue culture technology plays a very crucial role in multiplying and conserving medicinal plants, which are difficult to regenerate by conventional methods. Although tissue culture techniques have been developed for several medicinal plants but many of them need be to conserved[(3)]. By using the micro propagation technique, we can increase the rate of multiplication as well as produce pathogen-free plants. Medicinal plants are the backbone of traditional medicine because 3.3 billion people in underdeveloped countries utilize medicinal plants on a regular basis[(4)]. These medicinal plants consider as a rich resource of ingredients which can be used in synthesis and drug development[(5)].

Micropropagation involves the massive propagation of a plant from small explant tissue. A range of different explants can be used which will form adventitious shoots and/or embryos directly or indirectly via unorganized calluses[(6)]. Micropropagation is of special interest when applied to plants that requires many years to develop. Many plantlets can be produced from a small amount of stock plant in a short time, space requirements are small, plants are free of pathogens and production can be maintained continuously. Considering the medicinal importance and endangered status of the plant taxon *Anodendron paniculatatum*, an attempt has been made to achieve in vitro germination of it and plantlet establishment, and standardization of protocol for callus induction from cotyledonary leaf explants.

2 Methodology

2.1 Materials and Methods

For the present study, preliminarily field-based studies were conducted in the erstwhile Khammam district from 2019 to 2022 and selected medicinally important endangered plant *Anodendron paniculatum* A. DC. of Apocynaceae for the present study. Fresh plant parts like roots, stems, leaves, flowers, fruits, seeds, and nodal explants of *Anodendron paniculatum* were collected in a polythene bag from the Kinnerasani wildlife sanctuary area and the geographical region is recorded as $17^{o}\ 40'\ 23.00''$ N longitudes and $80^{o}\ 39'\ 00.10''$ E latitudes. The collected plant materials were authenticated by Prof. Md. Mustafa, Department of Botany, Kakatiya University, Warangal. The plant parts were dried under shade and made into herbarium specimens by following standard methods, and the voucher specimens were housed with accession numbers 1236 and 1237 at Kakatiya University Herbarium, Warangal (KUW) for further studies and reference. The plant materials were utilized as explants for the tissue culture and micropropagation studies.

2.2 Surface Sterilization of Seeds

Prior to collection, all the collected seeds from the field were stored at normal temperatures in the laboratory. Before seed surface sterilization, the seeds were hand-sorted to select healthy seeds, for each treatment 30 seeds were used. The seeds were soaked in distilled water for 10 hr and treated with different concentrations of various seed surface sterilization agents for different intervals of time in the laminar airflow cabinet as follows: i) sterilized distilled water for 15 min (control); ii) Mercuric chloride ($HgCl_2$) 0.1%, 0.2% and 0.3% w/v) for 3 and 6 min; iii) Ethanol 70% + 100 μL Tween-20 for 3 min; iv) Sodium hypochlorite (NaOCl) 2.5% and 4% (w/v) for 3 and 6 min, and v) Ethanol 70% (w/v) for 1 min and Sodium hypochlorite 2.5% and 4% (w/v) for 3 min. These seeds were subsequently rinsed with sterile distilled water about 3-4 times. Surface sterilized seeds were inoculated on $\frac{1}{2}$ strength MS medium supplemented with 1% sucrose and solidified with 0.8% agar (Hi-Media). The cultures were incubated in the culture room at 25 ± 2^{o}C under 16 hr daylight by cool-white fluorescent tube lights.

2.3 Inoculation

Surface sterilized seeds were transferred onto pre-sterilized Petri plates. Later, they were placed in culture vessels containing culture medium by removing cotton plug and closed quickly under aseptic environmental conditions. Inoculation was performed near the flame of the spirit lamp in the laminar airflow chamber to avoid contamination.

2.4 Incubation

Culture vessels after inoculation were incubated at 16/8 hrs. of photoperiod with 3000 lux intensity light and temperature about $25\pm2^{\circ}$C with 55 ± 5 percentage of relative humidity (RH). *In vitro*, plantlets were developed in the laboratory by seed germination procedure.

2.5 Induction of shoot and roots

To induce and elongate shoot, cut edges of the cotyledonary leaves of 21 days old seedling were excised and cultured on MS medium fortified with 3% sucrose (w/v) and different concentration of cytokinins BAP (0.0, 2.5, 5.0 and 10.0 μM) or Kn (0.0, 2.5, 5.0 and 10.0 μM) or TDZ (0.0, 2.5, 5.0 and 10.0 μM) either alone or in combination with auxins, IAA (1.25, 2.5 and 5.0 μM) or NAA (1.25, 2.5 and 5.0 μM) and GA3 (1.25, 2.5 and 5.0 μM). For the *in vitro* root induction, individual and healthy shoots (3-4 cm in length) were excised in the 7th week and were cultured onto basal and half-strength MS medium supplemented with various concentrations of auxins NAA, IAA and IBA and incubated in culture room (Figure 1).

3 Results and Discussion

The results of ANOVA [analysis of variance] (Table 1) indicated that the effect of seed sterilization treatment was significant ($p<0.01$) on germination rate and contamination, and germination percentage (GR, CP, and GP), mean germination time (MGT) and also on number of days to first germination (NDFG) of *Anodendron paniculatum*. The maximum percentage of germination (81.33%) was observed at 2.5% Sodium Hypochlorite for 3 min and a minimum of 32.66% at 0.2% Mercuric Chloride for 6 min, while control showed 22.00% of germination percentage. Among different treatments, the highest contamination percentage (66.66%) was recorded for Sterilized Distilled Water for 15 min and the lowest (4.00%) for 0.3% Mercuric Chloride for 3 min and 4% Sodium Hypochlorite for 6 min treatment. 2.5 % NaOCl for 6 min evolved the optimum

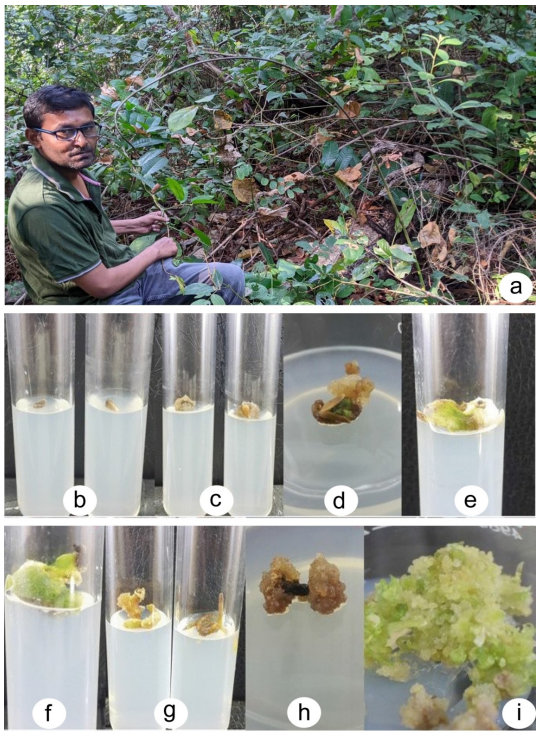

Fig 1. Regeneration of endangered medicinal plant *Anodendron paniculatum*(a) Collection of *A. paniculatum* material from the forest area; (b-h) Initiation and growth of shoot buds during 2nd to 4thweek; (i) Stem callus material of *A. paniculatum* cotyledonary leaf explants

growth rate (GR) with 12.04%. The correlations between MGT-GP and GR-MGT-GP were significant and positive. Whereas the correlations were negatively significant between MGT, GR, GP, and NDFT (Table 2). Increasing the time and concentration of $HgCl_2$ and NaOCl significantly reduced the contamination and also affected the germination of seeds in *Anodendron paniculatum*. The same results were in line with the findings in *Zehneria capillacea*[7].

Table 1. Effect of different surface sterilization methods on measured characteristics (Mean values ± Std. Error) in *Anodendron paniculatum*

Sterilant and Treatment Duration (min)	NDFG	GP	MGT	GR	CP
Sterilized Distilled Water, 15 min (control)	2.33 ± 0.33^{bcd}	22.00 ± 3.05^{g}	1.37 ± 0.30^{g}	2.36 ± 0.38^{e}	66.66 ± 1.76^{a}
0.1% $HgCl_2$, 3 min	2.00 ± 0.00^{cde}	50.66 ± 1.76^{d}	2.38 ± 0.16^{cdef}	7.41 ± 0.32^{c}	6.66 ± 0.66^{bc}
0.1% $HgCl_2$, 6 min	2.66 ± 0.33^{abc}	41.33 ± 1.76^{e}	2.82 ± 0.16^{cd}	3.92 ± 0.33^{de}	5.33 ± 0.66^{bc}
0.2% $HgCl_2$, 3min	2.66 ± 0.33^{abc}	38.66 ± 1.76^{ef}	1.94 ± 0.18^{fg}	4.33 ± 0.10^{d}	6.00 ± 1.15^{bc}
0.2% $HgCl_2$, 6 min	3.33 ± 0.33^{a}	32.66 ± 2.40^{f}	1.77 ± 0.10^{fg}	3.39 ± 0.36^{de}	^{bc} 5.33±0.66
0.3% $HgCl_2$, 3min	3.00 ± 0.00^{ab}	34.66 ± 3.33^{ef}	2.04 ± 0.23^{ef}	3.51 ± 0.28^{de}	4.00 ± 1.15^{c}
70% Ethanol + 100 μL Tween 20, 3 min	1.66 ± 0.33^{def}	39.33 ± 2.40^{ef}	2.22 ± 0.10^{def}	4.65 ± 0.45^{d}	5.33 ± 0.66^{bc}
2.5% NaOCl, 3min	1.33 ± 0.33^{ef}	81.33 ± 1.76^{a}	4.03 ± 0.40^{a}	10.82 ± 0.51^{ab}	8.00 ± 1.15^{b}
2.5% NaOCl, 6 min	1.00 ± 0.00^{f}	78.66 ± 2.40^{ab}	4.00 ± 0.40^{a}	12.04 ± 0.94^{a}	6.66 ± 0.66^{bc}
4% NaOCl, 3 min	1.66 ± 0.33^{def}	73.33 ± 2.90^{bc}	3.46 ± 0.12^{ab}	10.57 ± 0.91^{ab}	6.66 ± 0.66^{bc}
4% NaOCl, 6 min	1.66 ± 0.33^{def}	68.00 ± 3.05^{c}	2.94 ± 0.12^{bc}	9.76 ± 0.77^{b}	4.00 ± 1.15^{c}
70% Ethanol + 2.5% NaOCl, 3min	2.00 ± 0.00^{cde}	54.66 ± 2.90^{d}	2.67 ± 0.26^{cde}	6.89 ± 0.59^{c}	6.00 ± 1.15^{bc}

Continued on next page

Table 1 continued

70% Ethanol + 4% NaOCl, 3 min	2.00±0.00^{cde}	50.66±0.66^{d}	2.04±0.07^{ef}	8.10±0.51^{c}	5.33±0.66b^{c}

Means sharing the same letter within columns is not significantly different ($p \leq 0.05$) using Duncan's multiple range test; NDFG = Number of Days to first germination; GP = Germination Percentage; MGT= Mean Germination Time; GR = Germination Rate; CP = Contamination Percentage

Table 2. The phenotypic correlation coefficient of measured traits under various sterilization treatments in *Anodendron paniculatum*

	NDFG	GP	MGT	GR	CP
NDFG	1				
GP	-0.701**	1			
MGT	-0.620 **	0.909 **	1		
GR	-0.766 **	0.951 **	0.803 **	1	
CP	0.059	-0.426 **	-0.389 *	-0.359 *	1

**Significant correlation at 0.01 level (2-tailed) and * Significant correlation at 0.05 level (2-tailed). NDFG = Number of Days to first germination; GP = Germination Percentage; MGT = Mean Germination Time; GR = Germination Rate; CP = Contamination Percentage.

3.1 Effect of TDZ (thidiazuron) in combination with NAA/IAA on induction and multiplication of shoots from cotyledonary leaf explants of *Anodendron paniculatum*

Cotyledonary leaf explants cultured on MS medium with different combinations of auxin/cytokinin demonstrated shoot formation after 2 weeks of incubation. The rate of shoot induction varied depending on the combination and concentration of applied growth regulators (Table 3). Explants did not respond on plant growth regulator-free medium (control). Development of multiple shoots induction was observed from cut ends of the cotyledonary leaf explants in all the concentrations and combinations of PGRs used after two weeks of inoculation. TDZ (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) alone produced green callus for all concentrations tested, however, adventitious shoot buds were induced on MS medium supplemented with all combinations and concentrations of TDZ and NAA/IAA after 1-2 weeks. A lower concentration of TDZ (2.5 μM), as well as IAA (2.5 μM), showed more multiple-shoot induction from cotyledonary leaf explants of *Anodendron paniculatum*. Increased NAA concentration (5 μM) resulted in an increased frequency of shoot induction which is inversely proportional to the shoot regeneration frequency shown in the Himalayan Indica rice variety SR4 [8]. The optimum level of NAA (5 μM) with combination of TDZ and GA_3, yielded an average of 46.66 % respond with more number of multiple shoot formation per explant in comparison to other concentration of NAA tested. MS medium supplemented with TDZ (2.5 μM) in combination with IAA (2.5 μM) and GA_3 (1.25 μM) was most effective, providing multiple shoot regeneration for 79.33% of cotyledonary leaf explants associated with a high number of shoots per explants (20.00±1.15) and length of shoot (2.66±0.08 cm). Increased concentration of GA_3 resulted in a dropdown of shoot induction. TDZ directly promotes growth due to its biological activities in a fashion similar to that of an N-substituted cytokinin or it may induce the synthesis and accumulation of an endogenous cytokinin in different plants [9]. A synergistic effect of TDZ in combination with an auxin has been demonstrated to promote axillary shoot proliferation and shoot formation in plants where lower concentrations of TDZ result in greater axillary proliferation than cytokinin [10].

Table 3. Effect of TDZ (thidiazuron) in combination with different auxins and GA_3 on shoot bud proliferation from cotyledonary leaf explants in *Anodendron paniculatum*

Concentration of PGRs (μM)				Frequency of response (%)	The mean number of Shoots/Explant (±SE)	Mean Length of Shoots (cm) (±SE)
TDZ	NAA	IAA	GA_3			
2.5	2.5			42.33	11.00 ± 0.57	1.53 ± 0.88
2.5	5.0			45.00	13.33 ± 0.33	1.60 ± 0.57
5.0	2.5			40.33	12.00 ± 0.57	1.16 ± 0.88
5.0	5.0			41.00	9.67 ± 0.88	0.86 ± 0.08
7.5	2.5			34.00	9.67 ± 0.33	0.80 ± 0.05
7.5	5.0			31.33	9.33 ± 0.88	0.86 ± 0.03
2.5	5.0		1.25	46.66	16.67 ± 0.88	1.60 ± 0.05

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Table 3 continued

2.5	5.0		2.5	45.66	14.67 ± 0.88	1.56 ± 0.13
2.5		2.5		65.00	15.00 ± 0.57	1.73 ± 0.08
2.5		5.0		75.00	16.67 ± 0.33	2.53 ± 0.17
5.0		2.5		63.00	14.67 ± 0.66	1.60 ± 0.11
5.0		5.0		55.00	13.33 ± 0.33	1.66 ± 0.08
7.5		2.5		48.33	13.33 ± 0.88	1.63 ± 0.03
7.5		5.0		45.66	14.67 ± 0.66	1.60 ± 0.05
2.5		2.5	1.25	79.33	20.00 ± 1.15	2.66 ± 0.08
2.5		2.5	2.5	72.33	18.00 ± 1.15	2.46 ± 0.14

Mean sharing the same letter within columns are not significantly different ($p \leq 0.05$) using Duncan's multiple range test; PGRs = plant growth regulators; TDZ = thidiazuron; NAA = naphthalene acetic acid; IAA = indole 3-acetic acid; GA = gibberellic acid; SE = standard error.

3.2 Effect of BAP (6-benzyl amino purine) in combination with NAA/IAA on induction and multiplication of shoots from cotyledonary leaf explants of *Anodendron paniculatum*

In order to know the synergistic effect, the cotyledonary leaf explants of *A. paniculatum* were cultured on MS medium fortified with different concentrations of BAP (2.5 and 5.0 μM) in combination with 2.5 and 5.0 μM of IAA/NAA (Table 4). Direct shoot regeneration was observed in all these concentrations of BAP with combination of auxins used. MS medium with BAP and IAA compared to NAA significantly increased multiple shoots from cotyledonary leaf explants of *A. paniculatum*. The frequency of response, mean number of shoots and respective lengths were varied with different concentrations of BAP when used in combination with IAA or NAA. BAP (5.0 μM) along with IAA (2.5 μM) at a frequency of 47.66% has shown similar results in Andrographis paniculata[11]. Whereas, the addition of GA_3 (1.25 μM) in combination with. BAP (5.0 μM) and IAA (2.5 μM) have shown a maximum number of multiple shoot formation/explant (12.33) with length of shoot (1.53 ± 0.08 cm)[6]. The correlation between the average number of shoots per explant and average shoot length was significant and positive at the 0.01 level (2-tailed) (Table 4).

Table 4. Effect of BAP in combination with different auxins and GA_3 on shoot bud proliferation from cotyledonary leaf explants in *A. paniculatum*

Concentration of PGRs (μM)				Frequency of Response (%)	Mean Number of Shoots/Explant (±SE)	Mean Length of Shoots (cm) (±SE)
BAP	NAA	IAA	GA_3			
2.5	2.5			27.33	5.67 ± 0.33	0.70 ± 0.05
2.5	5.0			30.00	5.33 ± 0.33	0.66 ± 0.08
5.0	2.5			30.00	4.33 ± 0.88	0.50 ± 0.00
5.0	5.0			38.00	5.00 ± 0.57	0.63 ± 0.08
2.5	5.0		1.25	40.33	8.33 ± 0.88	0.83 ± 0.03
2.5		2.5		29.33	5.00 ± 0.57	0.66 ± 0.03
2.5		5.0		34.66	6.33 ± 0.88	1.20 ± 0.11
5.0		2.5		47.66	8.00 ± 0.57	1.33 ± 0.06
5.0		5.0		36.33	9.33 ± 0.33	1.06 ± 0.12
5.0		2.5	1.25	54.33	12.33 ± 0.88	1.53 ± 0.08

This means sharing the same letter within columns is not significantly different ($p \leq 0.05$) using Duncan's multiple range test; PGRs = plant growth regulators; TDZ = thidiazuron; NAA = naphthalene acetic acid; IAA = indole 3-acetic acid; GA = gibberellic acid; SE = standard error.

3.3 Effect of BAP/Kn in combination with NAA on multiple shoot induction from cotyledonary leaf explants of *Anodendron paniculatum*

Results of shoot multiplication in *A. paniculatum* at different concentrations of BAP/Kn in combination with NAA were tabulated (Table 5). During 4 weeks, on all tested treatments, shoots were induced directly from meristematic cotyledonary leaf explants. Analysed ANOVA test results, revealed that the PGR combinations and their concentrations highly affected the direct regeneration values using cotyledonary leaf explants in *Anodendron paniculatum*. BAP revealed a better response than Kn. When the concentration of Kn was exceeded from 7.5 μM there was a notable decrease in the frequency of response and shoot length from cotyledonary leaf explants. This indicated that Kn has a low effect when used at higher concentrations than

the optimum level[12]. On the media containing 10 μM BAP, (28%) of cotyledonary leaf explants showed shoot formation with 7.66 mean number of shoots per explant during 2 months. MS medium supplemented with 10 μM BAP and 2.5 μM NAA recorded the highest response (53.66%) with more number of shoots (9.66) and length of shoots (1.83 $\pm$ 0.03 cm) per explant in comparison to other concentrations of BAP and Kn have been examined. In contrast, further increase in NAA levels resulted decrease in shoot elongation. Therefore, there is a direct relationship between shoot length and the number of shoots proliferating from cotyledonary leaf explants regeneration of *A. paniculatum* as similar results were found in sesame[13].

Table 5. Effect of BAP/Kn alone or in combination with NAA on multiple shoot induction from cotyledonary leaf explants in *Anodendron paniculatum*

Concentration of PGRs (μM)			Frequency of response (%)	Mean number of Shoots/Explant ($\pm$SE)	Mean Length of Shoots (cm) ($\pm$SE)
BAP	Kn	NAA			
2.5			7.66	3.00 $\pm$ 0.57	1.10 $\pm$ 0.05
5.0			11.33	3.00 $\pm$ 0.57	1.20 $\pm$ 0.11
7.5			21.33	4.33 $\pm$ 0.66	1.33 $\pm$ 0.08
10.0			28.00	7.66 $\pm$ 0.88	1.43 $\pm$ 0.08
	2.5		5.33	2.33 $\pm$ 0.33	0.60 $\pm$ 0.05
	5.0		6.66	2.66 $\pm$ 0.66	1.10 $\pm$ 0.05
	7.5		10.66	4.33 $\pm$ 0.33	0.90 $\pm$ 0.05
	10.0		10.33	6.33 $\pm$ 0.33	0.70 $\pm$ 0.10
7.5		2.5	25.00	4.66 $\pm$ 0.33	1.23 $\pm$ 0.12
10.0		2.5	53.66	9.66 $\pm$ 0.88	1.83 $\pm$ 0.03
7.5		5.0	26.66	3.66 $\pm$ 0.33	0.66 $\pm$ 0.03
10.0		5.0	31.00	6.00 $\pm$ 0.57	0.83 $\pm$ 0.03
	7.5	2.5	31.33	5.0 $\pm$ 0.00	1.00 $\pm$ 0.05
	10.0	2.5	22.00	3.66 $\pm$ 0.33	0.96 $\pm$ 0.03
	7.5	5.0	19.66	4.00 $\pm$ 0.57	0.80 $\pm$ 0.05
	10.0	5.0	20.66	3.33 $\pm$ 0.33	0.63 $\pm$ 0.06

Means followed by the same letter are not significantly different using Duncan's multiple range tests ($p \leq 0.05$); PGRs = plant growth regulators; BAP = 6-benzylamino purine; Kn = kinetin; NAA = naphthalene acetic acid; SE = standard error.

The different system for seed surface sterilization and growth media composition not only has an effect on the uniform growth of seedlings but also affect the germination percentage. Hence, in our study, using 2.5% Sodium Hypochlorite for 3 min was a more suitable surface sterilization agent and uniformity growth of seedlings. In addition, $\frac{1}{2}$ strength MS medium with 1% (w/v) sucrose proved to be the best medium (81%) for germination and seedling development in *in-vitro* conditions[14,15]. More concentration of sucrose and MS medium strength caused to delay in seed germination and also decreased the percentage of seed germination, length of shoot, and length of root[16]. The analysis of data indicated the correlation between GP, GR, shoot length and root length was significant and positive.

3.4 *In vitro* rooting and Acclimatization

Well-developed *in vitro* regenerated plantlets along with rooting (4-6 cm in length) with six to eight leaves washed in distilled water to remove agar and were transferred to the pot containing sterilized garden soil, sand, and organic manure (2:1:1). Pots were covered with polyethylene bag to maintain relative humidity (80%) and kept in culture room at 25 $\pm$ 2°C under 16-h daylight. After 6 weeks plants were transferred to the greenhouse and maintained and eventually to the field.

Elongated shoots developed through cotyledonary leaf explants of *Anodendron paniculatum* were excised and transferred onto basal MS and $\frac{1}{2}$ strength MS medium supplemented with different concentrations of NAA/IAA/IBA (Table 6). Elongated shoots did not respond on MS medium without PGRs (control). In the second week of inoculation in rooting media, *in vitro* roots were observed from the basal region of micro shoots. Roots were developed in all concentrations of PGRs used. NAA was found to be superior to IAA and IBA for root formation and similar results were observed in sarpagandha[17]. The maximum percentage of response (84.33%) was recorded at $\frac{1}{2}$ strength MS medium with 5.0 μM NAA whereas the highest number of roots per shoot (6.66 $\pm$ 0.33) was induced at MS + 5.0 μM NAA[18]. The highest length of roots (3.66 cm) was recorded at basal MS medium fortified with 5.0 μM NAA and 7.5 μM NAA. Well-developed plantlets were transferred to the greenhouse and the survival rate was observed to be 75% to 80%.

Table 6. Effect of basal MS and $\frac{1}{2}$ MS supplemented with different concentrations of NAA, IAA, and IBA on in vitro rooting from regenerated shoots in *Anodendron paniculatum*

Concentration of PGRs			Frequency of Response (%)	Mean Number or Roots/Explant (±SE)	Mean Length of Roots (cm) (±SE)
NAA	IAA	IBA			
	MS+				
2.5			46.33	5.33±0.33	2.66±0.33
5.0			81.00	6.66±0.33	3.66±0.88
7.5			71.33	4.66±0.33	3.66±0.33
	2.5		38.66	2.66±0.33	0.43±0.03
	5.0		40.66	2.00±0.01	0.53±0.08
	7.5		56.33	2.00±0.57	0.90±0.05
		2.5	54.33	3.00±0.57	1.66±0.33
		5.0	65.33	6.33±0.66	3.00±0.57
		7.5	52.00	3.00±0.57	1.66±0.33
	$\frac{1}{2}$ MS+				
5.0			84.33	4.33±0.33	1.53±0.08
	7.5		58.33	3.33±0.33	1.33±0.08
		5.0	68.66	1.66±0.33	1.63±0.03

This means sharing the same letter within columns is not significantly different ($p \leq 0.05$) using Duncan's multiple range test; PGRs = plant growth regulators; NAA = naphthalene acetic acid; IAA = indole 3-acetic acid; IBA = indole 3-butyric acid; SE = standard error.

Various scientists worked on the antioxidant activity of the different parts of Apocynaceae members including the *Holarrhena antidysenterica* plant, *Tabernaemontana divaricata* leaves, *Carissa carandas* leaves, *Alstonia scholaris* flowers and *Anodendron paniculatum* adult plant and callus [19]. The previous studies demonstrated that the extracts of root, stem, and leaves of members of Apocynaceae showed antimicrobial activity against *Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa* [20].

4 Conclusion

The success rate of the micropropagation method depends on different factors like the culture medium, plant growth regulators, plant genotype, hygienic environment, and explant developmental stage [21] and the technique is highly important for mass production of rare, endangered, and threatened plant species. In the present investigation, we have provided the first report for direct regeneration from cotyledonary leaf explants in *Anodendron paniculatum*. Cotyledonary leaf explants were cultured on MS medium supplemented with different concentrations and combinations of auxin/cytokinin demonstrating shoot formation after 2 weeks of incubation. In the present study, MS medium supplemented with TDZ (2.5 μM) in combination with IAA (2.5 μM) and GA_3 (1.25 μM) was most effective, providing multiple shoot regeneration for *A. paniculatum* whereas, the addition of GA_3 (1.25 μM) in combination with BAP (5.0 μM) and IAA (2.5 μM) have shown a maximum number of multiple shoot formation per explant. MS medium supplemented with BAP (10 μM) and NAA (2.5 μM) were recorded the highest response with more number of shoot and length of shoot per explant in *Anodendron paniculatum*. Maximum percentage of response was recorded at $\frac{1}{2}$ strength MS medium with NAA (5.0 μM) whereas the highest number of roots per shoot was induced at MS + 5.0 μM NAA. The highest length of roots was recorded at basal MS medium fortified with NAA (5.0 and 7.5 μM). The present study for the first time standardized the protocol for reproducible and straightforward for *in vitro* micropropagation of *Anodendron paniculatum*.

Acknowledgments

The authors acknowledge their sincere thanks to the Head, the Department of Botany, for providing facilities to carry out the research work.

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