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In vitro Shoot Multiplication of Medicinally Important Plant *Andrographis lineata* Nees via Nodal Explants

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Authors' contributions

This work was carried out in collaboration between both the authors. Author VA carried out experimental work, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author TP planned and designed the investigation and managed the analyses of the study and the literature searches. Both the authors read and approved the final manuscript.

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ABSTRACT

Andrographis lineata Nees (Acanthaceae) is an endemic herb rich in flavonoids and diterpenoids which have pharmaceutical importance. Seedling explants source has not been studied for micropropagation and hence the present investigation has been taken up. An efficient protocol was developed for aseptic seed germination and *in vitro* shoot multiplication from different aseptic seedling explants. 30 days old aseptic seedlings grown on half strength MS medium were used as source of explants. Shoot tips, nodal segment, cotyledonary nodes, leaves, internodes and root segments were cultured on MS medium supplemented with BA 8.87 µM for testing for their responsivity. Nodal segments gave a maximum response when compared to other seedling explants and therefore were used in further experiments. Nodal segments were cultured on MS medium supplemented with various concentrations of cytokinins individually and in combination with auxins. Maximum number of 32.04 shoots per nodal explant were produced after 30 days of culturing on BA 8.87 µM + Indole-3-Butyric acid (IBA) 6.54 µM. Number of shoots further increased by sub culturing into fresh medium containing MS medium supplemented with BA 8.87 µM. Shoots were rooted on half strength MS medium and rooted plants were hardened in polycups containing sterile peat moss, farmyard manure and soil (1:1:1) with 75% survival rate.

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1. INTRODUCTION

Andrographis lineata Nees (Acanthaceae), an erect, perennial herb, distributed in South India, is used in herbal medicine. It also serves as good substitute for *Andrographis paniculata*. It is an endemic herb rich in flavonoids and diterpenoids which has pharmaceutical importance. Three flavonoids, diterpenoids and six known flavonoids were isolated from the whole plant of *A. lineata* [1]. *A. lineata* is used by traditional medicinal practitioners as mucolytic agent to treat all respiratory infections. The main active principle, andrographolide is extensively used in pharmacopy. Leaf powder of *A. lineata* is mixed with the cow or goat milk and taken orally for treatment of diabetes and also paste of this plant is applied externally on bitten site of scorpion string and snake bites [2]. In addition, leaf extract of *A. lineata* possesses antibacterial activity [3]. The medicinal properties of *Andrographis lineata* include antibacterial, antidiabetic, antipyretic, diuretic, hepatoprotective and anti-inflammatory activity [4].

Commercial propagation of this important medicinal and endemic plant species has not been attempted apparently due to the non availability of sufficient plant material, presently only wild population is exploited for extraction purposes. For conventional propagation seeds have poor seed viability and low germination rate which can not meet the demand for this valuable plant in time. Micropropagation is an important biotechnological tool to select, multiply and conserve *A. lineata*. Micropropagation of *Andrographis lineata* was investigated by Deepa et al. [5] and they used shoot tip as explants source. The protocol developed for *A. lineata* requires 4 months, additional elongation stage and more hormones which genuinely limit possible use for commercial propagation. The main aim of the present work was to find out the suitability of nodal explants from *in vitro* grown aseptic seedlings and for enhanced multiple shoot induction in short time and cost effective.

Tissue culture work on other species of the genus *Andrographis* includes *A. alata* [6], *A. neesiana* [7] and *A. paniculata* [8,9,10,11,12,13].

2. MATERIALS AND METHODS

Mature pods of *A. lineata* were collected from Palni hills of Tamilnadu, India. Healthy seeds were separated from pods and stored under room temperature. The seeds were washed thoroughly under running tap water for 5 min, treated with 1% v/v Tween 20 (Merck, India) for 30 minutes with constant agitation and washed with sterile distilled water until the traces of Tween 20 were removed. Under sterile conditions the seeds were immersed in 70% ethanol for 60 seconds followed by surface sterilization with 30% hydrogen peroxide for 5 minutes. After surface sterilization the seeds were finally rinsed thoroughly with sterile distilled water for 4-5 times. The sterilized seeds were inoculated aseptically on half strength MS [14] medium gelled with 0.6% agar.

Shoot tips, cotyledonary nodes, nodal explants, leaves, internodes and root segments of 30 days old aseptic seedlings were used for experiments. The seedling explants were inoculated on MS medium supplemented with 6-benzyladenine (BA) 8.87 µm to identify best seedling explants for further experiments. Since nodal explants were found to be better they were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins BA, Kinetin (KN), N^6-(Δ^2 – isopentenyl) adenine (2iP)) individually or in combination with auxins such as α – naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) (Table 1). The pH of the medium was adjusted with 1N NaOH or 1N HCl to 5.8. The medium was gelled with 0.8% w/v agar. 15 ml of medium was dispersed to each test tube (150 × 25 mm, Borosil, India) and closed tightly with aluminum foil. The medium was sterilized at 15 lbs pressure and 121ºC for 15 minutes. All the culture tubes were incubated at 25 ± 2ºC under 16 hours photoperiod of 45-50 µ mol/m^2/s irradiance provide by cool white fluorescent tubes (Philips, India). After four weeks of incubation the cultures were transferred to fresh medium containing MS medium supplemented with BA 8.87 µm in cultures bottles for further shoot multiplication. Microshoots with 5-6 nodes were excised from each culture passage and inoculated on full, half and quarter strength MS basal medium, Gamborg's B_5 medium (B_5) [15] and Woody plant medium (WPM) [16] gelled with 0.6% agar for *in vitro* rooting. For shoot initiation and shoot multiplication 3% sucrose and for *in vitro* rooting and aseptic seed germination 1% sucrose was added to culture medium.

The plantlets with well developed roots were taken out from culture medium and washed with sterile distilled water to remove the traces of agar. The plantlets were transferred to plastic pots with 5 cm diameter containing sterile peat moss, farmyard manure and soil (1:1:1) and covered with polythene cover. The plantlets were irrigated with quarter strength MS basal salt solution devoid of sucrose for every 3 days for 3 weeks. The plants were kept under shade for 4 weeks and then transferred to field conditions.

The experiments were conducted in a randomized block design and each was repeated twice with 20 replicates per treatment. Observations were recorded for every 4 weeks of culture. To detect the significance of differences among the treatments analysis of variance (ANOVA) was performed. The treatment means were compared using Tukey test at a 5% probability level.

3. RESULTS AND DISCUSSION

3.1 Selection of the Explant

Organogenic potential of a species depends upon the explants used and physiological status of the sources. Because most of the explants responded differently in their ability for shoot induction it is important to identify the correct explant for micropropagation. This may be due to differences in nutritional requirements and storage components among different parts of the same plant and also various levels of endogenous plant growth regulators. To test the best explant for shoot multiplication different aseptic seedling explants of *Andrographis lineata* such as cotyledonary nodes, nodal explants, shoot tips, internodes, leaves and root segments were used as explants.

In the present investigation explants were obtained from 30 days old aseptic seedlings. Different seedling explants were cultured on BA 8.87 µm. Positive morphogenetic response and readily development of multiple shoots were produced by shoot tips, cotyledonary nodes and nodal explants, whereas other seedling explants such as internodes, leaves and root segments were not able to produce multiple shoots (Fig. 1).

Among different seedling explants highest response was observed with nodal explants followed by cotyledonary nodes. Nodal segments produced 22.43 shoots per explant on MS medium supplemented with BA 8.87 µm. The lowest mean number of multiple shoots was produced by shoot tip explant on the same media. Maximum response of nodal explant was also reported in *A. neesiana* [7], *A. paniculata* [11,12], *Caralluma* [17]. But contrasting results were reported by Deepa et al. [5].

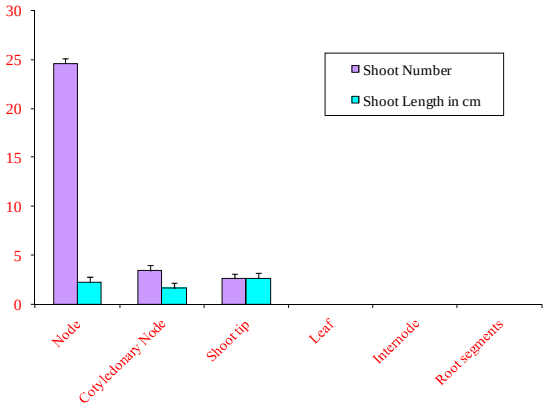

Fig. 1. Effect of different aseptic seedling explants of *Andrographis lineata* when cultured on MS medium supplemented with 8.87µM BA

3.2 Shoot Induction

The highest rate of micropropagation generally depends not only on the selection of explant but also on the identification of the correct combination of plant growth regulator [18]. Various concentrations of cytokinins such as BA, KN and 2iP each were tried for shoot induction in nodal segments of *Andrographis lineata.* The highest shoot induction was observed on the culture medium supplemented with BA. Among the different BA concentrations tested BA 8.87 µM showed 83% of response with a mean number of 22.43±0.38 shoots / explant and attained the length of 3.42±0.03 cm with at least 3-4 nodes (Fig. 3A). 2iP was found to be more effective when compared to KN (Table 1). Maximum number 9.23 shoots with shoot length of 1.87 cm were produced on MS medium with 2iP 14.76 µM. KN was inferior to 2iP not only in terms of percentage of response, but also in number of shoots / explant and shoot length. Effectiveness of BA over other cytokinins has been reported in *Andrographis paniculata* [11].

Axillary shoot proliferation in some species may be promoted by the presence of an auxin together with cytokinin in the medium. Assuming that combined effect of auxin and cytokinin could improve further shoot multiplication rate, combination of BA 8.87 µM with different concentration of IBA and NAA were studied.

Table 1. Effect of different concentrations of cytokinins (BA, KN, 2iP) individually and in combination with auxins (NAA, IBA) on shoot regeneration of nodal explant of *Andrographis lineata*

Plant growth regulators (µM)					Shoot sprouting frequency (%)	Mean shoot no. per explant ± S.E	Mean shoot length (cm) ± S.E
BA	KN	2iP	NAA	IBA			
2.22	-	-	-	-	53	2.27 ± 0.17^{h}	2.40 ± 0.03^{g}
4.44	-	-	-	-	76	4.43 ± 0.33^{g}	2.74 ± 0.02^{f}
8.87	-	-	-	-	83	22.43 ± 0.38^{c}	3.42 ± 0.03^{b}
13.31	-	-	-	-	80	20.80 ± 0.40^{cd}	2.74 ± 0.02^{f}
17.74	-	-	-	-	73	18.43 ± 0.45^{d}	2.54 ± 0.03^{g}
22.19		-	-	-	66	14.73 ± 0.31^{e}	2.11 ± 0.03^{h}
-	2.32	-	-	-	60	1.23 ± 0.11^{h}	1.84 ± 0.03^{i}
-	4.65	-	-	-	66	2.07 ± 0.19^{h}	2.08 ± 0.03^{h}
-	9.29	-	-	-	70	2.30 ± 0.16^{h}	2.77 ± 0.03^{f}
-	13.94	-	-	-	70	3.93 ± 0.31^{g}	2.37 ± 0.03^{g}
-	18.58	-	-	-	53	1.83 ± 0.17^{h}	1.80 ± 0.03^{i}
-	23.23	-	-	-	43	1.50 ± 0.13^{h}	1.37 ± 0.03^{k}
-	-	2.46	-	-	40	1.77 ± 0.12^{h}	2.92 ± 0.03^{e}
-	-	4.92	-	-	46	4.03 ± 0.24^{g}	2.67 ± 0.03^{fg}
-	-	9.84	-	-	70	5.17 ± 0.25^{g}	2.38 ± 0.03^{g}
-	-	14.76	-	-	76	9.23 ± 0.35^{f}	1.87 ± 0.03^{i}
-	-	19.68	-	-	53	4.90 ± 0.30^{g}	1.67 ± 0.03^{j}
-	-	24.61	-	-	46	3.47 ± 0.26^{g}	1.52 ± 0.03^{jk}
8.87	-	-	0.54	-	66	10.53 ± 0.42^{f}	2.95 ± 0.04^{e}
8.87	-	-	2.69	-	53	3.27 ± 0.22^{g}	2.44 ± 0.03^{g}
8.87	-	-	5.37	-	40	1.13 ± 0.15^{h}	1.19 ± 0.03^{l}
8.87	-	-	8.06	-	35	1.13 ± 0.15^{h}	1.19 ± 0.03^{l}
8.87	-	-	-	0.49	66	10.07 ± 0.40^{f}	3.13 ± 0.04^{d}
8.87	-	-	-	2.46	76	17.47 ± 0.27^{d}	3.30 ± 0.04^{c}
8.87	-	-	-	4.90	86	24.03 ± 0.39^{b}	3.58 ± 0.03^{a}
8.87	-	-	-	6.54	90	32.04 ± 0.32^{a}	3.50 ± 0.03^{a}

Means ± SE, n =50. Values followed by the different letter in the same column significantly different by the Tukey test at 0.05% probability level

Combinations of BA 8.87 µM with different concentration of NAA showed least number of shoots when compared to the individual cytokinins. Among four combinations of BA and NAA, BA 8.87 µM + NAA 0.54 µM produced 10 shoots per explant with basal callus. With increasing of concentration of NAA decrease in shoot number and shoot length was observed. The decrease in response, shoot number and shoot length was caused by more intensive callus formation with increasing concentration of NAA that prevents multiple shoot induction. Similar response was also observed during the propagation of *Gymnema sylvestre* [19,20].

Combination of BA 8.87 µM and IBA 0.49, 2.46 4.90 and 6.54 µM gave a contrasting result when compared to BA and NAA. Of the various treatments BA 8.87 µM + IBA 6.54 µM produced maximum number of 32.04 ± 0.32 shoots per explant with a shoot length of 3.50 ± 0.03 (Fig. 3B) (Table 1).

The best efficiency of BA and IBA for maximum shoot number in nodal segments was reported by several authors in *Ceropegia candelabrum* [21] and *Chonemorpha grandiflora* [22].

3.3 Shoot Multiplication

In shoot multiplication stage, primary shoots obtained from nodal segments were further increased by repeatedly subculturing shoots into fresh medium containing MS medium with BA 8.87 µM to generate large number of shoots. After 3 subcultures it was possible to obtain a significant number of new shoots (190 shoots/explant) with BA 8.87µM when compared

to 25 shoot/explants by Deepa et al. [5] in *A. lineata*. Shoots from culture tubes were transferred to culture bottles providing more space for growth of the cultures and also for maintenance of the cultures in viable state for more than 12 months.

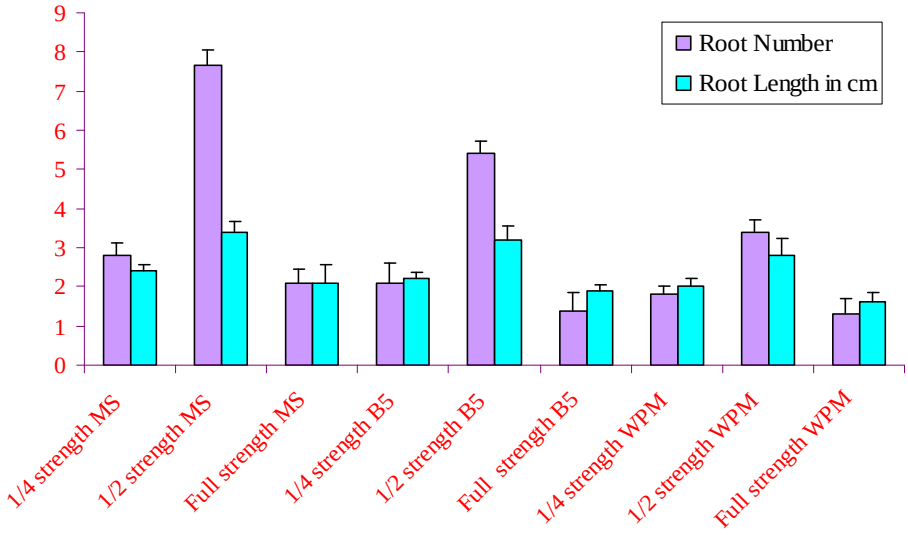

Fig. 2. Effect of various media strength on *in vitro* root induction from *in vitro* raised shoots if *Andrographis lineata* after 4 weeks of culture

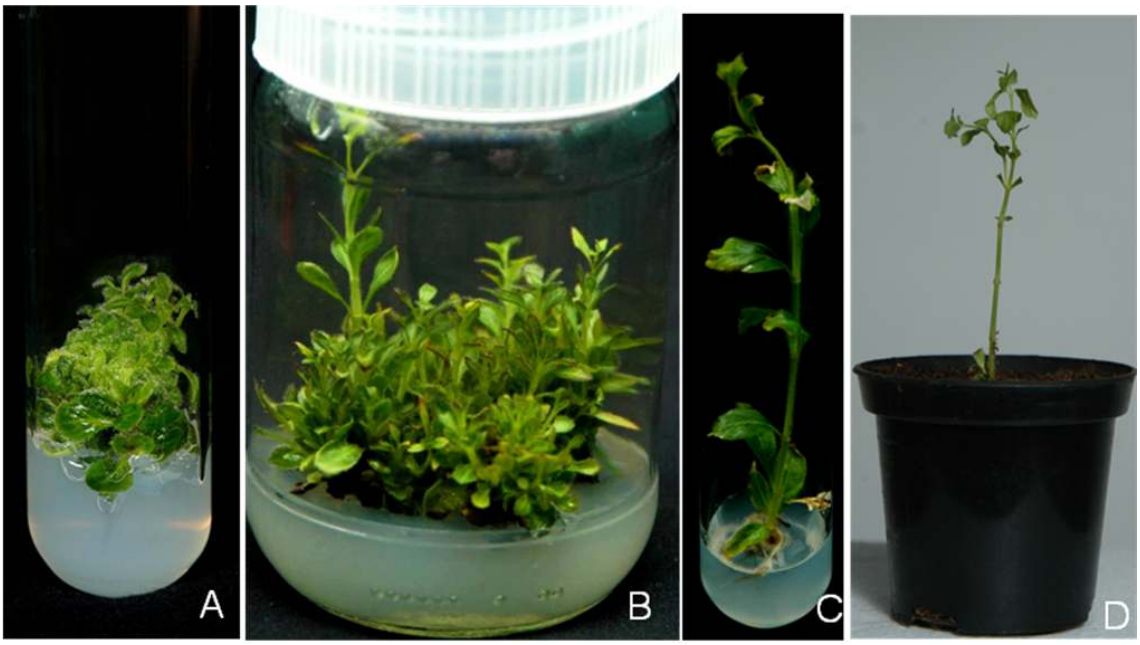

Fig. 3. *In vitro* propagation of *Andrographis lineata* Nees

A. Multiple shoots from nodal explant cultured on MS medium supplemented with 8.87 µM BA after 25 days of culture.

B. Multiple shoots from nodal explant cultured on MS medium supplemented with 8.87 µM BA + 6.54 µM IBA after 30 days of culture.

C. In vitro rooting on half strength MS medium after 30 days.

D. Acclimatization of in vitro raised plantlet of Andrographis lineata

3.4 *In vitro* Rooting

Microshoots which attained a length of 5-6 cm were excised and placed on rooting medium. In the present investigation different strength basal media without auxin were used for *in vitro* rooting. Maximum rooting efficiency was obtained on half strength MS basal medium when compared to different strength of MS, B_5 and WPM. The shoots when transferred onto auxin free full strength basal medium produced 2-3 roots. Reduction in MS salt concentration to half strength enhanced *in vitro* rooting (6-8 root per shoots) of micro shoots (Fig. 3C). But if there is further reduction in the concentration of MS salt to quarter strength there is decrease in rooting efficiency (Fig. 2). *In vitro* rooting only on half strength MS was also reported in *Andrographis paniculata* [12].

3.5 Hardening

After complete development of *in vitro* rooted plant they were taken out from culture tubes, washed with sterile distilled water and transferred to polycups filled with sterile peat moss, farmyard manure and soil (1:1:1). The potted plantlets covered with polythene sheet with small holes were kept in green house under high humidity. The potted plantlets were irrigated with quarter strength MS basal salt solution devoid of sucrose for every 3 days for 3 weeks (Fig. 3D). The pore size was gradually increased and finally the polythene sheet was removed so that plants get adjust to outer environment. The plants were kept under shade for 4 weeks and then transferred to field conditions. About 75% of plantlets were able to survive under field conditions.

4. CONCLUSION

The present protocol helps to produce large scale plantlet using nodal explants in short duration of time and cost effective. Such a protocol will provide the planting material to replenish the dwindling natural stocks of these plants. Moreover this reliable and efficient protocol can be further exploited for genetic manipulation experiments for enhanced production of biomass and andrographolide.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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