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Research Article

SHOOT PROLIFERATION AND MULTIPLICATION FROM NODES OF *ANDROGRAPHIS PANICULATA*

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ABSTRACT

Advanced biotechnological researches and exploitation of pharmacological potential of *Andrographis paniculata* Nees (Acanthaceae) require spontaneous and reliable in vitro propagation protocol**.** The purpose of the present study was to develop a reliable methodology for the high frequency direct shoot regeneration from nodal explants. Efforts were made for the in vitro shoot regeneration by collecting explants by two approaches: in vitro raised seeds and mother plants from greenhouse. The regeneration was carried out on the Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP), kinetin (Kn) and 2-isopentenyl adenine (2iP) at concentrations of 0.5 - 2.5 mg l^{-1} and BAP (2.0 mg l^{-1}) in combination with other cytokinins like Kn (0.5-2.0 mg l^{-1}) and auxins like 1-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) at concentrations of 0.1- 0.5 mg l^{-1} by using nodal explants. Among all the combinations tried, MS medium supplemented with BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) gave the maximum of 28.0 of shoots per explant. For the induction of roots, regenerated shoots were cultured on MS medium supplemented with NAA, IAA and indole-3-butyric acid (IBA) at concentrations of 0.5- 3.0 mg l^{-1}. 100% rooting was observed on transferring the cultures to full strength MS medium supplemented with IBA (3.0 mg l^{-1}).

KEY WORDS: 6-benzylaminopurine (BAP), Kinetin (Kn), Nodal Segment, Plant Regeneration, Murashige and Skoog (MS) medium.

INTRODUCTION

Over the decades, the bitter *Andrographis paniculata* has been the honeybunch for scientists and pharmacists all around the world. Its credit goes to the extraordinary medicinal properties of this amazing herb. *Andrographis paniculata*, a member of family Acanthaceae, known on the Indian subcontinent as Chirayetah and Kalmegh in Urdu and Hindi languages, respectively, is an erect annual herb, 1-3 ft high and is extremely bitter in taste. The plant is also known as 'Maha-tita', 'King of bitters' and known by various vernacular names. Incidentally, the genus *Andrographis* consists of 28 species of small annual herbs distributed in tropical Asia. Only a few species are medicinal, of which *Andrographis paniculata* is the most popular. Native populations of *Andrographis paniculata* are spread throughout South India and Sri Lanka [1].

Andrographis paniculata is used as a wonder drug in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and for multiple clinical applications in some other countries[2]. It is an important constituent of at least 26 ayurvedic formulations in Indian pharmacopoeia. Continuous usage of *Andrographis* in traditional medicinal practices led modern science to investigate about its medicinal importance. After years of research and findings, *Andrographis* was reported to possess anti hepatotoxic[3], antibiotic[4], antimalaria[5], antihepatic[6], anti-inflammatory[7] and anti-snake venom[8] properties to mention a few, besides its general use as an immune stimulant agent[9].The large medicinal profile of *Andrographis* is mainly because of the presence of a wide range of diterpenoid lactones[10,11] , flavonoids[12] and other miscellaneous compounds[13] in the plant. Andrographolide, a diterpenoid lactone is the major active constituent mainly present in aerial parts of the plant. Studies showed that Andrographolide exhibited various biological activities like anti-HIV[14], anticancer[15] and anti angiogenic activities[16]. A fair amount of research has also been done on the extraction and isolation of various secondary metabolites from *Andrographis,* which are of immense medicinal and therapeutic importance[17,18]. Therefore, the present study was undertaken to establish an economical and reliable micro propagation protocol of *Andrographis paniculata* as this work supports the commercial importance of this plant.

MATERIAL & METHODS

Explants preparation and surface sterilization

Healthy shoots were also collected from actively growing plants. Nodal segments with first three nodes and shoot tips of 1-3 month old plants were used as starting materials. Explants were washed in running tap water for 5 min and cut into segments (4.0–5.0 cm). Explants were treated with 0.1 % (w/v) Bavistin (BASF, India) for 15–18 min Segments were surface disinfected in 75 % (v/v) ethanol for 1 min, then 0.1 % (w/v) $HgCl_2$ (Hi-Media, India) for 5 min followed by rinsing 5-6 times with sterile water under aseptic conditions.

Nutrient medium and culture conditions

After discarding both ends, segments were vertically inserted in MS media (19). MS full strength with varied concentrations (0.5- 2.5 mg l^{-1}) of cytokinins (BAP/Kn) was used for culture initiation. The pH of medium was adjusted to 5.8 ± 0.02 and was autoclaved at 121 ^{o}C for 15 min. Explants were inoculated vertically in culture bottles and placed in culture room having high light intensity (40 –50 l mol m^{-2} s^{-1} PFD), provided by cool and white fluorescent tubes (Philips, India), at 26 ± 2 ^{o}C and 55– 60 % RH.

Multiplication of shoots

After initiating the in vitro cultures, shoots were further multiplied by repeated transfer of explants i.e. after harvesting in vitro regenerated

shoots. They were re-cultured on MS containing additives and different concentrations (0.5- 2.5 mg l^{-1}) of cytokinins (BAP/Kn/iP) alone or in combination with NAA or IAA (0.1 - 0.5 mg l^{-1}). To evaluate the effect of repeated transfer of mother explants on shoot proliferation, mother cultures were transferred up to four passages on fresh nutrient medium. Shoots were sub cultured after every 10-14 days. The cultures were kept under controlled conditions of temperature (26 ± 2 ºC), light (40–50 l mol m^{-2} s^{-1} PFD for 16 h day^{-1}) and RH (55–60 %).

***In vitro* rooting of regenerated shoots**

For root induction under *In vitro* conditions, green and healthy shoots were separated individually from cultures and transferred to different combinations of MS media with four concentrations (0.5, 1.0, 2.0 or 3.0 mg l^{-1}) of IAA , IBA and NAA. The cultures were incubated at 26 ± 2 ºC under diffused light (20–25 l mol m^{-2} s^{-1} PFD).The number of responding shoots and number of roots developed per shoot were recorded after 20–22 days of culture.

Hardening of *In vitro* regenerated shoots

In vitro rooted plantlets were excised carefully from culture bottles to avoid breaking of roots and washed with tap water. For hardening of plantlets developed from *In vitro* rooting, plantlets were transferred to poly-bags containing sand, farm yard manure (FYM), soil-rite and vermiculite in 1:1:1:1 ratio. After 60–65 days under green house conditions, these plantlets were transferred to the nursery.

Data analysis

Experiments were laid out in completely randomized block design (RBD) and repeated thrice. Each treatment had a minimum of fifteen replicates. The observations on number of shoots and percentage of rooted shoots were recorded after a regular time interval of seven days. The data were subjected to the standard deviation of the mean and single factor ANOVA (OPSTAT).

RESULTS & DISCUSSIONS

Andrographis paniculata as a medicinally and pharmaceutically interesting plant might strongly benefit from a reliable micro propagation protocol. In literature, there are few reports discussing regeneration potential of *Andrographis*[21,22]. On MS basal medium the explants failed to show any response and shriveled within two weeks. Similar results have also been reported in *Anethum graveolens*[23], where MS basal medium[24] alone was ineffective for the growth of young nodal segments. These findings suggest that endogenous levels of hormones present in these inflorescence explants are not sufficient enough to sustain their growth in the basal medium.

Shoot Induction and Multiplication

For initiation of shoot cultures, the percentage of response varied with the type of growth hormone used. Out of different combinations and concentrations of plant growth hormones tried, MS media fortified with 2 mg l^{-1} BAP proved to be the best media in which the axillary shoots at nodal region emerged out within a week of inoculation. There was quick response after the fourth day of inoculation when the nodal segments responded by an initial swelling of the dormant axillary buds followed by bud break. Shoot formation was observed within three weeks in the nodal explants at an optimized concentration of 2.0 mg l^{-1} BAP.

Further, effect of BAP (2 mg l^{-1}) alone and in combination with other cytokinins like Kinetin, 2iP (0.5-2.0 mg l^{-1}) and auxins (0.1-0.5 mg l^{-1} NAA, IAA) was tried to multiply the no. of shoots.

Impact of BAP with NAA

Explants were repeatedly transferred on MS medium with 2.0 mg l^{-1} of BAP and varied concentrations of NAA. This process of shoot amplification has been adopted by many workers [25,26]. On adopting this process, 12-16 shoots (4-5 cm length) were produced after two passages. However, the best response was at MS + BAP (2 mg l^{-1}) with NAA (0.5 mg l^{-1}) which gave 28.0 shoots after six weeks of culture. This media composition was found most suitable for shoot multiplication and elongation**.** Higher concentrations of BAP and NAA retarded the growth of shoot cultures.

Impact of BAP with Kn

Shoots cultured on MS containing varied concentrations of BAP and Kn initiated well and showed growth and vigor for two weeks, but the no. of shoots remained limited. At 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} Kn, the no. of shoots were observed to be 10.3 per explant. The raised concentrations of BAP and Kn in the medium inhibited the shoot proliferation from explants.

Impact of 2iP with NAA or IAA

Besides BAP, Kn and NAA, 2.5 mg l^{-1} of 2iP + 0.5 mg l^{-1} NAA on MS media also gave significant results yielding 23.4 shoots per explants. Role of 2iP in optimal growth of shoot is also observed in *Sophora tanokinensis* [27]. 2iP with IAA was also tried to observe the growth and multiplication of shoots, which was responsive but to a lesser extent.

Rooting

Although the promotive effect of auxins was achieved in eliciting rooting response[24], their type and level in the nutrient medium were found to vary from tissue to tissue and species to species [28]. The shoots regenerated from nodal explants failed to produce roots when cultured on full strength MS medium without any growth regulator even after four weeks of culture. Regenerated shoots were excised from the parent cultures and transferred onto full strength MS media supplemented with different combinations of IBA, IAA and NAA, at various concentrations (0.5- 3.0 mg l^{-1}) for root induction. The roots were induced directly from the shoot base without an intervening callus phase on media supplemented with NAA, IBA and IAA. Root induction occurred in 9 - 10 days of culturing with highest root induction (100%) on full strength MS medium containing IBA (3.0 mg l^{-1}) followed by 93.6% on MS+ IAA (3.0 mg l^{-1}). Similarly, *Beloperone plumbaginifolia*[29], *Adhatoda vasica*[30] and *A. paniculata*[31] showed maximum root formation in IBA.

Acclimatization

For acclimatization, well rooted plants were transferred were transferred to poly-bags containing sand, farm yard manure (FYM), soil-rite and vermiculite in 1:1:1:1 ratio, covered with a clear plastic bag and grown (28°C day, 20°C night, 16 h day-length with 70% relative humidity) for two weeks. Plants were misted manually with sterilized water once a day during this period to avoid desiccation. At the end of second week, the acclimatized plantlets were successfully established in the greenhouse with 85 % survival rate and transferred to the nursery.

The present study demonstrates a simple and efficient method for high frequency direct shoot regeneration from nodal explants of *Andrographis paniculata*. Data for percentage shooting and rooting were recorded and analyzed for test of significance.

Table 1: Effect of various growth hormones on multiplication of shoots of *Andrographis paniculata*

MEDIA CODE	BAP (mg l^{-1})	Kin (mg l^{-1})	NAA (mg l^{-1})	IAA (mg l^{-1})	2 iP (mg l^{-1})	MEAN NO. OF SHOOTS
MM0	0.0	0.0	0.0	0.0	0.0	0.00
MM1	2.0	0.5	-	-	-	8.3±0.3
MM2	2.0	1.0	-	-	-	10.3±0.3
MM3	2.0	-	0.5	-	-	**28.0 ± 3.8**
MM4	-	2.0	0.5	-	-	18.0±1.7
MM5	-	-	0.1	-	3.0	23.3±1.2
MM6	-	-	0.5	-	3.0	**23.5±1.9**
MM7	-	-	-	0.1	3.0	14.0±2.1
MM8	-	-	-	0.5	3.0	20.0±1.2

Table 2: Effect of growth hormones on rooting of *In vitro* grown shoots of *Andrographis.*

MEDIA CODE	IBA (mg l^{-1})	IAA (mg l^{-1})	NAA (mg l^{-1})	% RESPONSE	MEAN NO. OF ROOTING
AR0	0.0	0.0	0.0	0.00	0.00
AR1	0.5	-	-	83.33	5.25 ± 0.80
AR2	1.0	-	-	81.64	5.33 ± 0.94
AR3	2.0	-	-	80.21	4.50 ± 1.09
AR4	3.0	-	-	**100.00**	11.08 ± 0.7
AR5	-	0.5	-	75.51	5.16 ± 0.19
AR6	-	1.0	-	83.33	6.16 ± 0.89
AR7	-	2.0	-	91.66	9.91 ± 1.00
AR8	-	3.0	-	**93.06**	11.25 ± 0.41
AR9	-	-	0.5	66.00	2.41 ± 0.57
AR10	-	-	1.0	83.33	4.66 ± 0.71
AR11	-	-	2.0	91.66	8.75 ± 0.60
AR12	-	-	2.5	**61.17**	4.33 ± 0.58



Figure A. Initiation of shoots from nodal segments of *Andrographis paniculata* on MS +2.0 mg l^{-1} BAP.
Figure B. Multiplying shoots on MS supplemented with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA after four weeks.
Figure C-D. Cent per cent rooting on MS with 2.5 mg l^{-1} NAA.
Figure E. Full grown shoots shifted to greenhouse for hardening.

CONCLUSION

The superabundant medicinal account of *Andrographis paniculata* lures researchers to apply advanced biotechnological techniques for the benefits of medicinal world. Therefore, maintenance and conservation of natural reservoirs cannot be denied. The present study demonstrates a systematic micro propagation protocol of *Andrographis paniculata.* The method is spontaneous and maximum of 28.0 shoots per explant were regenerated on MS medium supplemented with BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). Proliferating shoot cultures were established by repeatedly sub-culturing the nodes on the same medium. It takes 3 to 4 months duration from initiation of tissue culture till ending with the transplanting of regenerated plantlets. Such a high regeneration frequency would be useful for mass propagation and multiplication of this valuable medicinal plant. Commercial utilization of this developed protocol is possible, as the nodal segments from *In vitro* raised shoots can further be used as explants for further multiplication, ruling out the dependence on natural flora.

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