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Plant regeneration protocol of *Andrographis paniculata* (Burm. f.) - an important medicinal plant

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Rapid direct plant regeneration of *Andrographis paniculata* was achieved from leaf and stem explants on Murashige and Skoog (MS) basal medium supplemented with 1.5 to 3.0 mg/l 6-benzyladenine (BA), 50 mg/l adenine sulfate (Ads) and 3% (m/v) sucrose. Inclusion of 1.0 mg/l 1-naphthalene acetic acid (NAA) in the culture medium along with BA + Ads promoted a higher rate of shoot bud regeneration. Maximum mean number of shoot bud per explant (28.6) was achieved on the MS medium supplemented with 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after six weeks of culture. The percent of regeneration varied depending on the culture medium. The elongated shoots were rooted within 9 to 11 days on ½ strength MS medium supplemented with 0.5 mg/l indole-3-butyric acid (IBA) or 1-naphthaleneacetic (NAA) acid with 2% sucrose. Maximum percentage of rooting (76.24%) was obtained on medium having 0.5 mg/l IBA and 2% sucrose. Basal region of the micro-shoots became callusing when transferred to higher concentrations of either IBA or NAA. The rooted plantlets were survived in the greenhouse. The *in vitro* raised plantlets were grown normally under soil condition. This result will facilitate the conservation and propagation of the important medicinal plant.

Key words: *In vitro*, shoot bud regeneration, growth regulators, medicinal plants.

INTRODUCTION

Andrographius paniculata (Burm. f.) is a valuable medicinal plant which belongs to the family Acanthaceae. The plant is distributed throughout tropical India and Sri Lanka and is commonly known as Kalmegh. It is used as laxative and to overcome difficulty in breathing, cough, edema, skin diseases, syphilitic eachexia, syphilitic ulcers, acidity and liver complaints (Sivarajan and Balachandran, 1994). The leaves and roots are used for the treatment of diabetes, malaria, cholera, dysentary, pneumonia, tuberculosis and scabies (Kirtikar and Basu, 1981). A number of secondary metabolites have been isolated from various parts of the plant namely, carvacrol, eugenol, myristic acid, hentriacontane, tritriacontane, oroxylon A and diterpenoids like andrograpanin, andropanoside, andrographolide and neoandrographolide (Rastogi and Mehrotra,1993; Sharma, 2003). Two flavonoids 2', 4', 6', 2, 3, 4-hexamethoxychalcone and 5-hydroxy-7, 2, 5-trimetrhoxyflavone together with a known flavones glycoside, echioidinin 5-0-beta-D-glucopyranoside were isolated from the whole plant of *Andrographis neesiana* (Pawar et al., 2011). The bitter principle of a diterpene lactone, Andrographolide which was originally extracted from *A. paniculata*, has a high demand to pharmaceutical industries, is largely met by

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Abbreviations: MS, Murashige and Skoog; **BA,** 6-benzyladenine; **Ads,** adenine sulfate; NAA, 1-naphthalene acetic; **IBA,** indole-3-butyric acid.

extraction of this compound from wild population. Conventional vegetative propagation is very difficult and propagation from seed is unreliable due to poor germination and deaths of many young seedlings under natural conditions (Anonymous, 1992) are some of the major hurdles to meet the ever growing demand of the industries. Thus, plant regeneration from different explants through *in vitro* is an alternative method for conservation and propagation of this species, but a few reports on such investigations on *A. paniculata* are available in literature (Prathanturarug et al., 1996; Martin, 2004).

The influence of plant growth regulators on plant regeneration has been reported in several medicinal plants by different investigators (Rout et al., 2000, Gopi et al., 2006; Faridah et al., 2011). The present investigation aimed to describe an efficient protocol for direct plant regeneration from leaf and stem explants of *A. paniculata*, an important medicinal plant.

MATERIALS AND METHODS

Plant material

Elongated shoots (2 to 4 cm long) were collected from field-grown plants of *A. paniculata* and brought to the laboratory with cut ends dipped in distilled water and subsequently, washed in a 2% (w/v) (Teepol, Qualigen, India) detergent solution. Further, surface sterilization was performed in a 0.1% (w/v) aqueous mercuric chloride solution for 5 min. After rinsing four to five times with sterile distilled water, stem (10 to 12 mm long) as well as leaf segment (0.25 cm^2) were used as explants source.

Culture medium and culture conditions

Both stem and leaf explants were placed on semi-solid Murashige and Skoog (MS, 1962) mineral salts plus 555 µM myo-inositol, 4.06 µM nicotinic acid, 2.43 µM pyridoxine-HCl, 0.296 µM thiamine-HCl supplemented with various concentrations of cytokinins, that is, 6-benzylaminopurine (BA: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l), adenine sulfate (Ads: 0, 25, 50 and 100 mg/l) and auxin like indole-3-acetic acid (IAA: 0.0, 0.25, 0.5, 1.0 and 2.0 mg/l), 1-naphthaleneacetic acid (NAA: 0.0, 0.25, 0.5, 1.0 and 2.0 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4-D: 0.0 0.25, 0.5, 1.0 and 2.0 mg/l) for callus induction and shoot bud regeneration.

The pH of the medium was adjusted to 5.8 prior to autoclaving. Each treatment was represented by 10 cultures and the experiment was repeated three times. The cultures were incubated under a 16-h photoperiod having a light intensity of 55 µE/m^2/s from cool, white fluorescent lamp at 25 ± 2°C.

Induction of rooting and acclimatization

Elongated shoots (2 to 3 cm long) were excised from the culture and transferred to ½ strength semisolid MS medium supplemented with different concentrations of IBA and/or NAA (0, 0.1, 0.25, 0.5, 1.0 and 1.5 mg/l) and 2% (w/v) sucrose for root induction. One excised shoot was cultured in each tube (25 × 150 mm) with 15 ml of the culture medium. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod with cool white fluorescent lamps. The percentage of shoots forming roots and the number of roots per shoot were examined periodically up to 3 weeks of culture. Rooted micropropagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing a mixture of soil, sand and dry cow-dung manure (1:1:1, w/v) and kept in the greenhouse for acclimatization. The plants were watered at every 2-day intervals. The survival rate was recorded one month after the transfer into pots.

Scoring of data and statistical analysis

All the cultures were examined periodically, and the morphological changes were recorded on the basis of visual observations. There were 10 cultures per treatment for organogenic calli production and plant regeneration. Then each experiment was repeated three times and subculture was carried out in a 6-week interval. The mean percentage of cultures producing shoot regeneration and mean number of shoots per culture were recorded after 6 weeks. The percentage of rooting and the average number of roots per shoot were recorded after six weeks. The data were analyzed statistically by the Duncan's multiple range test (Duncan, 1955). Means followed by the same letter within columns were not significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Green calli were initiated from leaf and stem explants of *A. paniculata* on the MS medium supplemented with different concentrations of BA or Kn alone or in combination with either NAA or 2,4-D. The initiation of callus and the subsequent proliferation could not be achieved on the medium without growth regulators. The media containing BAP alone or in combination with Ads showed a low frequency of orgonogenic calli initiation as compared to kinetin (Kn) alone or Kn + Ads (Table 1). The combination of Kn plus 2,4-D or NAA did not influence the shoot bud regeneration.

The medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA favoured the development of organogenic calli within six weeks of initial culture (Figure 1A). Initially, the organogenic calli were initiated in the upper surface of the explants and subsequently it proliferated into shoot buds. The medium containing BAP + Ads + NAA produced a significant number of shoot buds regeneration in leaf and stem derived calli as compared with BA + Ads (Table 1). The medium having 2,4-D or NAA alone had no effect on shoot bud regeneration (data not shown).

The regeneration of shoot buds from organogenic calli was varied on the basis of the culture medium composition. About 75.3% in case of leaf-derived calli and 63.4% in case of stem-derived cultures showed shoot bud regeneration in the medium having 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after six weeks of first subculture (Figure 1B). The increase of NAA concentration higher than 2.0 mg/l suppressed the rate of shoot bud regeneration and slow growth of the organogenic calli.

The maximum number of shoot buds (28.6) was obtained in the medium containing 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after four weeks of culture initiation

Table 1. Effect of different concentrations of cytokinins and auxins on shoot bud regeneration from leaf (A) and stem (B) derived calli of *A. paniculata* after 6 weeks of culture.

MS + growth regulators (mg/l)					Percent of organogenic calli development (mean ±S.E)*		Average number of shoot buds per culture (mean ±S.E)*	
BAP	Kn	Ads	NAA	2,4-D	A	B	A	B
0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	12.6±0.8^{b}	8.36±0.6^{a}	3.32±0.5^{c}	3.28±0.3^{b}
1.5	0	0	0	0	18.5±0.7^{c}	12.5±0.7^{b}	4.66±0.6^{d}	4.12±0.5^{c}
2.0	0	0	0	0	24.2±0.8^{d}	20.2±0.8^{f}	10.1±0.4^{h}	8.32±0.8^{f}
2.5	0	0	0	0	26.8±0.4^{e}	20.8±0.9^{f}	18.4±0.6^{k}	11.6±0.7^{h}
3.0	0	0	0	0	28.5±0.7^{f}	21.6±0.7^{g}	20.2±0.8^{l}	13.2±0.6^{j}
0	1.0	0	0	0	11.8±0.6^{a}	14.2±0.8^{c}	1.8 ±0.4^{a}	2.4±0.3^{a}
0	2.0	0	0	0	12.6±0.9^{b}	17.6±0.5^{d}	3.22±0.7^{c}	2.9±0.6^{a}
0	3.0	0	0	0	24.4±0.5^{d}	18.8±0.4^{e}	8.45±0.6^{f}	6.5±0.4^{e}
2.0	0	25.0	0	0	30.2±0.8^{g}	32.2±0.7^{k}	14.5±0.5^{j}	12.8±0.2^{i}
3.0	0	50.0	0	0	52.7±1.1^{k}	40.5±0.9^{n}	22.6±0.3^{m}	14.6±0.7^{k}
0	2.0	25.0	0	0	28.6±0.8^{f}	26.8±1.0^{j}	8.88±0.5^{f}	10.4±0.2^{g}
0	3.0	50.0	0	0	32.2±1.0^{h}	26.6±0.8^{j}	10.2±0.7^{h}	12.4±0.3^{i}
2.0	0	25.0	1.0	0	65.8±1.3^{l}	52.7±1.0o	24.8±0.6^{n}	15.9±0.7^{l}
2.0	0	25.0	0	1.0	25.6±1.1^{d,e}	24.2±0.8^{i}	9.7 ±0.3^{g}	6.8±0.4^{e}
3.0	0	25.0	1.0	0	72.8±1.2^{n}	61.8±1.0q	18.8±0.6^{k}	12.6±0.5^{i}
2.5	0	50.0	1.5	0	68.8±1.3^{m}	55.2±1.1p	22.7±0.8^{m}	15.5±0.6^{l}
3.0	0	50.0	1.0	0	75.3±2.1o	63.4±1.2r	28.6±0.8o	16.2±0.8^{m}
0	2.0	25.0	1.0	0	34.8±0.8^{i}	32.2±0.9^{k}	12.3±0.4^{i}	8.2±0.6^{f}
0	3.0	50.0	1.0	0	38.2±1.2^{j}	35.1±1.1^{l}	14.6±0.7^{j}	12.5±0.5^{i}
2.0	0	50.0	0	2.0	30.6±0.8^{g}	38.2±1.2^{m}	6.33±0.6^{e}	5.6±0.4^{d}
0	2.0	25.0	0	1.5	28.6±0.7^{f}	20.4±0.7^{f}	3.36±0.3^{c}	2.8±0.6^{a}
0	3.0	50.0	0	1.5	31.4±0.9^{g}	22.4±0.5^{h}	2.56±0.7^{b}	2.9±0.8^{a}

*Means of 10 replicates/treatment; repeated thrice; Mean followed by different letters are significantly different at the 5% level.

(Table 1). It indicates that cytokinin/auxin ratio being the principal players in the induction of shoot multiplication from explants. Similar observations indicating the effect of cytokinin and auxin on shoot multiplication were previously reported in *Clerodendrum colebrookianum* (Mao et al., 1995), *Plumbago* (Rout et al., 1999), and *Ocimum gratissimum* (Gopi et al., 2006). The present findings suggest a high frequency of shoot production from organogenic calli could be obtained by manipulating the growth regulators and culture condition. There were differences between treatments both in the percentage of cultures with response and in the mean number of shoot buds per culture.

Many authors reported that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but an inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot bud proliferation (Sharma et al., 1993; Sharma and Singh, 1997; Shasany et al., 1998; Rout et al., 2000; Rout, 2005). However, the molecular mechanisms through which auxin-cytokinin crosstalk act in concert to exert the shoot meristem induction are still poorly understood. A lower concentration of BA (< 3.0 mg/l) in the culture medium inhibited the growth of the shoot buds. The number of shoot buds per culture varied from 1.8 to 28.6 in case of leaf and 2.4 to 16.2 in case of stem in different treatments.

The rate of shoot bud regeneration increased as the number of subcultures increased. Similar observations were reported for *Gentiana kurroo* (Sharma et al., 1993) and *Plumbago* species (Rout et al., 1999).

Induction of rooting and acclimatization

Elongated shoots were excised and placed in half/ full strength MS medium supplemented with various concentrations of IBA or NAA for root induction. Full strength MS medium without growth regulators did not promote root induction; roots were observed in media containing ½ strength MS medium supplemented with NAA or IBA with 2% sucrose.

However, optimal rooting and growth of micro shoots were observed in medium containing 0.5 mg/l IBA or NAA with 2% sucrose after 9 to 11 days of culture without intervening callus (Table 2). The maxi-mum percentage of rooting (76.2%) was obtained in the medium containing

Figure 1. Direct plant regeneration from leaf and stem explants of *Andrographis paniculata.* A) Organogenic calli development from leaf explant on medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA after 6 weeks of initial culture. B) Shoot bud regeneration (arrows) from rganogenic calli on medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA after 6 weeks of subculture. C) Root initiation from micro shoots of *Andrographis paniculata* after 3 weeks of culture of ½ strength MS medium supplemented with 0.5 mg/l IBA + 2% sucrose. D) Plantlets established in the pot.

0.5 mg/l IBA within three weeks of culture (Figure 1C). Root development was; however, slow at higher concentrations of IBA or NAA. The rooted plantlets were transferred into pots for acclimatization. About 60% of the rooted plantlets survived in the pot one week after the transfer. The plants were grown normally (Figure 1D).

Table 2. Effect of different concentrations of NAA and IBA on root induction from excised shoots of *Andrographis paniculata* after three weeks of culture.

½ MS + auxin concentrations (mg/l)		Days to rooting	Percent of rooting (mean ±SE)*
NAA	IBA		
0	0	0	0
0.25	0	11 - 12	25.42±1.2[a]
0.50	0	10 - 11	62.24±1.3[f]
1.0	0	12 -13	42.12±1.4 +[c]
0	0.25	10 -11	52.24±1.2[e]
0	0.50	9 - 10	76.24±2.1[g]
0	1.00	12-13	46.28±1.4+[d]
0.25	0.50	13 -14	40.16±1.5+[c]
0.50	0.25	12-13	36.22±1.1+[b]

*10 micro-shoots/treatment; repeated thrice; + - callusing at the basal end. Mean followed by different letters are significantly different at the 5% level.

In conclusion, a successful production of shoot bud regeneration from leaf and stem explants and induction of roots from excised root were dependent on the nutrient medium and the culture conditions. This study might provide new opportunities for mass propagation and conservation of an important medicinal plant, *A. paniculata.*

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