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IN VITRO REGENERATION OF *EUPATORIUM TRIPLINERVE* VAHL- AN IMPORTANT MEDICINAL HERB THROUGH AXILLARY BUD CULTURE

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ABSTRACT

Eupatorium triplinerve Vahl. commonly known as Ayapana belongs to the family Asteraceae is a rich source of coumarin which is widely prescribed drug warferin. The present study aimed to develop an efficient protocol for in vitro regeneration through the culture of axillary bud explant. Murashige and Skoog (MS) medium as basal medium and supplemented with different concentrations and combinations of 6-benzyl amino purine (BAP), α-naphalene acetic acid (NAA) and indole butyric acid (IBA) were employed in order to shoot induction, multiplication and rooting. The maximum percentage of shoot induction with 4.93 ± 0.06 of shoots was produced on the medium fortified with 17.76 µM/L of BAP and 2.69 µM/L of NAA. The well initiated shoots were sub cultured on fresh medium with same composition for further multiplication of shoots. The medium enriched with 22.20 µM/L of BAP and 2.69 µM/L of NAA was found to be efficient for maximum percentage of shoot multiplication with the highest number of shoots (26.00 ± 1.15) and mean shoot length of 4.00 ± 0.28 cm. The effective root induction with maximum number of roots was obtained on the half strength MS liquid medium supplemented with 9.84 µM/L of IBA. The well rooted healthy plantlets were hardened under greenhouse condition and establish successfully with 90% of survival. This protocol may be useful for the large-scale production of this plant and other medicinal plants.

KEY WORDS

Axillary buds, Eupatorium triplinerve, In vitro regeneration, Medicinal herb

INTRODUCTION

Medicinal plants have been the subject of man's curiosity since time immemorial [1]. Plants play a prominent role in the introduction of new therapeutic agents and also drugs from the higher plants continue to occupy an important niche in modern medicine [2]. *Eupatorium triplinerve* Vahl. commonly known as ayapana belonging to the family Asteraceae (Compositae). *E. adenophorum* (L.) Spr., is used in the treatment of stomach aches and to prevent bleeding [3]. *E.triolinerve* is rich in naturally occurring coumarin (7-methoxy coumarin) which may support to explain why the plant is used in medicine as an antitumor remedy. A decoction of the plant and the juice of the leaves are considered detergent in Philippines and applied to foul ulcers. In considering the adverse effects of synthetic drugs the western population is looking for natural remedies, which are safe and effective [4,5]. *E. adenophorum* is conventionally propagated by vegetative cuttings.

Plant tissue culture refers to as *in vitro*, which is an important tool in both basic and applied studies as well as in commercial application [6,7]. *In vitro* cultures are now being used as a tool for the study of various basic problems in plant sciences. *In vitro* propagation method offers highly efficient tool for germplasm conservation and mass multiplication of many threatened plant species [8,9]. There are limited reports regarding seed propagation or micropropagation through tissue culture [10]. *In vitro* micropropagation through axillary bud multiplication has been reported in many medicinal

plants [11,12,13]. The present study aimed to develop an efficient protocol for *in vitro* regeneration of *Eupatorium triplinerve* through the culture of axillary bud explant.

MATERIALS AND METHODS

Source and preparation of explants

The healthy mother plants of *E. triplinerve* for this study were collected form herbal nursery maintained by forest deparment of Tamil nadu, India and maintained in the garden of Pachaiyappa's College, Chennai, Tamilnadu. The mother plants were identified and authenticated by Taxonomist in Department of Botany, Pachaiyappa's College, Chennai. Axiliary buds were excised and used as explants for this study. The explants were washed with running tap water to remove the dust particle on the surface of the plant material and add few drops of soap solution (Teepol) and wash thoroughly with tap water. The washed explants were transferred in to Laminar Air Flow chamber (LAF) where the surface sterilization was carried out with the help of disinfectant mercuric chloride ($HgCl_2$). The axillary buds were subjected to surface sterilization with 0.1% of (w/v) of $HgCl_2$ for 3 minutes. Under the aseptic condition the axillary buds were resized about 0.5-1.0 cm long for inoculation (Fig-1a).

Nutrient medium

Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media. In this study, Murashige and Skoog [14] medium was used basal medium for all the experiments. The MS basal medium fortified with different concentrations of 6-benzyl amino purine (BAP) (4.44 - 26.64µM/L) alone and in combination with 2.69 µM/L of α- naphalene acetic acid (NAA) was employed for shoot induction and multiplication (Table-1). The full and half strength MS basal liquid medium supplemented with indole butyric acid (IBA) (4.92 - 17.22 µM/L) were used for rooting (Table-2). The pH of the media was adjusted to 5.8 and all the media used were autoclaved for 20 minutes at 121°C and 15 psi of pressure.

Inoculation and culture condition

The axillary bud explants were excised and resized was about 0.5-1.0 cm length and inoculated on MS basal medium fortified with different concentrations and combinations of PGRs. All the cultures were incubated under the temperature of 25 ± 2°C and the light intensity of 2000 - 4000 Lux. The photoperiod regime for cultures was 16 hr light and 8 hr dark and the relative humidity was maintained between 50 - 60%.

Initiation of shoots and subculture

Axillary bud explants were inoculated in each culture bottles containing MS basal medium fortified with BAP (4.44 - 26.64 µM/L) alone and in combination with 2.69 µM/L of NAA for initiation of shoots. The young multiple shoots initiated were removed and sub cultured on fresh medium with same composition for further multiplication of shoots. The cultures were observed regularly and the results were recorded for every sub culture. The number and the length of shoots were recorded in 4 weeks old culture to assess the best treatment.

Root induction and Hardening

After the multiplication phase, the *in vitro* developed shoot lets were separated (5 - 6 cm in length) and transferred to the medium is constituted with half and full strength of MS basal liquid medium supplemented with various concentrations of IBA (4.92 - 17.22 µM/L) for root induction. The number and the length of roots were recorded to assess the best treatment. The well developed and healthy plant lets with roots were taken out and washed thoroughly and then transferred to paper cups containing sterile soil and vermiculite in the ratio of 1:1 for primary hardening. After primary hardening they were transferred 4.93±0.06 to mud pot containing sand, soil and vermiculite in the ration of 1:1:1 secondary hardening under the shade house.

Statistical analysis

All the tests were carried out in triplicates and the data were analyzed statistically using the SPSS 16.0 software (SPSS Inc., Chicago, USA) and the mean values are expressed as Mean ± SE. The significance of differences among means was carried out at $P< 0.05$ probability level using Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Initiation and multiplication of shoots

The sterilized axillary bud explants were inoculated on MS basal medium supplemented with various concentrations of BAP (4.44 - 26.64 µM/L) alone and in combination with 2.69 µM/L of NAA for initiation of shoots. The medium supplemented with 17.76 µM/L of BAP and 2.69 µM/L of NAA was showed maximum percentage (89.66 ± 0.88) of initiation and produced 4.93 ± 0.066 number of shoots with mean length of 3.90 ± 0.20 cm (Table-1; Fig-1b).

Table - 1: Effect of plant growth regulators on initiation and multiplication of shoots

Medium	Plant growth regulators		Initiation of shoots			Multiplication of shoots		
	BAP (µM/L)	NAA (µM/L)	Percentage of shoot induction	Number of shoots per explant	Shoot length (cm)	Percentage of shoot multiplication	Number of shoots per micro shoot	Shoot length (cm)
MS-1	4.44	-	27.66±0.33^{a}	1.16±0.16^{a}	3.50±0.28^{ef}	20.33±0.88^{a}	4.16±0.16^{a}	3.50±0.00^{a}
MS-2	4.44	2.69	35.33±0.88^{b}	2.10±0.20^{b}	3.00±0.00^{d}	31.00±1.00^{b}	6.00±0.00^{ab}	4.03±0.31^{ab}
MS-3	8.88	-	37.66±0.33^{bc}	2.93±0.06^{c}	3.90±0.20^{f}	26.00±1.00^{ab}	6.00±0.57^{ab}	6.50±0.28^{d}
MS-4	8.88	2.69	41.00±0.57^{d}	3.00±0.00^{c}	2.91±0.08^{cd}	35.33±0.33^{b}	8.00±0.28^{bc}	6.00±0.00^{cd}
MS-5	13.32	-	38.66±0.66^{cd}	3.06±0.17^{c}	3.10±0.20^{de}	30.33±0.33^{b}	8.00±0.00^{bc}	6.00±0.57^{cd}
MS-6	13.32	2.69	56.00±0.57^{e}	4.06±0.17^{d}	2.86±0.06^{cd}	35.66±0.66^{b}	10.33±1.45^{c}	6.00±0.28^{cd}
MS-7	17.76	-	75.33±1.45^{f}	4.76±0.14^{e}	3.16±0.16^{de}	50.33±3.17^{c}	15.66±2.02^{d}	5.00±0.00^{bc}
MS-8	17.76	2.69	89.66±0.88^{i}	4.93±0.06^{e}	2.00±0.00^{a}	59.66±2.90^{d}	22.00±1.73^{e}	6.00±0.57^{cd}
MS-9	22.20	-	78.33±0.88^{g}	4.60±0.10^{e}	2.23±0.14^{ab}	70.33±2.60^{e}	24.00±0.00^{ef}	4.33±0.88^{ab}
MS-10	22.20	2.69	83.33±1.66^{h}	4.00±0.00^{d}	2.50±0.00^{bc}	90.00±2.88^{g}	26.00±1.15^{f}	4.00±0.28^{ab}
MS-11	26.64	-	25.00±0.00^{f}	4.03±0.14^{d}	2.00±0.00^{a}	74.33±8.08^{ef}	21.66±2.02^{e}	5.00±0.57^{bc}
MS-12	26.64	2.69	77.66±1.45^{fg}	3.06±0.06^{c}	2.46±0.20^{abc}	10.33±3.17^{f}	24.33±0.88^{ef}	3.53±0.33^{a}
	F-value		564.147	77.172	15.213	6.444	53.616	6.673
	P-value		0.00	0.00	0.00	0.00	0.00	0.00

The values represent the Mean±SE of ten replicates and all experiments were repeated thrice, mean difference of significant is at the 0.05 level. Means with different letter within column are significantly different from each other at P ≤ 0.05.

In the present study, among these different concentrations and combinations of PGRs, the medium supplemented with the combination of BAP and NAA was comparatively more efficient than that of individual effect of BAP on maximum percentage of shoot induction with highest number of shoots per explants. Hence, the medium fortified with 17.76 µM/L of BAP and 2.69 µM/L of NAA was found to be suitable one for efficient shoot initiation.

The young multiple shoots initiated were sub cultured on fresh medium with same composition for further multiplication of shoots. The multiplication of shoots was noticed in all media combinations but with varied response in terms of number and length of shoots (Table-1). The maximum percentage 90.00 ± 2.88 of shoot multiplication was observed on the medium enriched with 22.20 µM/L of BAP and 2.69 µM/L of NAA. This medium produced highest number of shoots (26.00 ± 1.15) with mean length of 6.50 ± 0.28 cm (Table-1; Fig-1c). Some reports of various authors in *E. triplinerve* was contrary and support the results of present study such as Usha and Karpagam [15] reported that 18 shoots were produced on MS medium fortified with 4.44 µM/L of BA only in *E. triplinerve*. Only 8.1 mulptiple shoots/node recorded on the medium supplemented with 8.87 µM/L of BAP and 2.46 µM/L of IBA [16]. Samydurai *et al.,* [17] reported that, only 5 shoots/explants were observed on MS medium fortified with 0.2 mg/L BAP and 0.02 mg/L GA_3.

Hence, this combination is considered to be the most suitable one for the successful multiplication of shoots. In the present study, among the various media combinations, BAP + NAA was found to be the most favorable than individual effect of BAP for multiplication of shoots because this combination was markedly enhancing the number and length of the shoots.

Root induction and Hardening

The *in vitro* developed shoots were separated (5-6 cm in length) and transferred to rooting medium for root induction. The medium is constituted with half strength of MS liquid medium supplemented with different concentrations of IBA (4.92 - 17.22 µM/L). The root induction was observed in all media combinations, but the response was varied based on the concentration of plant growth regulators (Table-2). The half strength MS liquid medium supplemented with 9.84 µM/L of IBA was responded to the maximum percentage of root induction (90.00 ± 3.17). This medium produced highest mean number of roots (30.33 ± 0.88) per shoot with mean length of 5.50 ± 0.00 cm (Table-2; Fig: 1d). This result contrary to the report of Usha and Karpagam [15] that minimum number of roots obtained on half strength MS agar medium fortified with 2.46 µM/L of IBA. *Ex vitro* rooting was also been reported in *Eupatorium triplinerve* [16]. Samydurai *et al.,* [17]

reported that half strength MS liquid medium supplemented with 0.2 mg/L of IBA was efficient for rooting.

Table - 2: Effect of IBA on root induction

Medium	IBA (µM/L)	Percentage of root induction (%)	Number of Roots per shoots	Root length (cm)
MS-13	4.92	59.66 ± 2.60^{a}	24.33 ± 0.88^{bc}	2.00 ± 0.00^{a}
MS-14*	4.92	76.33 ± 0.88^{b}	26.00 ± 1.15^{c}	3.60 ± 0.10^{b}
MS-15	9.84	80.33 ± 3.17^{b}	25.00 ± 0.00^{c}	1.43 ± 0.23^{a}
MS-16*	9.84	90.33 ± 3.17^{c}	30.33 ± 0.88^{d}	5.50 ± 0.00^{c}
MS-17	17.22	77.00 ± 0.57^{b}	22.00 ± 0.00^{ab}	2.00 ± 0.00^{a}
MS-18*	17.22	80.00 ± 2.30^{b}	20.00 ± 1.15^{a}	2.10 ± 0.55^{a}
F-value		17.880	17.900	37.057
P-value		0.00	0.00	0.00

Note: * Half strength MS medium.

The values represent the Mean±SE of ten replicates and all experiments were repeated thrice, mean difference of significant is at the 0.05 level. Means with different letter within column are significantly different from each other at P ≤ 0.05.

In root induction full and half strength of MS basal liquid medium supplemented with various concentration of IBA were tried. The results clearly indicated that, the half strength MS was effective towards efficient root induction, when compare to full strength MS basal medium. The well developed and healthy plant lets with roots were taken out and washed thoroughly (Fig: 1e) and then transferred to paper cups containing sterile soil and vermiculite in the ratio of 1:1 for primary hardening (Fig: 1f). After primary hardening they are transferred to mud pot containing sand, soil and vermiculite in the ration of 1:1:1 secondary hardening under the shade house for 4 weeks. Transferred plants showed 90% of survival rate after the hardening period of 4 weeks (Fig: 1g).

The plant growth regulator plays a vital role in controlling the growth and development of plants. Auxin and cytokinin were the two important PGRs widely used for morphogenetic manifestation under *in vitro* condition. In the present study, the effect of growth regulators like BAP, NAA and IBA were tested. In general, cytokinins stimulate shoot induction and bud formation in plant tissue culture and BAP has been considered to be one of the most active cytokinins in organogenic differentiation in plant tissue culture [18,19]. Extensive work was carried out on the synergistic effect of auxin and cytokinin in plant regeneration by Skoog and Miller [20]. The cytokinin is required in optimal quantity for shoot proliferation in many genotypes but an inclusion of low concentration of auxin along with cytokinin increases the rate of shoot multiplication in several experiments [21,22].

In the present investigation, axillary buds were used as explants. The axillary buds were capable of directly producing multiple shoots on MS medium supplemented with various concentrations and combinations of cytokinins (BAP) and auxin (NAA). The nodal segment was found to be the suitable explant for the initiation of shoots. Ajithkumar *et al*., [23] reported that, the reason for the suitability of nodal segment is attributed due to the presence of protected axillary buds, which are not damaged during surface sterilization. Various authors were suggested the axillary buds' explants for propagation of other medicinal plants, such as *Rouwolfia serpentina* [24]; *Holarrhena antidyseterica* [25]; *Eupatorium triplinerve* [15,17].

Figure – 1: *In vitro* Regeneration of *Eupatorium triplinerve* Vahl. a. Healthy axillary bud explants, b. Shoot initiation, c. Shoot multiplication, d. Root induction, e. Shoot lets with well-developed roots, f. Shoot lets under primary hardening, g. Secondary hardened plant with vigorous growth.

CONCLUSION

The aim of present study was achieved and the successful production of multiple shoots and *in vitro* root formation were dependent on the nutrient medium and the culture conditions. The explants axillary buds were found to be suitable for successful regeneration. This protocol may help to the large-scale production of this important medicinal plant and this study might provide new opportunities for clonal propagation and germplasam conservation of *Eupatorium triplinerve* Vahl.

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