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In vitro axillary bud multiplication of an important medicinal plant-*Ayapana triplinervis* vahl

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Abstract
An efficient protocol was developed for the micropropagation of *Ayapana triplinervis* through axillary bud multiplication using nodal explants. The excised nodal segments were inoculated to MS media supplemented with various concentrations and combinations of PGRs. Murashige and Skoog's medium fortified with 0.5 mgL^{-1} Kinetin was the best combination for the shoot proliferation of *Ayapana triplinervis.* An average of 15 shoots was formed from each explant within 60 days of incubation. Rooting was observed in all the media combinations tested; however most effective root formation was observed in MS medium supplemented with 1.0 mgL^{-1} IBA. The *in vitro* developed plantlets were hardened and successfully established in pots with 100% survival rate.

Keywords: axillary bud, *Ayapana triplinervis*, hardening, *In vitro,* kinetin

Introduction
Ayapana triplinervis Vahl. (Syn. *Eupatorium ayapana*, *Eupatorium tripliverve*) belonging to the family Asteraceae is an erect perennial herb with aromatic properties. It is familiar in India as well as in other tropical countries. The plant has antiseptic, stomachic, antitussive, diaphoretic, antiulcerous, hemostatic (Rajasekaran *et al*., 2010) [13], antitumerous, anticoagulant, hepatoprotective (Bose *et al*., 2007) [2], astrigen and emollient properties (Gauvin-Bialecki & Marodon, 2008) [7]. *A. triplinervis* contains a variety of coumarins; mainly 'ayapanin and ayapin', which have anticoagulant properties, so it is used as the precursor for drugs such as 'Warfarin' (Bose, 1937) [1]. The antiulcer effect and radical scavenging activity of *A. triplinervis* extract was used to treat against ulcerative colitis in mice (Krishnan *et al*., 2014) [9]. The active secondary compound present is 7- methoxy coumarin called as 'herniarin or ayapanin' and rest of the constituents found in leaves are 6, 7- dimethoxy coumarin (ayapin), carotene, vitamin C and stigmasterol (Bose & Roy, 1936) [3]. In addition to these, other coumarins like hydragetin, daphnetin, daphnetin-7-methyl ether dimethyl ether and umbelliferone are present in the plant (Chaturvedi & Mulchandani, 1989) [4]. The 7-methoxy coumarin has antitumerous activity and is toxic to multi drug resistant cancer cells (Kawase *et al*., 2005) [8] and inhibitory against chemicals dispensed from leukemic cells (Watanabe *et al*., 2005) [15], it also has anti-nociceptive activity (Cheriyan *et al*., 2017) [5]. Phytochemical analysis of *Ayapana triplinervis* revealed various phytochemicals except steroids and saponins (Mamatha & Thangavel, 2018) [10]. The objective of the present study was to develop an efficient protocol for the rapid propagation of this important medicinal plant using nodal segments.

Materials and Methods
Plant Material and Surface Sterilization
Nodal segments were collected from the shoots of *A. triplinervis* Vahl. Growing in the Botanical garden of St. Joseph's college (Autonomous), Devagiri, Calicut. The nodal segments (NSs) were thoroughly washed under running tap water for 30 minutes and then vigorously washed with Extran (5% v/v) for 10 minutes. Then the explants were rinsed with distilled water and were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 8 minutes, followed by washing 3-4 times with sterile distilled water. After surface sterilization the nodes were trimmed at cut ends and reduced the size to 1 – 1.5 cm before inoculation to sterilized media.

Culture Medium
MS medium (Murashige & Skoog, 1962) [12] was used throughout the study and it was supplemented with varying concentrations and combinations of plant growth regulators (PGRs). 8 gL^{-1} agar was used to solidify the media and 30 gL^{-1} sucrose was used as the source of carbon. PGRs such as BAP, Kinetin (Kn), NAA and IBA in different concentrations and combinations were used. pH of the media was adjusted to 5.8 with 0.1 M NaOH and 0.1 M HCl, culture media were sterilized by autoclaving at 1 atmosphere pressure and 121°C for 20 minutes. After inoculation, the cultures were incubated at 25 ± 2°C with 16h photoperiod under white tube lights. These conditions were maintained throughout the multiplication procedure.

Shoot Multiplication
For axillary bud proliferation explants were cultured on MS media supplemented with different concentrations of PGRs like Kn, BAP, NAA and IBA, either individually or in combinations. A total of 14 combinations of PGRs with MS media were used. The best medium for axillary bud multiplication was selected based on the number of shoots formed per explant and the length of the shoots formed.

Rooting
For root induction, *in vitro* raised shoots were cultured on MS media supplemented with various concentrations and combinations of NAA/IBA.

Acclimatization
Fully grown *in vitro* raised plantlets were taken out from the culture vessels and washed with sterile distilled water for

removing the agar and then transferred to paper cups containing auotclaved sand: soil (1:1). The cups were covered with transparent polythene bags to maintain humidity and to prevent drying of the plants. The polythene bag was removed after 10 days. Finally, the plantlets were transplanted to the garden pots with potting mixture.

Statistical Analysis of Data

All the experimental trials were conducted with 12 replicas for each treatment. The observations of the cultures were done regularly and also every morphological change was recorded at regular time periods. The results were analysed statistically using SPSS Version 16.0 and data were compared using ANOVA and Duncan's multiple range test. Graph was constructed using MS Excel.

Results and Discussion

Shoot Multiplication

Nodal explants were cultured on MS media containing various concentrations and combinations of PGRs and the results were evaluated for 60 days of growth period (Table 1, Figures 1&2). 1 – 2 shoots (1.7±0.64) were formed from each explant in MS basal media without any plant growth regulators (control). When cultured in media fortified with cytokinins increased growth rate and increase in the number of shoots per explant were observed. Best axillary bud multiplication was obtained when cultured on MS medium fortified with 0.5 mgL^{-1} Kinetin, this combination induced the maximum multiplication of shoots (13.9±7.86) than other concentrations and combinations of PGRs. After 60 days of incubation, the number (13.9±7.86) and length of shoots (4.4±1.04cm) were high in 0.5 mgL^{-1} Kinetin fortified medium. However length of shoots (5.08±1.94cm) were highest in MS medium supplemented with 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} NAA, this could be due to the synergistic effect of cytokinin and auxin in shoot elongation. Previously Martin, 2003 reported synergistic effect of cytokinin and auxin in shoot elongation and axillary bud multiplication of *A. Triplinervis*. But in the present study highest shoot multiplication was observed in media supplemented with cytokinin alone. Kinetin in combination with auxins NAA and IBA induced the production of less number of shoots, large number of roots and callus formation. Similar results showing highest shoot multiplication of *A. Triplinervis* in medium supplemented with cytokinins alone was reported earlier by Usha & Karpagam, 2017 [14]. In the present study BAP with NAA combination was not effective in axillary bud proliferation (0.8±0.46) of *A. Triplinervis*, this combination only induced very low amount of callus (brown coloured). The MS medium containing 1.0 mgL^{-1} BA with 0.5 mgL^{-1} IBA induced the production of less number of shoots (1.5±0.57). At higher concentrations of NAA (1.0 mgL^{-1}) the number of shoots declined and a tuft of roots were formed from the basal cut ends.

Fig 1: *In vitro*, culture establishment of *A. triplinenervis*. (a) Habit; Culture established in medium containing (b) 0.5 mg La^{-1} Kinetin; (c) 0.5 mg L^{-1} BA: (d) 0.5 mg t^{-1} NAA: Acclimatized plantlets (e) in sterile soil al lab conditions and (f) well established plantlet in Garden pot.

Table 1: Effect of PGRs on shoot multiplication from nodal segments of *A. triplinervis* in MS medium

KIN (mgL^{-1})	BA (mgL^{-1})	NAA (mgL^{-1})	IBA (mgL^{-1})	No. of Shoot	Shoot length (cm)
0	0	0	0	1.7±0.64	4.8±1.16
0.5	0	0	0	13.9±7.86	4.4±1.04
0	0.5	0	0	0.9±0.63	4.7±2.05
0	0.5	0.5	0	0.8±0.46	3.04±1.66
0	1.0	1.0	0	1.1±0.63	5.08±1.94
0	1.0	0.5	0	1.7±0.93	3.5±1.51
0.1	1.0	0	0	3.2±2.09	1.7±0.97
1.0	0.1	0	0	18.1±11.85	0.6±0.35
0.5	1.0	0	0	3.08±2.18	2.3±0.71
1.0	0.5	0	0	2.4±1.32	1.5±0.58
0.5	0	0.5	0	1.9±1.11	3.2±1.39
1.0	0	0.5	0	0.9±0.57	4.2±2.3
0	1.0	0	0.5	1.5±0.57	2.9±1.05
0	0	0	0.5	1.4±0.78	4.2±1.54
0	0	0	1.0	1.4±0.64	3.6±1.37

Data in each column represents mean ± standard deviation. Significant differences denotes as $p< 0.05$ according to Duncan's multiple range test (Duncan, 1955) [6].

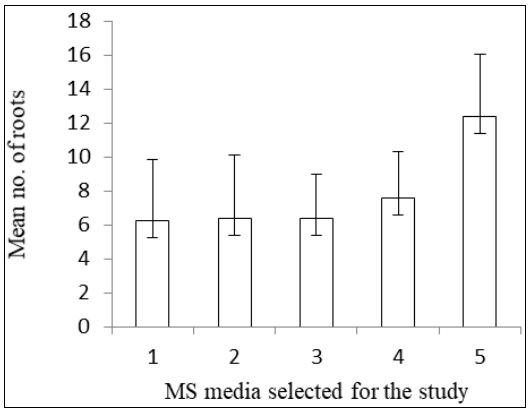

Fig 2: Root formation in *in vitro* plantlets of *A. triplinervis* in MS media with various PGRs (1) 0.5 mgL^{-1} NAA, (2) 1.0mgL^{-1} NAA, (3) 1.0 mgL^{-1}

Rooting

In the present study spontaneous root formation was observed from *in vitro* raised shoots of *A. triplinervis* in all the different combinations of media used. Rooting was visible after 5-6 days of inoculation and 90% of *in vitro* raised shoots produced 3-4 roots in the media used for shoot multiplication without the addition of auxins like NAA or IBA. This may be due to the presence of enormous amount of endogenous auxins within the explants. Slight brown callusing at the base with tuft of thin and hair like roots (6.36±3.74) were formed in MS medium supplemented with 1.0 mgl-1 NAA. MS media fortified with IBA improved the root growth and reduced the time duration for root induction. 1.0 mgL-1 IBA in MS medium was best for *in vitro* rooting (12.36±3.67) of *A. triplinervis.* Similar results showing efficiency of IBA in root induction of *A. triplinervis* was reported earlier by (Martin, 2003) [11].

Acclimatization

In vitro raised plantlets were successfully transferred to the *ex vitro* conditions and they showed good growth and stayed healthy. Their shoots flourished and leaves became bigger and greenish under field conditions. The plants produced by microprpagation were morphologically and physiologically similar to mother plants.

Conclusion

The protocol developed for the *in vitro* axillary bud multiplication of *A. triplinervis* is best and convenient for the rapid propagation of this important medicinal plant. MS medium fortified with 0.5 mgL^{-1} Kinetin was the best combination for the rapid shoot production of *A. triplinervis*. The present work developed an efficient protocol for the propagation of contamination free plants with in a limited time period. By using the *in vitro* raised plants other stress related studies such as enhancement and the assessment of secondary metabolite concentrations in the plant parts for the pharmaceutical pruposes of *A. triplinervis* could be conducted.

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