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Rapid and Efficient Plant Regeneration from Nodal Explants of *Artemisia annua* L.

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Abstract

An efficient plant regeneration protocol was developed for *Artemisia annua*, an aromatic medicinal herb. Nodal explants inoculated on MS supplemented with 4.44 μM BAP showed better growth response and produced 116.2 ± 0.1 micro-shoots of an average length 1.9 ± 0.3 cm after 35 days culture. The cluster of small shootlets were cultured on shoot elongation medium supplemented with 1.44 μM GA_3 and 10% coconut milk (CM) showed shoot elongation up to 4.6 ± 0.7 cm. Roots were induced after transfer to half strength MS supplemented with 2.46 μM IBA produced 14.3 ± 0.2 roots with an average height of 4.3 ± 0.53 cm after 30 days. The rooted plantlets were transferred for hardening, 80 per cent of plantlets survived and resumed growth in the mixture of soil, vermiculite and farm yard manure (1 : 1 : 1).

Introduction

Artemisia annua L. a valuable medicinal plant belongs to Asteraceae. It is grown commercially in many parts of Europe, America, China, Turkey, Iran, Afghanistan and Australia (Bhakuni et al. 2001, Allen et al. 1997). In India, it is being cultivated on an experimental basis in temperate as well as subtropical conditions (Mathur and Kumar 1996, Prasad et al. 1997, Ram et al. 1997). All *Artemisia* species produce aromatic oils and important compounds, such as artemisinin produced by *Artemisia annua*, very effective against both *Plasmodium vivax* and *Plasmodium falciparum*, the latter being responsible for cerebral malaria (Klayman 1985, Sy and Brown 2001). Artemisinin is a rare sesquiterpene lactones end peroxide isolated from *A. annua*, it was recommended as a medicine against malaria. The yield of the active compound from wild or cultivated plants (leaves

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or flower) is low and varies from 0.01 to 0.06% but may reach 0.5 % in certain Chinese varieties (Klayman 1985, Ferreira et al. 1997). Populations raised from seeds are characterised by morphological variation in plant height, leaf biomass and flowering dates, thus, resulting in low and variable yield of the active constituents (Elhag et al. 1992). Micropropagation and organogenesis of different *Artemisia* species have been previously established by using several parts of plants, in order to obtain a large number of plants, such as *A. scorpia* (Aslam et al. 2006), *A. vulgaris* L. (Govindaraj et al. 2008), *A. mutellina* Vill. (Mazzetti and Donato 1998). *In vitro* culture of *A. annua* has been attempted through organogenesis (Lualon et al. 2008). The present study aims at developing a simple, rapid, economical, and high frequency regeneration protocol from nodal explants of *A. annua* for potential application in large scale propagation.

Material and Methods

Healthy plants of *Artemisia annua* L. collected from Ooty Botanical Garden (Ooty), Tamil Nadu, India and were raised in pots containing soil and farm yard manure (1 : 1) under greenhouse conditions at Department of Biotechnology of authors' College. Nodal segments were collected from potted plants, brought to the laboratory and processed. For surface sterilization, the explant were cleaned thoroughly under running tap water for 20 min; washed with a solution of Tween 20 (2 drops in 100 ml of water) for 1 min, and again washed with sterile distilled water. The cleaned explants were finally treated with 0.1% (w/v) $HgCl_2$ for 4 min under aseptic conditions and washed five times with sterile distilled water to remove traces of $HgCl_2$.

After surface sterilization, explants were trimmed to 0.8 - 1.0 cm and inoculated on MS supplemented with individual concentrations of BAP (1.11, 2.22, 4.44, 6.66 and 8.88 μM), TDZ (1.14, 2.27, 4.54, 6.77 and 9.08 μM) and Kn (0.46, 2.32, 4.65, 9.20 and 13.75 μM) for shoot multiplication. At the end of the experiment, percentage of shooting, shoot length and the number of shoots per explant were recorded after 35 days in culture. Small shoots were transferred to MS supplemented with different concentrations of GA_3 (0.72 - 2.89 μM) with CM (10.0%) for shoot elongation.

The proliferated shootlets (4.6 cm in length) were excised from cultures and transferred to half strength MS supplemented with IBA (0.49, 0.98, 2.46, 4.92 and 12.30 μM) for *in vitro* rooting. Root number and length were recorded after 30 days in culture. Well developed plantlets were rinsed thoroughly with sterile water to remove residuals and potted with a mixture of red soil, vermiculite and farm yard manure (1 : 1 : 1), covered with transparent polyethylene bags to

ensure high humidity. After 15 days, the fully acclimatized plantlets were transplanted to plastic pots (80 mm diameter).

For all the above studies, MS supplemented with 3% (w/v) sucrose was used for all *in vitro* culture studies. The pH of the medium was adjusted to 5.6 ± 0.2 prior to adding 0.9 % (w/v) agar, and autoclaved at 121°C for 15 min. Cultures were maintained at 25 ± 1°C under 16 hr photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol $m^{-2}s^{-2}$ provided by cool white fluorescent tubes (Phillips, India) and with 60 - 65% relative humidity. The plant growth regulators (PGRs) were filter sterilized using 0.2 μm filter (Minisart®, Sartorius,VivaScience AG, Hannover, Germany) prior to addition to culture media.

Each experiment was repeated three times and each treatment had six replicates. The data were analysed using ANOVA, and the means were compared using the DMRT using SPSS (SPSS version 16.0) at 5% level of significance ($p < 0.05$).

Results and Discussion

Multiple shoots developed from nodal explants cultured on MS supplemented with BAP (1.11 - 8.88 μM), TDZ (1.14 - 9.08 μM) and Kn (0.46 - 13.75 μM). In the present study, individual concentration of BAP seems to influence the induction of shoots in *Artemisia annua.* Initiation of multiple shoots in most of the treatments was observed within three weeks of culture. High number of micro-shoots developed in MS containing 4.44 μM BAP showed better growth response (80%) and produced 116.2 ± 0.1 small shootlets per explant with an average length of 1.9 ± 0.3 cm after 35 days of culture (Table 1, Fig. 1A- C). The cluster of small shootlets were cultured on shoot elongation medium supplemented with 1.44 μM GA_3 and 10% CM showed shoot elongation up to 4.6 ± 0.4 cm (Table 2, Fig. 1D).

Higher concentration of BAP (8.88 μM) resulted in gradual decrease in the number of shoots per explants. BAP has been considered to be one of the most active cytokinins in organogenic differentiation in plant tissue culture (Gururaj et al. 2007, Fracaro and Echeverrigaray 2004, Janarthanam et al. 2009). Coconut milk (CM) is an undefined complex mixture of organic substances that has been successfully employed for culturing different plant species (Arditti and Ernst 1993, Suttle 1996, Janarthanam et al. 2011). It is evident in their report that CM stimulated the development of shoot buds. In general, CM is known to promote callus formation and also plays a suppressive role in the formation of shoots buds. However, in the present investigation the results were contrary to the general trends and coincided with the findings of Maity et al. 2005. Nodal

explants transferred to MS containing TDZ (1.14 - 9.08 μM), TDZ 4.54 μM produced 17 small shoots and an average length of 1.7 cm after 35 days, whereas the higher concentration of TDZ (9.08 μM) produced dark green callus and compact structures which ultimately turned brown and failed to develop into normal shoots (Table 1). In KN alone (0.46 - 9.20 μM) supplemented medium was not effective.

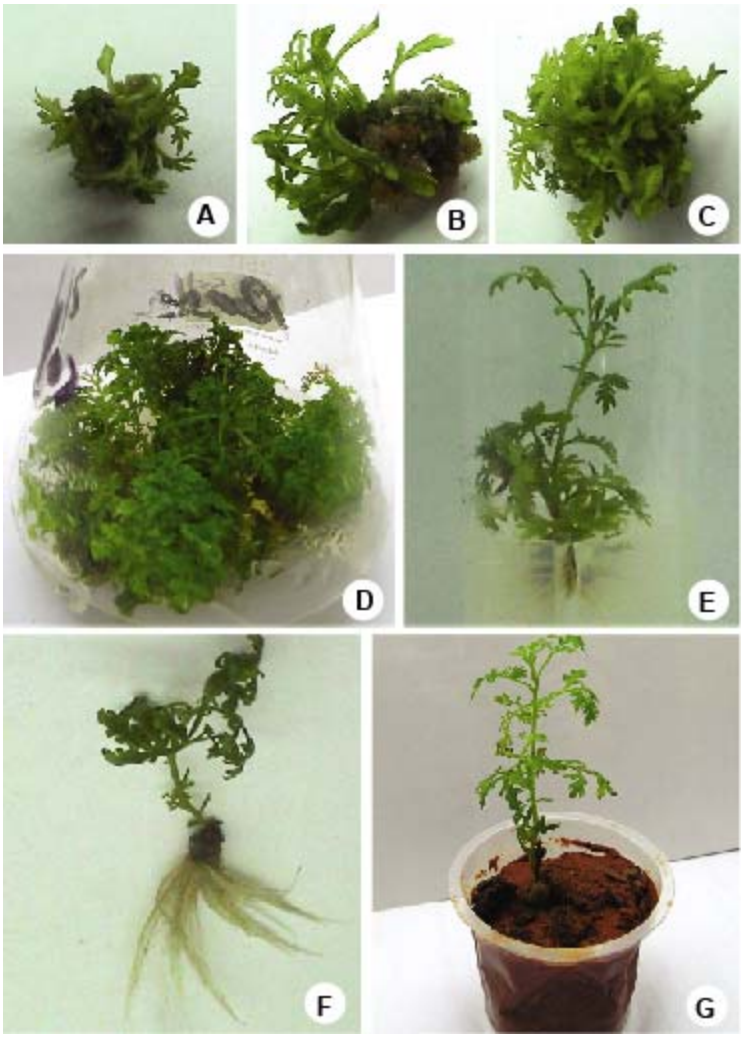

Fig 1. A-F. Regeneration of multiple shoot from nodal explants of *Artemisia annua*. A-B. Nodal explants inoculated on MS supplemented with BAP 4.44 μM after two weeks of culture. C. Proliferation of multiple shoots from nodal explants at 35 days of cultured on MS containing BAP 4.44 μM. D. Cluster of small shootlets were cultured on shoot elongation medium supplemented with 1.44 μM GA_3 and 10% CM. E. Healthy *in vitro* shootlets inoculated on half strength MS containing 2.46 μM IBA. F. A well established plant. G. Well established and hardened in *vitro* plants successfully transferred to the paper cups.

Table 1. Effect of BAP on shoot regeneration from nodal explants of *Artemisia annua*.

Growth regulators (μM)		Response (%)	Number of shoot per explant	Shoot length (cm)
BAP	1.11	39.8 ± 2.9	17.6 ± 0.4	1.2 ± 0.3
	2.22	46.8 ± 3.5	24.0 ± 2.1	1.3 ± 0.1
	4.44	80.0 ± 5.0	116.2 ± 0.1	1.9 ± 0.3
	8.88	52.8 ± 3.9	29.1 ± 0.4	1.1 ± 0.1
TDZ	2.27	34.9 ± 3.2	14.2 ± 2.8	1.1 ± 0.2
	4.54	40.0 ± 2.5	17.1 ± 1.1	1.7 ± 0.2
	6.77	37.7 ± 4.6	16.5 ± 2.3	1.4 ± 0.5
	9.08	28.6 ± 3.3	11.9 ± 1.3	1.3 ± 0.3
Kn	0.46	8.6 ± 0.8	4.3 ± 1.0	1.1 ± 0.1
	2.32	15.9 ± 3.3	6.1 ± 1.6	1.3 ± 0.1
	4.65	23.9 ± 2.9	10.3 ± 1.6	1.4 ± 0.1
	9.20	5.3 ± 6.9	2.0 ±1.4	1.0 ± 0.3

Results represent mean ± SD of three replicated experiments and data were recorded after 35 days of culture.

Table 2. Effect of different concentrations of GA_3 and CM on MS on shoot elongation from *in vitro* microshoot of *Artemisia annua*.

Growth regulators GA_3 (μM)	Concentration of CM (%)	Mean number of shoots/explant	Mean shoot length
Control		116.2 ± 0.1	1.9 ± 0.3
0.72	5	116.2 ± 0.1	1.9 ± 0.3
	10	116.2 ± 0.1	2.3 ± 0.3
	15	116.2 ± 0.1	2.0 ± 0.2
1.44	5	116.2 ± 0.1	3.1 ± 0.3
	10	116.2 ± 0.1	4.6 ± 0.4
	15	116.2 ± 0.1	3.4 ± 0.5
2.89	5	116.2 ± 0.1	2.2 ± 0.2
	10	116.2 ± 0.1	2.8 ± 0.5
	15	116.2 ± 0.1	2.3 ± 0.2

Control: MS with BAP 1.0 mg/l. Results represent mean ± SD of three replicated experiments. Data were recorded after 35 days of culture.

Individual shoots from a multiple shoot complex were separated after 28 days of culture and transferred to half strength of MS supplemented with IBA (0.49 - 12.30 μM). The root induction was initiated after two weeks of culture, and after four week s, the root system was well developed (Fig. 1E,F). The

maximum rooting response (75%) was achieved on medium supplemented with IBA (2.46 μM), with an average of 14 roots per *in vitro* shoot (Table 3). Higher concentration of IBA (12.30 μM) resulted in gradual decrease in the number of shoots per explants.

Table 3. Effect of different concentration of IBA in half strength MS on rooting response of *Artemisia annua*.

IBA (μM)	% response	Roots/shoot	Root length (cm)
0.49	30.0 ± 5.0	2.6 ± 0.5	3.06 ± 0.3
0.98	31.6 ± 2. 8	2.6 ± 1.2	3.06 ± 0.5
2.46	75.0 ± 5. 0	14.3 ± 0.2	4.3 ± 0.53
4.92	45.0 ± 5. 0	2.6 ± 0.6	2.30 ± 0.3
12.30	36.6 ± 2. 8	2.0 ± 1.0	2.00 ± 0.2

Results represent mean ± SD of three replicated experiments and data were recorded after 30 days of culture.

In the present study root induction was obtained with lower concentration of IBA 2.46 μM and produced significantly higher rooting this could be attributed to the nature of *in vitro* shootlets from nodal explants grown on BAP. Eighty percent plantlet survival was seen after hardening of the regenerated *Artemisia annua* in red soil, vermiculite and farmyard manure (1 : 1 : 1) for three weeks. However, the rate decreased as some plants died over the next 4-5 weeks after transfer to soil. It was observed that very gradual acclimatization of *in vitro* grown plants to the external environment is most essential to *Artemisia annua.* Seventy per cent of the plants transferred to pots survived and resumed growth (Fig. 1G).

In conclusion, the results showed the ability of the internodal explant explants to produce higher number of shootlets without any intervening callus phase, where all the plantlets were uniform in height and growth. Hence, the present authors propose this protocol a simple, economical, rapid and highly reproducible to obtain more plantlets within a short period of time.

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