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Standardized protocol for the *in vitro* culture of *Artemisia annua* L. – A medicinal plant at high altitudes of Nilgiris, the Western Ghats.

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ABSTRACT:

A reliable protocol for callus induction and organogenesis, and successful plantlet survivability through hardening were developed for leaf explants of the medicinal plant species, *Artemisia annua* L. MS medium containing the auxin, NAA at 0.9mg/l is determined to be the optimum concentration for callus induction. Higher shooting (20.67/callus) was performed in the medium supplemented with BAP and GA_3 at 0.5 and 1.0 mg/l respectively while subculturing the callus. Maximum number of roots (12.00 roots/shoot) was noted to be obtained in the medium containing IBA at 0.9mg/l. The *in vitro* regenerated plantlets were successfully acclimatized (86% survivability rate) in the hardening medium encomposing vermiculate, coir waste and forest litter in the ratio of 1:1:1 by volume.

Keywords:
In vitro culture, Medicinal plant, *Artemisia annua*, Nilgiris, Western Ghats, India.

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INTRODUCTION

Artemisia annua L. (Asteraceae) is a medicinal herb inhabiting at the high altitudes of Nilgiris, Western Ghats at open habitats. Presence of high content of bioactive alkaloids like artemisinin in *A.annua* receives attention towards pharmacological industries as it is used for the treatment of malarial fever (Duke *et al.,* 1987; Chen *et al.,* 1991). As a result of over exploitation, the species become lower in population size at the high altitudes of Nilgiris (Paulsamy *et al.,* 2008). Conventional method of propagation of this species through seed was also not successful (Paulsamy, 2005). Hence, *in vitro* culture by employing tissue culture technology has been attempted for this species to enable bulk production.

MATERIALS AND METHODS

The leaf explants of *Artemisia annua* were collected from the healthy individuals at Nilgiris and washed thoroughly with tap water, then they were cut into small discs of 0.8cm diameter and then treated with a surfactant, tween 20 (5% w/v) for 5 minutes. After repeated washes in double distilled water, to eliminate the fungal contamination, the explants were treated with Carbendazim (50% w/v), fungicide (10%) for 15 minutes and rinsed with double distilled water for 2 or 3 times. To eliminate bacterial contamination explants were also treated with 5% antibiotics (ampicillin and rifampicin) for 30 minutes followed by three rinses in sterile double distilled water. Furthermore, surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 minutes followed by 3-4 rinses in sterilized double distilled water inside the Laminar air flow chamber. Leaf discs were horizontally placed in Petri dishes containing MS (Murashige and Skoog, 1962) medium fortified with various combinations and concentrations of different growth regulators *viz.,* BAP, NAA, 2,4-D and TDZ for callus induction. The pH of the medium was adjusted between 5.6 and 5.8 before autoclaving at 121^{o}C for 20 min. The culture was incubated at a constant temperature of 25$\pm$2^{o}C with 14h photoperiod (3000 lux) and 8h darkness. Callus from these primary cultures were transferred to MS medium containing different concentrations of BAP, IAA and GA_3 for shoot induction. After the origin of multiple shoots, elongated shoots of 2 cm long were excised from the culture and transferred to MS medium supplemented with different concentrations of IBA, IAA and NAA for root initiation. After two weeks, the percentage of shoot forming roots, roots per shoot length were assessed. Rooted shoots were thoroughly washed to remove the adhering gel and planted in polythene bags containing different hardening media and kept in greenhouse for acclimatization. The pots were watered at one day interval and supplied with ½ strength MS salts, twice a week by spraying. The survival rate of plantlets was recorded for one month after transferring to polythene bags. Triplicates were maintained for all experiments.

RESULTS AND DISCUSSION

The number of days required for callus induction from the leaf explants of the study species, *Artemisia annua* is noted to be varied from 10 to 30 days according to the combinations and concentrations of the growth regulators *viz.,* BAP, NAA, 2,4-D, IBA and TDZ in the MS medium (Table 1). It may be explained that the specific growth hormones at appropriate concentrations can play a major role to induce callus besides the other

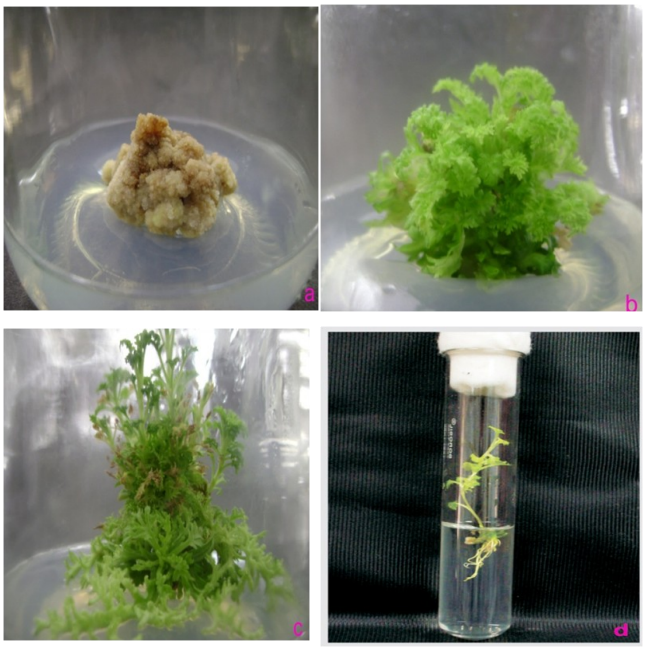

Figure 1. Successful *in vitro* culture of *Artemisia annua* from the leaf explants.
a, Effective callus formation in MS medium containing 0.9 mg/l of NAA.
b, Higher response of callus for shoot formation in MS medium fortified with BAP at 2mg/l.
c, High degree of multiple shoot formation while subculturing onto MS medium supplemented with BAP and GA_3 at 2.7 and 0.9 mg/l respectively.
d, More pronounced root formation during subculturing onto MS medium containing 0.9mg/l of IBA.

factors (Ananthi *et al.,* 2011). The amount of leaf explant responding for callus formation was ranging between 10 and 84% (Table 1). MS medium fortified with NAA at 0.9 mg/l initiated 98.66% of leaf discs for callus formation (Fig. 1a) followed by 0.7mg/l of NAA initiated 84% of leaf discs for callusing and 0.5mg/l of NAA and TDZ each initiated 73% of discs for callusing. The other combinations and concentrations of growth hormones in the medium initiated around only 20 to 50% of leaf discs of *A.annua* for callus formation. Baskaran and Jayabalan (2005) explained that the differential response of same or different explants for callus formation could be due to the nature of tissue, degree of totipotency and composition of medium with respect to micronutrients and hormones. Further it is explained that the variation in response of discs in terms of callus initiation may be due to the variation in distribution of endogenous level of growth regulators as observed in many other plants (Farternale *et al.,* 2002; Senthilkumar and Paulsamy, 2010b). It was noted that the NAA alone or in combination with TDZ generally have the efficiency of initiation at high percentage of (>50%) leaf explant for callus formation. It indicates the higher requirement of certain auxins like NAA alone or in combination with low quantity of cytokinins like TDZ for callus formation of the study species, *A. annua.* Karappusamy and Pullaiah (2007) for the species, *Bupleurum distichophyllum* and Senthilkumar and Paulsamy (2010a) for the species, *Ageratum conyzoides* also have reported effective callus formation from the leaf explants in the medium containing high quantity of NAA. Mariani *et al.,* (2011) reported the requirement of the cytokinin like compounds, TDZ for effective callus formation in the ornamental plant, *Aglaonema sp.* The colour

Table 1. Effect of different concentrations of growth regulators on per cent callus induction from leaf, node and intermodal explants of the species, *Artemisia annua.*

Growth regulator (mg/l)					Days required for callus formation after inoculation	Callus formation (%)	Colour of the callus
BAP	NAA	2,4-D	IBA	TDZ	Leaf explant	Leaf explant	Leaf explant
0.5	0	0	0	0	10	10.00±2.00[a]	G
1.0	0	0	0	0	12	13.66±1.52[a]	G
1.5	0	0	0	0	14	18.00±2.00[b]	G
2.5	0	0	0	0	16	21.33±1.53[bc]	G
3.0	0	0	0	0	18	23.33±1.58[c]	G
0	0.1	0	0	0	18	25.00±2.00[c]	G
0	0.3	0	0.5	0	21	29.00±2.00[cd]	G
0	0.5	0	1.0	0	20	30.00±1.00[d]	DG
0	0.7	0	1.5	0	23	49.00±2.00[d]	DG
0	1.0	0	2.0	0	22	57.33±2.51[e]	DG
0.5	0	0.5	0	0	18	39.00±1.00[f]	G
1.0	0	1.0	0	0	17	44.33±2.08[d]	DG
1.5	0	1.5	0	0	15	46.66±1.52[d]	DG
2.0	0	2.5	0	0	21	49.33±2.08[d]	LG
2.5	0	3.0	0	0	19	51.33±2.08[d]	LG
3.0	0	3.5	0	0	23	55.00±1.00[e]	B
0	0.1	0	0	0	14	39.66±1.53[f]	LG
0	0.3	0	0	0	18	49.66±1.55[d]	LG
0	0.5	0	0	0	20	46.66±1.59[d]	B
0	0.7	0	0	0	21	84.00±4.35[g]	LB
0	0.9	0	0	0	30	98.66±2.51[h]	LB
0	0.5	0	0	0.1	22	54.33±1.58[e]	DB
0	0.5	0	0	0.2	19	54.66±0.57[e]	DB
0	0.5	0	0	0.3	17	56.66±2.08[e]	DB
0	0.5	0	0	0.4	25	58.00±3.00[e]	DB
0	0.5	0	0	0.5	23	73.00±4.00[i]	LB

G-Green, DG- Dark green, LG- Light green, B-Brown, DB-Dark brown, LB- Light brown
Means in column followed by different letter(s) are significantly different at 5% level according to DMRT.

of the calli was showing wide degree like green, dark green, light green, brown, dark brown and light brown according to the combinations and concentrations of the growth regulators in the MS medium (**Table 1**).

The results of the subculturing experiments by using the secondary explant, leaf derived callus showed that the cytokinin, BAP alone in higher concentration (>1.5mg/l) (Fig. 1b) or BAP in combination with GA_3 have enhanced the response of calli for shoot formation by 98 and 90% respectively (Table 2). In addition, greater number of 20 shoots/callus was also noted to be produced while subculturing the calli on MS medium with BAP and GA_3 at 2.7 and 0.9 mg/l respectively (Table 2) (Fig. 1c). However, the higher shoot length of 10cm was achieved in the MS medium fortified with BAP alone at 2 mg/l (Table 2). All these facts indicate that the cytokinin, BAP is the most essential growth regulator for effective shooting of the study species, *A.annua*. It is of common fact that cytokinin is the major growth hormone involved in shoot formation in many plant species (Vijaykumari *et al.,* 2001; Roy *et al.,* 2008; Senthilkumr and Paulsamy, 2010a; Sunder and Jawahar, 2011).

The rooting attributes of *A.annua* while subculturing the secondary explant shoots were well pronounced in the MS medium supplemented with the auxin, IBA alone at higher concentrations from 0.5 to 0.9 mg/l (Table 3). The IBA concentration at 0.9 mg/l initiated 85% shoots for root formation (Fig. 1d) followed by 0.7mg/l initiated 80% and 0.5 mg/l initiated 71% shoots for root formation. The number of roots per shoot were also observed to be higher (12 roots/shoot) in the MS medium containing 0.9mg/l IBA for the study species, *A.annua.* Similarly, the root length was greater (5.1cm) during the subculturing of *in vitro* cultured shoots for roots on MS medium with IBA at 0.9 mg/l. All these facts showed that the auxin, IBA is the most required growth regulator for shooting characters of the study species, *A.annua*. It agrees with the concept that auxins are the plant hormones endogenously or exogenously inducing root formation in majority of plant species (Van

Table 2. Effect of different concentrations of growth regulators on shoot initiation, shoot number and shoot length after subculturing the leaf derived callus of the species, *Artemisia annua.*

Growth regulator (mg/l)			Culture response (%)	No. of shoots/callus	Shoot length (cm)
BAP	IAA	GA_3			
0.5	0.0	0.0	57.33±2.08^{ag}	08.66±1.52^{a}	07.57±0.35^{a}
1.0	0.0	0.0	65.66±3.51^{b}	09.00±2.00^{a}	07.93±0.57^{a}
1.5	0.0	0.0	85.33±3.05^{c}	12.00±1.00^{b}	08.83±0.74^{a}
2.0	0.0	0.0	98.00±2.00^{d}	14.00±1.00^{b}	10.20±0.26^{b}
0.5	0.2	0.0	20.33±3.05^{e}	06.33±1.53^{a}	04.80±0.30^{c}
1.0	0.4	0.0	29.66±1.53^{e}	07.33±1.53^{a}	06.50±0.20^{ca}
1.5	0.6	0.0	48.33±2.08^{af}	09.67±1.54^{a}	07.00±0.20^{a}
2.0	0.8	0.0	62.33±4.51^{b}	14.00±2.00^{b}	07.37±0.15^{a}
3.0	1.0	0.0	93.00±2.00^{d}	17.66±1.56^{cd}	08.50±0.36^{a}
0.3	0.0	0.1	45.00±2.00^{f}	12.33±1.53^{b}	05.03±0.25^{c}
0.6	0.0	0.2	50.33±3.21^{f}	15.00±2.00^{bd}	05.13±0.15^{c}
0.9	0.0	0.3	54.00±3.00^{a}	15.67±1.53^{bd}	05.23±0.15^{c}
1.2	0.0	0.4	55.33±2.08^{a}	16.67±1.53^{bd}	05.20±0.10^{c}
1.5	0.0	0.5	55.33±1.53^{a}	17.00±2.00^{cd}	05.30±0.10^{c}
1.8	0.0	0.6	59.67±3.05^{g}	18.00±2.00^{cd}	05.67±0.15^{c}
2.1	0.0	0.7	70.33±1.53^{b}	19.67±1.57^{cd}	05.90±0.20^{c}
2.4	0.0	0.8	80.00±1.00^{c}	19.00±2.00^{cd}	05.90±0.10^{c}
2.7	0.0	0.9	90.00±2.00^{e}	20.33±2.08^{cd}	06.67±0.15^{ca}
0.5	0.0	1.0	94.67±2.08^{e}	20.67±1.58^{cd}	07.10±0.20^{a}
1.0	0.2	1.0	46.33±2.52^{af}	07.00±2.00^{a}	02.87±0.20^{d}
1.5	0.4	1.0	50.67±2.52^{f}	06.67±1.53^{a}	02.93±0.15^{d}
2.0	0.6	1.0	51.33±2.52^{f}	06.00±1.00^{a}	03.00±0.10^{dc}
2.5	0.8	1.0	59.33±2.08^{g}	07.00±1.00^{b}	03.47±0.25^{dc}
3.0	1.0	1.0	74.00±2.00^{b}	11.33±1.53^{a}	04.70±0.20^{c}

Means in columns followed by different letter(s) are significantly different at 5% level according to DMRT.

Table 3. Effect of different concentrations of growth regulators on root number, rooting percentage and root length after subculturing the leaf calli derived shoots of the species, *Artemisia annua.*

Growth regulator (mg/l)			Shoots rooted (%)	No. of roots/shoot	Root length (cm)
IBA	IAA	NAA			
0.1	0.0	0.1	19.00±1.00^{a}	05.00±0.81^{a}	2.10±0.20^{a}
0.2	0.0	0.2	20.66±1.52^{a}	07.00±0.82^{b}	2.70±0.10^{ab}
0.3	0.0	0.3	22.67±2.51^{a}	08.00±0.85^{b}	3.03±0.15^{b}
0.4	0.0	0.4	27.33±1.57^{b}	10.66±1.24^{c}	3.73±0.16^{b}
0.5	0.0	0.5	29.00±1.00^{b}	08.66±1.26^{b}	2.80±1.90^{ab}
0.0	0.1	0.1	22.33±1.52^{a}	07.33±1.27^{b}	3.46±0.15^{b}
0.0	0.2	0.3	26.00±1.00^{b}	10.00±2.44^{c}	3.53±0.15^{b}
0.0	0.3	0.5	29.67±1.54^{b}	09.33±1.21^{c}	3.63±0.15^{b}
0.0	0.4	0.7	40.00±1.00^{ce}	07.00±1.64^{b}	3.80±0.10^{b}
0.0	0.5	0.9	46.00±1.01^{d}	09.00±1.63^{c}	4.06±0.17^{b}
0.0	0.1	0.0	39.00±1.05^{ce}	10.67±1.28^{cd}	2.17±0.21^{a}
0.0	0.2	0.0	41.67±2.08^{ce}	08.67±1.23^{b}	2.70±0.20^{ab}
0.0	0.3	0.0	42.67±1.54^{ce}	10.00±1.63^{c}	3.00±0.13^{b}
0.0	0.4	0.0	44.68±1.52^{df}	09.00±2.16^{c}	2.80±0.10^{ab}
0.0	0.5	0.0	48.33±1.27^{f}	08.67±1.25^{b}	3.13±0.25^{b}
0.1	0.0	0.0	50.66±2.51^{f}	07.00±1.63^{b}	3.20±0.27^{b}
0.3	0.0	0.0	60.00±1.00^{g}	09.66±1.26^{c}	4.03±0.15^{bd}
0.5	0.0	0.0	71.00±2.00^{h}	11.66±1.27^{cd}	4.43±0.31^{bd}
0.7	0.0	0.0	80.00±1.00^{i}	10.33±1.20^{cd}	4.80±0.20^{d}
0.9	0.0	0.0	85.00±1.00^{j}	12.00±0.81^{d}	5.10±0.10^{d}
1.0	0.0	0.1	41.66±1.52^{ce}	11.33±1.27^{cd}	3.26±0.15^{b}
1.0	0.0	0.2	47.00±2.00^{f}	09.00±1.63^{c}	3.60±0.20^{b}
1.0	0.0	0.3	50.66±2.08^{f}	07.33±1.25^{b}	3.73±0.20^{b}
1.0	0.0	0.4	56.00±1.00^{g}	05.00±0.81^{a}	3.73±0.15^{b}
1.0	0.0	0.5	60.00±1.00^{g}	06.00±0.83^{a}	3.90±0.10^{b}

Means in columns followed by different letter(s) are significantly different at 5% level according to DMRT.

Eck and Kitto, 1992). Similar kind of findings of effective root formation by the influence of various types of auxins in many plant species have been reported elsewhere (Mallikadevi and Paulsamy, 2009; Mahesh *et al.,* 2010; Loc *et al.,* 2011; Mungole *et al.,* 2011; Rajput *et al.,* 2011).

The hardening experiments showed that high degree of acclimatization was achieved by performing 78% of plantlet survivability in the hardening medium encomposed by red soil, sand and vermicompost in the ratio of 1:1:1 by volume. Hence, before transplanting the plantlets, hardening must be done in this prescribed encomposed medium for higher survivability of plantlets. However, field observations can be made after transplantation to know the rate of survivability in the open environmental conditions.

The present paper describes a prime and easy-to-use protocol for large scale production of plantlets of *A.annua* through leaf culture and the method is useful for the *ex situ* conservation of this species as well. In addition, the findings of the present investigation provide a baseline data for further research in this species.

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