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Research Article

Mass Propagation of a Threatened Medicinal Plant, *Artemisia nilagirica* (C.B. Clarke) Pampan Inhabiting high Hills of Nilgiris, the Western Ghats

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ABSTRACT

Protocol for *in vitro* culture by using leaf explants of the threatened medicinal plant species, *Artemisia nilagirica* found in open habitats of Nilgiris, the Western Ghats at high altitudes was developed. The MS medium supplemented with growth hormones, like BAP and NAA at concentrations of 2.5 and 0.5mg/l respectively was found to be the optimum for higher frequency of callus formation. Maximum number of shoots (11 shoots/callus) was observed in MS medium fortified with BAP, NAA and IAA at 1.0, 1.0 and 0.3mg/l respectively. MS medium with IBA at 1.0 mg/l alone produced higher number of roots during subculturing (15 roots/callus). The plantlets obtained were successfully transferred in the hardening medium containing garden soil, farmyard manure and sand in the ratio of 2:1:1 by volume in which 80% survivability was achieved.

***Key words**: Artemisia nilagirica*, medicinal plant, Nilgiris, the Western Ghats

Abbreviations

2, 4-D 2, 4-Dichlorophenoxyacetic acid
BAP 6-Benzyladenine
IAA Indole-3-acetic acid
IBA Indole-3-butyric acid
Kn Kinetin
NAA Naphthaleneacetic acid
TDZ Thiodizuron

INTRODUCTION

Artemisia nilagirica (C.B. Clarke) Pampan. is a valuable medicinal plant of Asteraceae included in threatened category. The bioactive compounds like volatile oils, sesquiterpene lactones and flavonoids reported in the species are having insecticidal, antimicrobial and antiparastical properties (Borzabad *et.al.,* 2010). In traditional medicine, this plant is being widely used for the treatment of diabetes, epilepsy, depression, insomonia and anxiety stress (Walter *et. al.*, 2003). All the parts of the plant are used as antihelmintic, antiseptic, antispasmodic, carminative, cholagogue, digestive, expectorant, purgative and stimulant also. The essential oils of the plant were reported to exhibit, 90% mosquito repellency against the mosquito, *Aedes aegypti* that transmits yellow fever (Ram and Mehrotra, 1995). A paste or powder form of the leaves is applied for the treatment of skin diseases (Kapoor, 2000). Due to the over exploitation for its medicinal uses, the species become lower in population size in high altitudes of Nilgiris, (Paulsamy *et. al.*, 2008). Early report showed that the conventional method of propagation of this species through seed is also not successful (Paulsamy, 2005). The aim of the present investigation was to develop methods for *in vitro* propagation to conserve the species and to enable bulk production.

MATERIALS AND METHODS

The leaf explants of *A. nilagirica* were collected from the healthy individuals at Nilgiris and washed thoroughly with tap water, they were then cut into small discs of 0.8cm diameter and then treated with a surfactant, tween 20 (5% w/v) for 5 minutes. After repeated washing in double distilled water, to eliminate the fungal contamination, the explants were treated with Carbendazim (50% w/v) fungicide (10%) also for 15 minutes and rinsed with double distilled water 2 or 3

times. To eliminate bacterial contamination the explants were 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 Laminor air flow chamber.

Leaf explants 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, Kn and TDZ for callus induction. The pH of the medium was adjusted to 5.6 - 5.8 before autoclaving at 121°C for 20 min. The culture was incubated at a constant temperature of 25±2°C with 14h photoperiod (3000 lux) and 8h darkness. Callus from these primary cultures were transferred to MS medium containing different concentrations of BAP, NAA and IAA 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 and root length were noted. 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 one month after transfer 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, *A. nilagirica* is noted to be varied from 15 to 30 days according to the combinations and concentrations of the growth regulators *viz.*, BAP, NAA, 2,4-D, Kn and TDZ in the MS medium (Table 1). It may be explained that the specific growth hormones at appropriate concentrations can play major role to induce callus besides the other factors (Ananthi *et al.*, 2011). The amount of leaf explant responding for callus formation was ranging between 10 and 97% (Table 1). MS medium fortified with BAP and NAA at 2.5 mg/l and 0.5mg/l initiated 97% of leaf explants for callus formation (Fig. 1a) followed by BAP and 2,4-D at 1.0mg/l and 0.9mg/l which initiated 79% of leaf explants for callusing and 0.5mg/l of TDZ in which 65% of discs produced callus. The other combinations and concentrations of growth hormones in the medium initiated only around 10 to 60% of leaf discs 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 formation 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 BAP alone or in combination with NAA generally have the efficiency of initiation at high percentage of (>50%) leaf explant for callus formation. Ganesan and Paulsamy (2011) reported the higher requirement of certain auxins like NAA alone or in combination with low quantity of cytokinins like TDZ for callus formation in another species, *Aretimisa annua.* Callus potential has been reported to vary from species to species and often differes in varieties of same species (Pradeep Kumar and Ranjitha Kumari, 2010). Karappusamy and Pullaiah (2007) for the species, *Bupleurum distichophyllum* and Senthilkumar and Paulsamy (2010a) for the species, *Ageratum conyzoides* also reported effective callus formation from the leaf explant 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 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 IAA enhanced the response of calli for shoot formation by 97 and 90% respectively (Table 2). In addition, greater number of 11 shoots/callus was also noted to be produced while subculturing the calli on MS medium with BAP,NAA and IAA at 1.0,1.0 and 0.3 mg/l respectively (Table 2) (Fig. 1c). However, the higher shoot length of 12cm was achieved in the MS medium fortified with BAP and IAA at 3.0 mg/l and 0.6 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, *Artemisia nilagirica*. It is of common fact that cytokinin is the major growth hormone involved in shoot formation in many plant species (Vijaykumari *et al.*, 2001; Sujatha *et al.*, 2008; Roy *et al.*, 2008; Senthilkumar and Paulsamy, 2010a; Ganasen and Paulsamy, 2011; Sunder and Jawahar, 2011).

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

Growth regulator (mg/l)					Days required for callus formation after inoculation	Callus formation (%)	Colour of the callus
BAP	NAA	2,4-D	Kn	TDZ	Leaf explant	Leaf explant	Leaf explant
0.5	0.0	0.0	0.0	0.0	-	-	-
1.0	0.0	0.0	0.0	0.0	15	10.00±1.00	G
1.5	0.0	0.0	0.0	0.0	17	16.66±2.08	G
2.5	0.0	0.0	0.0	0.0	18	27.00±2.00	G
3.0	0.0	0.0	0.0	0.0	19	30.66±2.51	G
0.5	0.1	0.0	0.0	0.0	22	35.00±1.00	G
1.0	0.2	0.0	0.0	0.0	23	40.33±1.52	G
1.5	0.3	0.0	0.0	0.0	25	46.00±1.00	DG
2.0	0.4	0.0	0.0	0.0	19	60.00±2.00	DG
2.5	0.5	0.0	0.0	0.0	30	97.00±2.00	DG
0.5	0.0	0.5	0.0	0.0	17	16.00±1.00	G
1.0	0.0	0.9	0.0	0.0	21	79.67±1.53	DG
1.5	0.0	1.3	0.0	0.0	23	29.00±1.00	DG
2.0	0.0	1.7	0.0	0.0	16	34.33±1.52	LG
2.5	0.0	2.1	0.0	0.0	25	37.67±1.57	LG
3.0	0.0	2.5	0.0	0.0	22	40.00±1.00	B
0.0	0.5	0.0	0.5	0.0	17	10.33±1.52	LG
0.0	0.5	0.0	1.0	0.0	16	19.00±1.00	LG
0.0	0.5	0.0	1.5	0.0	21	21.00±1.00	B
0.0	0.5	0.0	2.0	0.0	20	21.00±2.00	LB
0.0	0.5	0.0	2.5	0.0	22	24.33±1.52	LB
0.0	0.0	0.0	0.0	0.1	25	26.33±1.53	DB
0.0	0.0	0.0	0.0	0.2	24	29.00±1.00	DB
0.0	0.0	0.0	0.0	0.3	20	38.00±1.00	DB
0.0	0.0	0.0	0.0	0.4	19	45.33±1.25	DB
0.0	0.0	0.0	0.0	0.5	23	65.00±1.00	LB

G-Green, DG- Dark green, LG- Light green, B-Brown, DB-Dark brown, LB- Light brown

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 nilgirica.*

Growth regulator (mg/l)			Culture response (%)	No. of shoots /callus	Shoot length (cm)
BAP	NAA	IAA			
0.1	0.0	0.0	08.00±1.00	03.00±1.00	6.96±0.15
0.2	0.0	0.0	13.00±1.10	03.34±0.58	7.00±0.20
0.3	0.0	0.0	16.01±1.20	04.35±1.15	5.26±0.15
0.4	0.0	0.0	20.00±1.00	04.39±2.08	6.68±0.18
0.5	0.0	0.0	30.00±1.09	04.68±1.52	4.96±0.17
0.6	0.0	0.0	21.66±2.08	04.00±1.00	3.23±0.16
0.7	0.0	0.0	24.35±1.53	03.00±1.00	7.60±0.10
0.8	0.0	0.0	26.67±1.54	03.36±0.58	5.07±0.14
0.9	0.0	0.0	30.35±1.52	04.00±1.00	4.73±0.21
0.5	0.1	0.1	33.10±2.01	05.00±1.00	7.07±0.21
1.0	0.2	0.2	37.02±2.04	06.34±1.56	5.67±0.25
1.5	0.3	0.3	38.34±1.54	07.68±1.59	6.93±0.18
2.0	0.4	0.4	39.00±1.00	07.33±1.60	3.27±0.15
2.5	0.5	0.5	41.33±1.55	08.96±1.52	5.80±0.20
0.5	0.75	0.1	46.00±1.58	08.33±1.55	6.73±0.21
1.0	1.0	0.3	50.00±2.08	11.00±1.01	5.30±0.20
1.5	1.25	0.5	60.00±1.00	03.00±1.05	6.03±0.23
2.0	1.50	0.7	70.00±1.30	04.35±1.27	5.73±0.38
2.5	1.75	0.9	80.20±1.00	05.04±1.09	6.46±0.31
3.0	2.0	1.1	90.33±1.58	04.69±1.58	7.20±0.75
0.5	0.0	0.1	51.36±2.51	05.00±1.05	6.53±0.35
1.0	0.0	0.2	54.00±1.00	07.00±1.00	5.33±0.20
1.5	0.0	0.3	60.00±1.00	01.38±0.58	4.00±0.10
2.0	0.0	0.4	70.35±1.59	01.68±0.59	6.63±0.15
2.5	0.0	0.5	92.35±1.57	02.00±1.00	8.03±0.18
3.0	0.0	0.6	97.00±2.00	04.00±1.00	12.00±0.21

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 nilagirica.*

Growth regulator (mg/l)			Shoots rooted (%)	No. of roots/shoot	Root length (cm)
IBA	IAA	NAA			
0.1	0.0	0.0	26.33±1.56	09.67±0.57	4.93±0.15
0.2	0.0	0.0	40.00±1.00	13.33±1.52	5.26±0.14
0.3	0.0	0.0	46.68±1.58	12.00±2.65	3.63±0.15
0.4	0.0	0.0	50.33±1.58	11.00±2.00	2.00±0.10
0.5	0.0	0.0	56.00±1.00	10.66±2.08	5.00±0.20
0.6	0.0	0.0	64.67±1.54	09.69±3.05	4.00±0.12
0.7	0.0	0.0	68.34±1.58	08.33±1.54	3.90±0.33
0.8	0.0	0.0	83.00±1.00	13.00±1.00	5.23±0.25
0.9	0.0	0.0	90.00±1.00	10.69±1.54	4.66±0.32
1.0	0.0	0.0	95.00±2.00	15.00±1.00	6.00±0.20
0.0	0.2	0.0	16.33±1.58	07.00±1.00	4.20±0.12
0.0	0.3	0.0	23.33±1.57	10.68±2.08	3.46±0.16
0.0	0.4	0.0	40.33±1.45	12.00±1.00	5.03±0.16
0.0	0.5	0.0	42.68±1.62	13.33±1.58	5.50±0.26
0.0	0.6	0.0	48.00±1.00	12.36±0.57	4.93±0.15
0.0	0.7	0.0	15.67±2.08	05.36±1.54	1.13±0.04
0.0	0.8	0.0	20.00±1.00	06.68±1.48	1.30±0.10
0.0	0.9	0.0	24.33±1.52	06.00±1.00	1.26±0.20
0.0	1.0	0.0	28.00±1.00	08.69±1.54	1.50±0.10
0.0	0.0	0.1	40.33±1.58	09.68±1.56	1.76±0.18
0.0	0.0	0.2	50.00±1.00	10.69±1.25	2.03±0.16
0.0	0.0	0.3	53.38±1.54	11.34±1.58	2.50±0.20
0.0	0.0	0.4	55.35±1.46	13.68±1.59	2.90±0.25
0.0	0.0	0.5	66.38±1.57	07.00±2.00	3.16±0.18
0.0	0.0	0.6	72.68±1.53	06.67±1.57	3.50±0.10

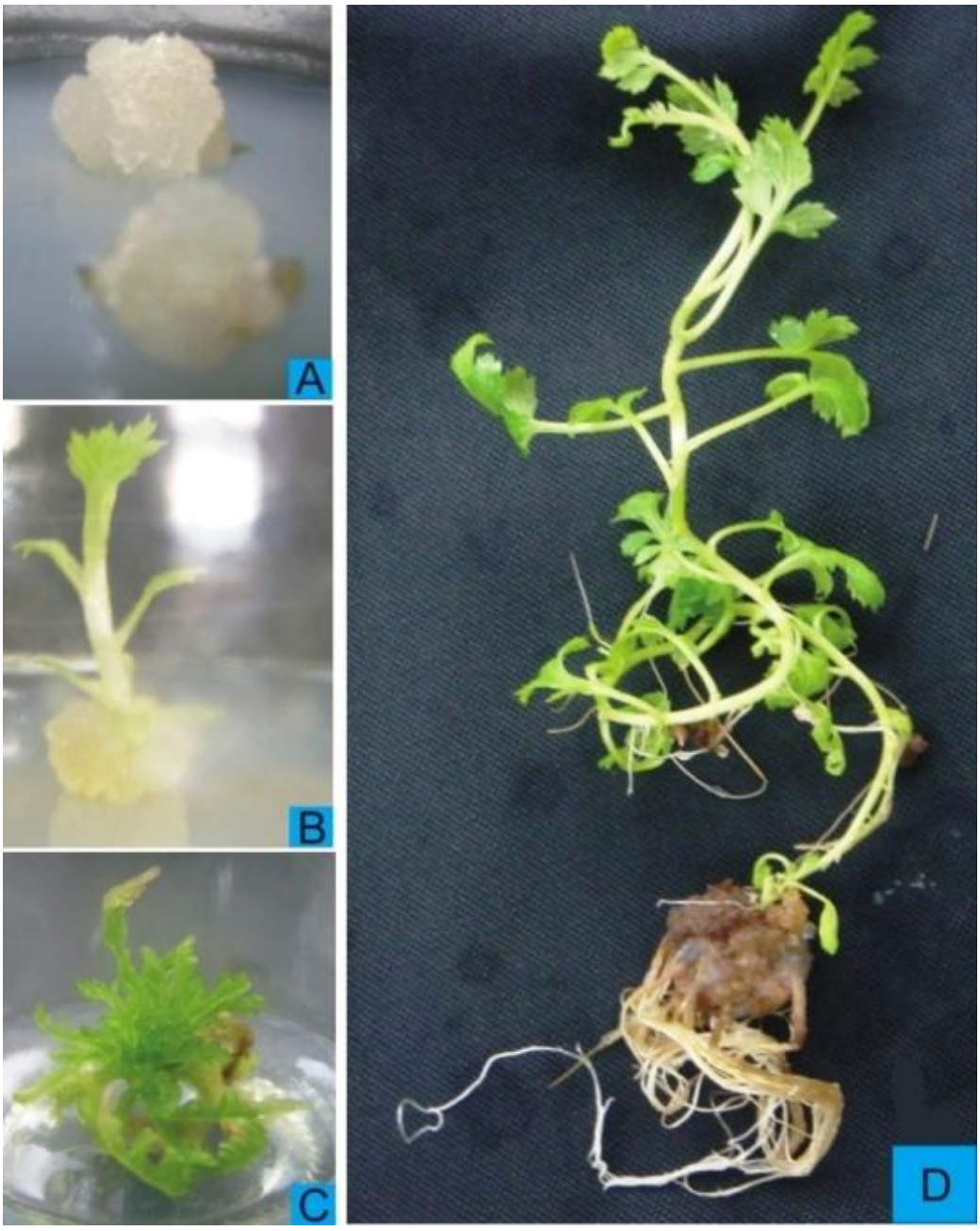

Figure 1: Successful *in vitro* culture of *Artemisia nilagirica* from the leaf explants. A - Effective callus formation in MS medium containing BAP and NAA at 2.5 mg/l and 0.5mg/l. B - Higher response of callus for shoot formation in MS medium fortified with BAP and IAA at 3.0 mg/l and 0.6 mg/l. C - High degree of multiple shoot formation while subculturing onto MS medium supplemented with BAP, NAA and IAA at 1.0, 1.0 and 0.3 mg/l respectively. D - More pronounced root formation during subculturing onto MS medium containing 1.0 mg/l of IBA.

The rooting attributes of *A. nilagirica* while subculturing the secondary explants, shoots were well pronounced in the MS medium supplemented with the auxin, IBA alone at higher concentrations from 0.5 to 1.0 mg/l (Table 3). The IBA concentration at 1.0 mg/l initiated 95% shoots for root formation (Fig. 1d) followed by 0.9mg/l initiated 90% and 0.8 mg/l initiated 83% shoots for root formation. The number of roots per shoot was also observed to be higher (15 roots/shoot) in the MS medium containing 1.0 mg/l IBA for the study species, *A. nilagirica.* Similarly, the root length was greater (6.0cm) during the subculturing of *in vitro* cultured shoots for roots on MS medium with IBA at 1.0 mg/l. All these facts showed that the auxin , IBA is the most required growth regulator for shooting characters of the study species, *A.nilagirica*. It agrees the concept that auxins are the plant hormones endogenously or exogenously inducing root formation in majority of plant species (Van 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 (Soniya and Sujitha, 2006; 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 encompassing red soil, sand and vermicompost in a 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 must be made after transplantation to know the rate of survivability in the open environmental conditions.

The present paper describes a standardized protocol for large scale production of plantlets of *Artemisia nilagirica* through leaf discs and hence the mass production of the species. It will be useful for the conservation of wild individuals in high hills of Nilgiris and to meet the demand as well.

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