AcademicPres

Available online at www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2013, 5(3):338-345

Notulae Scientia Biologicae

Influence of Additives on Enhanced *In vitro* Shoot Multiplication of *Orthosiphon aristatus* (Blume) Miq.

Swarna JAYAKUMAR, Ravindhran RAMALINGAM*

T.A.L Samy Centre for Plant Tissue Culture and Molecular Biology, Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam, Chennai 600 034, Tamil Nadu, India; raviloyola1998@gmail.com (corresponding author)*

Abstract

Orthosiphon aristatus is a valuable medicinal plant and different parts of the plant are pharmaceutically used for the treatment of various diseases. The present study was designed to develop an efficient protocol for micropropagation of *O. aristatus* from nodal explants and to study the influence of additives on the enhancement of the number of shoots per explant. Among the different types of additives used, 10% coconut water and 30 mg/L glutamine added to Murashige and Skoog's (MS) medium supplemented with 1.0 mg/L 6-benzyl amino purine (BAP) and 0.5 mg/L kinetin (KIN) was found to be most effective. Maximum number of shoots (44.07 ± 0.38) with 100% shooting response and shoot length of 7.47 ± 0.10 cm was recorded. *In vitro* rooting of the microshoots was achieved on half-strength MS medium containing 0.5 mg/L indole-3-butyric acid (IBA), producing an average of 30.27 ± 0.36 roots and 6.02 ± 0.20 cm root length. The rooted shoots were acclimatized with 100% survival rate on coco pith: soil (3:1) planting substrate and was successfully transferred to field conditions. The hardened plants exhibited homogeneity and no morphological variations were observed among the regenerants and the mother plants. Thus, the procedure described is a quick and reliable method which could be applied for efficient large-scale propagation, genetic transformation assays and secondary metabolite production.

Keywords: Java tea, Lamiaceae, micropropagation, MS medium, PGRs

Introduction

Orthosiphon aristatus (Blume) Miq. is an erect, slender and perennial shrub belonging to the Lamiaceae family. It is popularly known as kidney tea plant or java tea. It is a medicinal herb widely cultivated in temperate and tropical regions, especially in South-East Asia and Australia. The plant is also grown as an ornamental for its unique flowers, white or purplish in colour, with far-exerted filaments resembling a cat's whiskers (Indubala and Ng, 2000).

According to traditional ethnobotany, *O. aristatus* has been widely used for the treatment of various kidney and urinary bladder diseases because of its diuretic activity (Englert and Harnischfeger, 1992). The presence of orthosiphonin and high percentage of potassium salts helps in keeping uric acid and urate salts in solution, thus preventing calculi deposition. The plant has also been medically implicated in the treatment of diabetes, eruptive fever, epilepsy, gallstones, hepatitis, rheumatism, jaundice, hypertension, gout and hepatitis (Wiart, 2002).

O. aristatus is mainly valued for its leaves and to fulfil the ever-growing demand of the market for the production of leaves, the plant has been commercially exploited. The flowers are usually removed when they appear as they are supposed to deprive the leaves of their active constituents. This subsequently hinders seed formation and plant production (Anonymous, 1976). Cultivation of this species on large scale is affected as the conventional propagation method is beset with problems of scanty and delayed rooting of seedlings and vegetative cuttings. Thus conservation of this species is required to ensure its sustainable utilization. Therefore, development of a rapid clonal multiplication procedure of this medicinally important herb has become imperative in order to reduce the existing pressure on natural populations and to supply constant plant materials to pharmaceutical industries for the preparation of herbal products.

Micropropagation methods, which strictly maintain clonal fidelity, are particularly applicable to this plant. Very few reports are available on the micropropagation of this species (Elangomathavan *et al*., 2003; Leng and Lai-Keng, 2004). But, till date there are no reports on the influence of additives to enhance shoot multiplication. Coconut water (CW) contains growth hormones and is liberally made use in tissue culture. Addition of CW into the media enhanced the shoot growth and development of medicinal plants propagated *in vitro* (Tefera and Wannakrairoj, 2004). Amino acids such as glutamine and asparagine were frequently employed in the culture medium as an organic nitrogen source (Franklin and Dixon, 1994). Many reports have claimed the use of exogenous glutamine to be beneficial for *in vitro* culture, increasing the regeneration rate and biomass of the explants (Ogita *et al.*, 2001; Rao *et al.*, 2001; Vasudevan *et al.*, 2004).

Thus the aim of the present investigation was to establish an efficient and improved protocol by enhancing *in*

Received 16 April 2013; accepted 17 July 2013

vitro shoot multiplication of *O. aristatus* using various additives. Subsequently the most effective hormonal concentration for root induction and *ex vitro* hardening was also determined.

Materials and methods

Medium and culture conditions

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) used in this study was prepared by adding 3% (w/v) sucrose to MS basal salts and vitamins. Different concentrations of plant growth regulators (PGRs) were supplemented to the medium and the pH was adjusted to 5.7 before adding 0.8% (w/v) agar. MS medium devoid of growth regulators served as control. Molten medium (10 mL) was dispensed into culture tubes and plugged with non absorbent cotton plugs. Culture bottles containing 50 mL medium were tightly closed with polypropylene caps. The tubes and bottles containing the media were autoclaved at 121°C for 15 min. The cultures were incubated at 24 ± 2°C in light with 16 h photoperiod supplied by white fluorescent tube lights, with a light intensity of 50 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Plant material and explant preparation

Orthosiphon aristatus plants were collected from the medicinal plant garden at Irula Tribal Women's Welfare Society, Chengalpattu, Chennai, India. Botanical identification was performed by Dr. D. Narashiman, Associate Professor, Centre for Floristic Research, Department of Plant Biology & Plant Biotechnology, Madras Christian College, Chennai and a voucher specimen has been deposited in the Loyola College Herbarium (LCH 64). Surface sterilization of nodal explants was performed by washing the explants under running tap water for 20 min. It was then washed with 1% (v/v) non-ionic surfactant solution and rinsed thoroughly with sterile water. The explants were disinfected by soaking in 0.2% (w/v) bavistin (Carbendazim 50% WP fungicide) for 5 min and washed five times with sterile distilled water. Surface decontamination was performed under aseptic conditions by immersing the explants in 0.1% (w/v) mercuric chloride for 3 min followed by rinsing 4-5 times with sterile distilled water. Nodal explants were trimmed to 10-12 mm in size and they were used as the explants for micropropagation.

Multiple shoot induction and proliferation

For multiple shoot induction, nodal explants were inoculated on MS medium supplemented with different hormones such as 6-benzyl amino purine (BAP) and kinetin (KIN) (0.5, 1.0, 2.0 and 4.0 mg/L) individually and in combination with auxins such as α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) (0.1, 0.25 and 0.5 mg/L). A combination of both the cytokinins (BAP - 1.0 mg/L and KIN - 0.25, 0.50, 0.75 and 1.0 mg/L) was also used for this study. Subculturing was done twice onto their respective medium after a 4-week interval. Total number of shoots per explant and length of the shoots were measured after 8 weeks of culture.

Influence of additives on shoot multiplication

To study the influence of various additives on shoot proliferation, explants were cultured on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L KIN along with different concentrations and combinations of peptone (50, 100, 150, 200 mg/L; w/v), asparagine and glutamine (10, 20, 30 and 40 mg/L; w/v), watermelon juice and coconut water (5, 10, 15 and 20%; v/v). MS medium containing 1.0 mg/L BAP + 0.5 mg/L KIN, 10% CW in combination with glutamine or asparagine (20 and 30 mg/L) was also used to study the efficiency on inducing multiple shoots. Subculturing was done two times in the same medium after a 4-week interval. The number and length of the shoots per explant were recorded after eight weeks of culture.

Root induction

For root initiation, the proliferated shoots, 2-3 cm in length were separated, excised and inoculated on root induction medium. To determine the effect of MS salt concentration on root induction, half-strength MS and full-strength MS medium containing 3% sucrose (w/v) was used. The medium was supplemented with various concentrations (0.1, 0.5, 1.0, 2.0 and 3.0 mg/L) of indole-3-acetic acid (IAA), IBA and NAA individually. After 4 weeks of culture, the total number of roots produced per shoot and length of the roots were measured.

Acclimatization

Rooted plants (4-5 cm) were carefully removed from the test tubes and washed with sterile water to remove any agar medium adhering to the roots. The plantlets were transferred to paper cups (6 cm in diameter and 11 cm high) filled with mixtures of sterilized sand and soil (1:1), coco pith and soil (3:1) and saw dust and soil (3:1) (v/v). They were regularly supplied with 1/4 strength MS basal salt solution devoid of sucrose to prevent any fungal contamination. To prevent desiccation and maintain high humidity, the plants were covered with transparent polythene bags (10 × 8 cm) and grown in the culture room at 24 ± 2°C with 16 h photoperiod, provided by cool white fluorescent light with an intensity of 50 μmol m^{-2} s^{-1} PPFD. The relative humidity was gradually reduced by opening the bags and after 3 weeks, the plants were removed from the cups and transferred to pots filled with 1:1 mixture of soil and organic manure (w/w). These acclimatized plantlets were transferred and established in the field. The survival percentage of *ex vitro* plants was evaluated after 6 weeks and any variation in morphological characters was noted.

Data collection and statistical analysis

All experiments were performed with a minimum of ten explants per treatment and each experiment was repeated three times. The data were collected after 8 weeks for shoot multiplication and 4 weeks for rooting experiments. For shoot proliferation, the mean (%) of explants responding, mean number of shoots per explant and average shoot length (cm) were measured. For root induction, mean number of roots and average root length (cm) were calculated. The data were analysed statistically using IBM SPSS statistics 19 (SPSS Inc., Chicago, USA) and the mean values were expressed as mean ± SE of three experiments. The significance of differences among means was carried out using Duncan's Multiple Range Test (DMRT) at a 5% probability level.

Results and discussion

Influence of plant growth regulators on multiple shoot induction

Multiple shoots were successfully induced from nodal explants of *Orthosiphon aristatus*. The morphogenetic responses of nodal explants to various cytokinins alone (BAP and KIN) and in combination with auxins (NAA and IBA) are summarized in Tab. 1. Nodal explants (Fig. 1a) that were cultured on MS medium devoid of PGRs (control) induced 2.40 ± 0.26 shoots. However, the multiplication rate and shoot number were markedly increased in cultures supplemented with PGRs. The percentage response, average number of shoots per explant as well as the mean length of shoots varied with the type of growth regulator as well as its concentration.

Among the two cytokinins tested, BAP was found to be more efficient than KIN with respect to initiation and subsequent proliferation of shoots (Tab. 1). Of the various concentrations of BAP used, 1.0 mg/L proved to be most effective (86.67%) with an average of 22.33 ± 0.32 shoots and 4.60 ± 0.02 cm shoot length per culture. When KIN alone (2.0 mg/L) was added, 80% shooting response was recorded, producing an average of 19.40 ± 0.45 shoots and 3.47 ± 0.02 cm shoot length. Upon increasing the concentration of the PGRs, a reduction in the number of shoots per culture was observed. Similar to our study, multiple shoots have been induced from nodal explants of *O. spi-*

Tab. 1. Effect of different concentrations and combinations of PGRs on multiple shoot induction from nodal explants of *O. aristatus* on MS medium after 8 weeks of culture

Combination and concentration of PGRs (mg/L)				Nodal explants		
Cytokinins		Auxins		% Response	Average number of shoots per explant	Average length of shoots (cm)
Control				40.00	2.40 ± 0.26 ^{a}	1.25 ± 0.04 ^{a}
BAP	KIN	NAA	IBA			
0.5	-	-	-	66.67	15.23 ± 0.32 ^{c}	3.40 ± 0.02 ^{h}
1.0	-	-	-	86.67	22.33 ± 0.32 ^{mn}	4.60 ± 0.02 ^{m}
2.0	-	-	-	80.00	18.70 ± 0.33 ^{hi}	4.15 ± 0.01 ^{kl}
4.0	-	-	-	63.33	12.07 ± 0.26 ^{c}	2.81 ± 0.02 ^{ef}
-	0.5	-	-	70.00	13.77 ± 0.29 ^{d}	2.55 ± 0.02 ^{d}
-	1.0	-	-	76.67	16.27 ± 0.36 ^{efg}	3.18 ± 0.02 ^{g}
-	2.0	-	-	80.00	19.40 ± 0.45 ^{hijk}	3.47 ± 0.02 ^{h}
-	4.0	-	-	60.00	9.97 ± 0.38 ^{b}	2.34 ± 0.08 ^{c}
1.0	0.25	-	-	100.0	23.53 ± 0.43 ^{o}	4.63 ± 0.05 ^{m}
1.0	0.5	-	-	100.0	27.30 ± 0.54 ^{p}	5.43 ± 0.06 ^{o}
1.0	0.75	-	-	90.00	20.03 ± 0.44 ^{jk}	3.72 ± 0.02 ^{i}
1.0	1.0	-	-	76.67	15.20 ± 0.31 ^{e}	2.49 ± 0.01 ^{d}
1.0	-	0.1	-	80.00	18.57 ± 0.45 ^{h}	3.10 ± 0.07 ^{g}
1.0	-	0.25	-	96.67	24.13 ± 0.39 ^{o}	4.79 ± 0.03 ^{n}
1.0	-	0.5	-	90.00	19.90 ± 0.38 ^{ijk}	4.03 ± 0.02 ^{jk}
1.0	-	-	0.1	86.67	23.20 ± 0.33 ^{no}	4.22 ± 0.04 ^{l}
1.0	-	-	0.25	80.00	20.53 ± 0.40 ^{kl}	3.67 ± 0.02 ^{i}
1.0	-	-	0.5	66.67	16.97 ± 0.45 ^{g}	2.82 ± 0.02 ^{ef}
-	2.0	0.1	-	83.33	15.70 ± 0.33 ^{ef}	1.76 ± 0.06 ^{b}
-	2.0	0.25	-	73.33	21.27 ± 0.33 ^{lm}	3.51 ± 0.02 ^{h}
-	2.0	0.5	-	70.00	18.80 ± 0.36 ^{hij}	2.71 ± 0.05 ^{e}
-	2.0	-	0.1	76.67	17.43 ± 0.46 ^{g}	3.40 ± 0.04 ^{h}
-	2.0	-	0.25	86.67	19.47 ± 0.48 ^{hijk}	3.92 ± 0.09 ^{j}
-	2.0	-	0.5	73.33	16.83 ± 0.44 ^{fg}	2.87 ± 0.07 ^{f}

Note: Values represent mean values ± standard error of 10 explants per treatment of three repeated experiments.% Response values represent mean percentage response. Means followed by the same letter within columns are not significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT)

Fig. 1. Multiple shoot induction from nodal explants of *Orthosiphon aristatus*. a Initiation of nodal explant (*Bar* 0.5 cm); b Shoot multiplication on MS medium supplemented with 1.0 mg/L BAP + 0.5 mg/L KIN, 10% coconut water and 30 mg/L glutamine, 2 weeks after initiation (*Bar* 0.5 cm); c Proliferation of shoots on the same medium, after 4 weeks (*Bar* 1.0 cm), and d after 8 weeks of culture (*Bar* 2.0 cm); e *In vitro* rooting of shoots on half-strength MS medium with IBA (0.5 mg/L) after 4 weeks (*Bar* 1.0 cm); f Acclimatization of plantlet in culture room using different planting substrates with 1/4 strength MS basal salt solution (w/o sucrose) (*Bar* 5.0 cm); g Hardening of plant in nursery (*Bar* 7.0 cm); h Plant in pot under natural sunlight (*Bar* 6.0 cm); i *Ex vitro* plant, successfully established under field conditions after 3 months (*Bar* 12.5 cm)

ralis on MS medium containing BAP (Elangomathavan *et al*., 2003; Leng and Lai-Keng, 2004). The superiority of the cytokinin BAP, used for the induction of multiple shoots, has been reported in several plants in the Lamiaceae family such as *Pogostemon heyneanus* (Hembrom *et al*., 2006), *Salvia brachyodon* (Misic *et al*., 2006) and *Melissa officinalis* (Tavares *et al*., 1996).

MS medium supplemented with a combination of BAP (1.0 mg/L) and KIN in different concentrations was found to be effective in enhancing the number of shoots and shoot length per explant (Tab. 1). Maximum response (100%) was observed in medium supplemented with BAP and KIN at 1.0 and 0.5 mg/L respectively, producing an average of 27.30 ± 0.54 shoots and 5.43 ± 0.06 cm shoot length per culture. Higher concentration of cytokinin combinations reduced the number of shoots and shoot length. The combined effect of BAP and KIN on efficient shoot induction has been well documented and proved in *Capsicum chinense* (Sanatombi and Sharma, 2008) and *Bambusa balcooa* (Negi and Saxena, 2011).

The efficiency of the optimal concentration of BAP and KIN with auxins (NAA and IBA) was also evaluated for multiple shoot induction (Tab. 1). BAP with NAA was found to be an effective combination for shoot regeneration and multiplication. Nodal explants cultured on MS medium supplemented with BAP (1.0 mg/L) and NAA (0.25 mg/L) exhibited 96.67% shoot regeneration with a maximum of 24.13 ± 0.39 shoots and a mean length of 4.79 ± 0.03 cm. A combination of KIN (2.0 mg/L) with NAA (0.25 mg/L) induced 21.27 ± 0.33 shoots and 3.51 ± 0.02 cm shoot length. Shooting response was lower when IBA was added along with BAP or KIN. The results cor-

roborate with the earlier findings in plants such as *Centella asiatica* (Tiwari *et al.*, 2000) and *Chlorophytum arundinaceum* (Lattoo *et al.*, 2006) where the addition of low-level of auxin with cytokinin promoted shoot proliferation.

Influence of additives on multiple shoot induction

In vitro growth and development of *O. aristatus* were highly influenced by additives and the results have been presented in Tab. 2. The present study revealed that a combination of CW with glutamine or asparagine positively influenced multiple shooting in *O. aristatus* (Tab. 2). MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L KIN, 10% CW and glutamine or asparagine significantly increased the number of shoots per explant (Tab. 2). Glutamine at 30 mg/L produced an average of 44.04 ± 0.38 shoots per explant with a mean length of 7.47 ± 0.10 cm (Fig. 1b, c, d) whereas 20 mg/L asparagine resulted in 40.43 ± 0.38 shoots per explant and 6.89 ± 0.05 cm shoot length after 8 weeks of culture. Increasing the concentration of additives decreased the regeneration ability.

Several reports have confirmed the beneficial effects of additives for micropropagation (Komalavalli and Rao, 2000; Baskaran and Jayabalan, 2005). Adding extra reduced nitrogen to the culture medium, in the form of amino acids (glutamine and asparagine), is beneficial for shoot development (Jain and Babbar, 2003). In the present investigation, glutamine and asparagine significantly improved shoot multiplication. Similar studies have been reported for other plant species such as *Syzygium cumini* (Jain and Babbar, 2003), *Cuccumis sativus* (Vasudevan *et al.*, 2004) and *Embelia ribes* (Annapurna and Rathore, 2010). The reduction of nitrate to ammonia is a limiting factor in the cells (Gamborg, 1970). Thus, the enhancement of growth rate in the presence of glutamine could

Tab. 2. Effect of different concentrations of additives on multiple shoot induction on MS medium supplemented with BAP (1.0 mg/L) + KIN (0.5 mg/L) after 8 weeks of culture

Concentration of additives	% Response	Average number of shoots per explant	Average length of shoots (cm)
Coconut Water (CW,%)			
5	83.33	26.20 ± 0.26^{j}	4.72 ± 0.08^{i}
10	90.00	29.67 ± 0.29^{k}	5.47 ± 0.08^{j}
15	80.00	21.00 ± 0.23^{i}	4.07 ± 0.10^{h}
20	66.67	14.63 ± 0.27^{f}	3.24 ± 0.18^{f}
Watermelon Juice (%)			
5	83.33	18.03 ± 0.31^{g}	3.78 ± 0.06^{g}
10	76.67	15.40 ± 0.20^{f}	3.08 ± 0.11^{ef}
15	70.00	11.07 ± 0.28^{cd}	2.67 ± 0.50^{c}
20	60.00	7.57 ± 0.11^{a}	1.91 ± 0.03^{b}
Asparagine (mg/L)			
10	90.00	19.23 ± 0.22^{h}	3.12 ± 0.11^{ef}
20	96.67	31.20 ± 0.25^{l}	5.83 ± 0.10^{k}
30	86.67	25.87 ± 0.42^{j}	4.47 ± 0.05^{h}
40	66.67	10.80 ± 0.31^{c}	2.89 ± 0.06^{de}
Glutamine (mg/L)			
10	96.67	21.23 ± 0.24^{i}	4.58 ± 0.05^{hi}
20	96.67	29.30 ± 0.24^{k}	5.40 ± 0.06^{j}
30	100.0	35.70 ± 0.22^{m}	6.35 ± 0.07^{m}
40	80.00	13.27 ± 0.21^{e}	3.02 ± 0.10^{ef}
Peptone (mg/L)			
50	70.00	11.73 ± 0.38^{d}	1.30 ± 0.04^{a}
100	76.67	15.10 ± 0.30^{f}	1.86 ± 0.04^{b}
150	83.33	18.03 ± 0.25^{g}	2.71 ± 0.04^{c}
200	63.33	8.40 ± 0.32^{b}	1.11 ± 0.05^{a}
CW (10%) + Glutamine (mg/L)			
20	100.0	36.93 ± 0.24^{n}	6.47 ± 0.08^{m}
30	100.0	44.07 ± 0.38^{p}	7.47 ± 0.10^{o}
CW (10%) + Asparagine (mg/L)			
20	100.0	40.43 ± 0.38^{o}	6.89 ± 0.05^{n}
30	100.0	31.43 ± 0.24^{l}	6.06 ± 0.08^{l}

Note: Values represent mean values ± standard error of 10 explants per treatment of three repeated experiments. % Response values represent mean percentage response. Means followed by the same letter within columns are not significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT)

be explained on the basis that it provides a readily available source of nitrogen to the plant cells. Also, glutamine is relatively non-toxic, hence it helps in enabling the cells to maintain high growth rate for a longer period (Gamborg *et al.*, 1968). A maximum of 32.25 ± 1.06 shoots when cultured on MS medium supplemented with 2.22 µM BAP alone has been reported (Elangomathavan *et al.*, 2003). But in the current study, glutamine was found to be a suitable nitrogen source for markedly enhancing the number of shoots, thus establishing an improved procotol for rapid *in vitro* shoot multiplication.

Rooting of regenerated shoots

The presence of auxins at low concentration in half-strength MS medium was found to be more efficient in inducing better rooting response when compared with full-strength MS. Of the three auxins (NAA, IBA and IAA) tested, IBA (0.5 mg/L) was found to be more effective for rooting by producing an average of 30.27 ± 0.36 roots and 6.02 ± 0.20 cm root length per culture (Fig. 1e; Tab. 3). Successful rooting (28.83 ± 0.93 roots per explant) of *O. aristatus* shoots on half-strength MS medium supplemented with 4.9 µM IBA has been reported (Elangomathavan *et al.*, 2003). *In vitro* rooting was noticeably influenced by the concentration of the auxins as well the strength of the MS medium. Similar to our current study, rooting in MS medium supplemented with IBA has been reported to be efficient in several plants belonging to the Lamiaceae family such as *Isodon wightii* (Thirugnanasampandan *et al.*, 2009), *Ocimum basilicum* (Siddique and Anis, 2008) and *Coleus blumei* (Rani *et al.*, 2006).

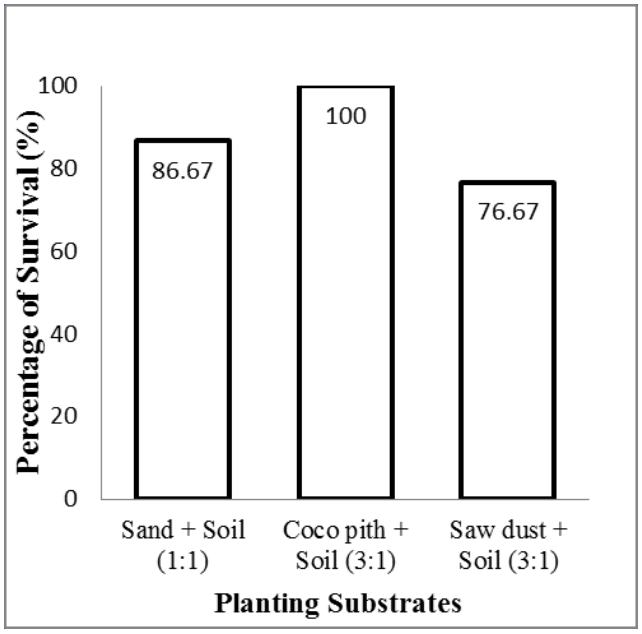

Fig. 2. Effect of different planting substrates used during acclimatization of *in vitro* grown plantlets, 6 weeks after establishment under field conditions. Percentage of survival values represents mean percentage response of 10 plantlets per treatment of three repeated experiments

Acclimatization and establishment of plantlets

The rooted microshoots were successfully hardened in the culture room (24 ± 2°C) in paper cups containing sterilized planting substrates (Fig. 1f). After 3 weeks, these plantlets were transferred into pots containing natural garden soil and organic manure and maintained in the nurs-

Tab. 3. Effect of MS salt concentration and different auxins on root induction from *in vitro* raised microshoots after 4 weeks of culture

Concentration of auxins (mg/L)			Half-strength MS		Full-strength MS	
			Average number of roots per shoot	Average length of roots (cm)	Average number of roots per shoot	Average length of roots (cm)
Control			1.00 ± 0.47 ^{a}	0.48 ± 0.11 ^{a}	0.60 ± 0.16 ^{a}	0.26 ± 0.05 ^{a}
IBA	IAA	NAA				
0.1	-	-	24.80 ± 0.22 ^{p}	4.12 ± 0.07 ^{n}	16.77 ± 0.25 ^{l}	3.46 ± 0.09 ^{lm}
0.5	-	-	30.27 ± 0.36 ^{r}	6.02 ± 0.20 ^{r}	21.10 ± 0.38 ^{n}	4.09 ± 0.11 ^{n}
1.0	-	-	27.00 ± 0.34 ^{q}	5.17 ± 0.19 ^{q}	23.87 ± 0.30 ^{o}	4.87 ± 0.14 ^{pq}
2.0	-	-	18.47 ± 0.25 ^{m}	3.07 ± 0.15 ^{ijk}	12.50 ± 0.22 ^{i}	3.01 ± 0.06 ^{ij}
3.0	-	-	14.47 ± 0.20 ^{j}	2.29 ± 0.09 ^{fg}	9.03 ± 0.27 ^{fg}	2.59 ± 0.07 ^{gh}
-	0.1	-	8.23 ± 0.28 ^{ef}	2.43 ± 0.11 ^{fgh}	4.17 ± 0.31 ^{c}	1.43 ± 0.16 ^{bc}
-	0.5	-	13.10 ± 0.31 ^{i}	3.04 ± 0.12 ^{ijk}	9.23 ± 0.43 ^{g}	2.28 ± 0.11 ^{fg}
-	1.0	-	17.40 ± 0.29 ^{l}	3.67± 0.06 ^{lm}	11.07 ± 0.38 ^{h}	3.08 ± 0.13 ^{ijk}
-	2.0	-	5.03 ± 0.33 ^{cd}	2.20 ± 0.06 ^{ef}	7.60 ± 0.43 ^{e}	1.83 ± 0.20 ^{de}
-	3.0	-	2.60 ± 0.17 ^{b}	1.66 ± 0.07 ^{cd}	2.20 ± 0.18 ^{b}	1.10 ± 0.10 ^{b}
-	-	0.1	15.40 ± 0.18 ^{k}	2.18 ± 0.17 ^{ef}	10.30 ± 0.37 ^{h}	3.32 ± 0.06 ^{jkl}
-	-	0.5	19.27 ± 0.29 ^{m}	3.40 ± 0.04 ^{klm}	17.43 ± 0.42 ^{l}	4.33 ± 0.07 ^{no}
-	-	1.0	25.07 ± 0.23 ^{p}	4.63 ± 0.06 ^{op}	15.60 ± 0.46 ^{k}	3.70 ± 0.05 ^{m}
-	-	2.0	10.67 ± 0.17 ^{h}	2.78 ± 0.17 ^{hi}	8.33 ± 0.31 ^{efg}	2.13 ± 0.16 ^{ef}
-	-	3.0	5.87 ± 0.22 ^{d}	1.86 ± 0.14 ^{de}	4.87 ± 0.25 ^{c}	1.27 ± 0.14 ^{b}

Note: Values represent mean values ± standard error of 10 shoots per treatment of three repeated experiments

Means followed by the same letter are not significantly different at 5% probability level using Duncan's Multiple Range Test (DMRT)

ery (Fig. 1g). After six weeks, the plants were transplanted and established in field conditions under natural sunlight (Fig. 1h, 1i). Of the three different types of planting substrates examined, the percentage survival (100%) of the plantlets was highest in coir pith: soil (3:1; v:v) (Fig. 2). The regenerants grew well and all the plants transferred for hardening showed homogeneity. No morphological variation was observed among the *ex vitro* plants and the mother plants.

Conclusions

The study demonstrates a promising and cost effective protocol that will help in the rapid multiplication, large-scale production and conservation of *O. aristatus*, a valuable medicinal plant. Influence of additives on enhanced shoot multiplication has been established for the first time using nodal explants. MS medium supplemented with BAP (1.0 mg/L) and KIN (0.5 mg/L) in combination with 10% coconut water and 30 mg/L glutamine was found to be most effective in inducing multiple shoots when compared to other additives. Half-strength MS medium with 0.5 mg/L IBA produced maximum rooting response and the complete plantlets were successfully established under field conditions with 100% survival rate. Axillary bud proliferation, without any intervening callus phase, supposedly leads to the production of genetically stable, true-to-type plantlets. Thus, the described procedure could be used for *en masse* propagation, which will be helpful for the study of phytomedicine production and genetic transformation.

References

Annapurna D, Rathore TS (2010). Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F. Plant Cell Tissue Organ Cult 101:269-277.

Anonymous (1976). The Wealth of India: A dictionary of raw materials and industrial products, Vol VII (N-Pe), CSIR, New Delhi, India, 109-110 p.

Baskaran P, Jayabalan N (2005). An efficient micropropagation system for *Eclipta alba*-A valuable medicinal herb. In Vitro Cell Dev Biol Plant 41:532-539.

Elangomathavan R, Prakash S, Kathiravan K, Seshadri S, Ignacimuthu S (2003). High frequency *in vitro* propagation of Kidney Tea Plant. Plant Cell Tissue Organ Cult 72:83-86.

Englert J, Harnischfeger G (1992). Diuretic action of aqueous *Orthosiphon* extract in rats. Planta Med 58:237-238.

Franklin CI, Dixon RA (1994). Initiation and maintenance of callus and cell suspension cultures, 1-29 p. In: Dixon RA, Gonzales RA (Eds.). Plant Cell Culture - a practical approach. 2nd Edition, Oxford University Press Inc., NY.

Gamborg OL, Miler RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158.

Gamborg OL (1970). The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol 45:372-375.

Hembrom ME, Martin KP, Patchathundikandi SK, Madassery J (2006). Rapid *in vitro* production of true-to-type plants of *Pogostemon heyneaus* through dedifferentiated axillary buds. In Vitro Cell Dev Biol Plant 42:283-286.

Indubala J, Ng L (2000). Herbs: The green pharmacy of Malaysia. Vinpress Sdn. Bhd. Kuala Lumpur, 76-77 p.

Jain N, Babbar SB (2003). Regeneration of "juvenile" plants of black plum, *Syzygium cuminii* Skeels, from nodal exp of mature trees. Plant Cell Tissue Organ Cult 73:257-263.

Komalavalli N, Rao M (2000). *In vitro* micropropagation of *Gymnema sylvestre*-A multipurpose medicinal plant. Plant Cell Tissue Organ Cult 61:97-105.

Lattoo SK, Bamotra S, Dhar RS, Khan S, Dhar AK (2006). Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker.- an endangered medicinal herb. Plant Cell Rep 25:499-506.

Leng LW, Lai-Keng C (2004). Plant regeneration from stem nodal segments of *Orthosiphon stamineus* Benth., a medicinal plant with diuretic activity. In Vitro Cell Dev Biol Plant 40:115-118.

Misic D, Grubisic D, Konjevic R (2006). Micropropagation of *Salvia brachyodon* through nodal explants. Biol Plant 50:473-476.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497.

Negi D, Saxena S (2011). Micropropagation of *Bambusa balcooa* Roxb. through axillary shoot proliferation. In Vitro Cell Dev Biol Plant 47:604-610.

Ogita S, Sasamoto H, Yeung EC, Thorpe TA (2001). The effects of glutamine on the maintenance of embryogenic cultures of *Cryptomeria japonica*. In Vitro Cell Dev Biol Plant 37:268-273.

Rani G, Talwar D, Nagpal A, Virk G (2006). Micropropagation of *Coleus blumei* from nodal segments and shoot tips. Biol Plant 50:496-500.

Rao SGV, Chandra R, Polisetty R (2001). Role of amino acids in evolution of ethylene and methane and development of microshoots in *Cajanus cajan*. Biol Plant 44(1):13-18.

Sanatombi K, Sharma GJ (2008). *In vitro* propagation of *Capsicum chinense* Jacq. Biol Plant 52:517-520.

Siddique I, Anis M (2008). An improved plant regeneration system and ex vitro acclimatization of *Ocimum basilicum* L. Acta Physiol Plant 30:493-499.

Tavares A, Pimenta M, Goncalves M (1996). Micropropagation of *Melissa officinalis* L. through proliferation of axillary shoots. Plant Cell Rep 15:441-444.

Tefera W, Wannakrairoj S (2004). Micropropagation of Karwan. Sci Asia 30:9-15.

Thirugnanasampandan R, Mahendran G, Narmatha Bai V (2009). High frequency *in vitro* propagation of *Isodon wightii* (Bentham) H. Hara. Acta Physiol Plant 32:405-409.

Tiwari KN, Sharma NC, Tiwari V, Singh BD (2000). Micropropagation of *Centella asiatica* (L.), a valuable medicinal herb. Plant Cell Tissue Organ Cult 63:179-185.

Vasudevan A, Selvaraj N, Ganapathi A, Kasthurirengan S, Anbazhagan VR, Manickavasagam M (2004). Glutamine: a suitable nitrogen source for enhanced shoot multiplication in *Cucumis sativus* L. Biol Plant 48:125-128.

Wiart C (2002). *Orthosiphon stamineus* Benth. Medicinal Plants of Southeast Asia. Prentice Hall, Kuala Lumpur, 265 p.