BIOLOGIA PLANTARUM 57 (2): 210-218, 2013 DOI: 10.1007/s10535-012-0280-2

In vitro regeneration of *Aristolochia tagala* and production of artificial seeds

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Abstract

Protocols for *in vitro* plant multiplication from somatic tissues and production of artificial seeds through encapsulation of nodes were developed for *Aristolochia tagala* Cham., a rare and valuable medicinal plant, as a measure of conservation and as a prerequisite for genetic transformation procedure. A maximum number of adventitious shoots were regenerated from leaf-derived callus on Murashige and Skoog (MS) medium containing 6-benzylaminopurine (BAP; 2 μM), α-naphthaleneacetic acid (NAA; 0.5 μM), and phloroglucinol (PG; 10μM). Nodes collected from *in vitro* established shoot cultures were encapsulated in 3 % (m/v) sodium alginate and 1 % (m/v) calcium chloride. Multiple shoots were successfully regenerated from the encapsulated nodes cultured on MS medium supplemented with 3 μM BAP and 0.5 μM kinetin (KIN). Regenerated shoots from callus and artificial seeds were successfully rooted and acclimated to greenhouse conditions. Since roots of *A. tagala* are primarily used in traditional medicine, a protocol for regenerating roots directly from the leaf derived callus was also developed. Maximum root length was obtained when the callus was cultured in MS medium supplemented with KIN (1 μM), indole acetic acid (IAA; 0.5 μM), NAA (0.1 μM), and PG (10 μM). Biochemical parameters were studied in calli grown with and without PG in the medium to establish a correlation between these parameters and shoot morphogenesis. An increment of antioxidant enzymes (peroxidase and catalase) and metabolites (sugars and proteins), and a decrease in the amount of polyphenol oxidase was observed in the calli which were grown in the presence of PG.

Additional key words: auxins, cytokinins, encapsulated nodes, indirect regeneration, organogenesis, phloroglucinol, plant preservation.

Introduction

Aristolochia L. is a large genus of *Aristolochiaceae* with about 120 species distributed throughout tropical and subtropical countries. *Aristolochia tagala* Cham., a climbing shrub, is distributed in India, Sri Lanka, China, Malaysia, Burma, Java, and Australia, and is a rare medicinal plant (Murugan *et al.* 2006). Roots of *A. tagala* are extensively used in traditional medicine. Due to indiscriminate harvesting, the species has become rare in its natural habitat (Ravikumar and Ved 2000).

Propagation of *A. tagala* relies solely on seeds. However the viability of the seeds is very low due to the presence of scanty endosperm (Biswas *et al.* 2007). It has been shown that shoot organogenesis from callus cultures can be used as an effective method for multiplication of medicinal plants (Castillo and Jordan 1997). Also, efficient tissue culture based propagation and regeneration systems are important prerequisites for the development of *Agrobacterium tumefaciens*-mediated transformation (Bliss *et al.* 2009). Additionaly, cell and protoplast cultures and long-term culture of dedifferentiated callus offer new sources of genetic variation (Novak 1992).

In the present study, we have chosen adventitious shoot regeneration as a measure for conservation and as a prerequisite for developing a genetic transformation of *A. tagala.* Somaclonal variation through cell or callus

Received 24 February 2012, *accepted* 20 July 2012.
Abbreviations: AC - activated charcoal; BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole acetic acid; IBA - indolebutyric acid; KIN - kinetin; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PG - phloroglucinol.
Acknowledgements: The authors express their sincere thanks to Dr. R. Gopalan, Botanical Survey of India, Coimbatore, Tamil Nadu and Dr. Venkata Subramaniam, Institute Of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, for their valuable assistance in collection and identification of the plant.
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cultures also could be an important source for genetic improvement of *A. tagala.* We have even made an attempt to encapsulate the *in vitro* derived nodal segments of *A. tagala* for the production of artificial seeds as well as determining the *in vitro* requirements for the production of a maximum number of multiple shoots from the encapsulated nodes. Since roots of *A. tagala* are primarily used in traditional medicine, we have also described a protocol for production of adventitious roots directly from the callus of *A. tagala.* Activity of peroxidase, catalase, polyphenol oxidase, and content of reducing sugars, nonreducing sugars, and proteins were studied in calli grown with and without phloroglucinol in the medium to establish a correlation between these parameters and shoot morphogenesis.

Materials and methods

A. tagala was collected from Kolli hills which is situated in the south western edge of the Eastern Ghats, India. The plant was identified by Dr. R. Gopalan (Botanical Survey of India, Southern Circle, Coimbatore, India) and a voucher specimen was deposited in the herbarium of the Botanical Survey of India. To avoid causalities, some of the plants were maintained in greenhouse at the Department of Botany, Bharathiar University, Coimbatore, India.

The explants, namely nodes (1 - 1.5 cm long), shoot tips (3 - 5 mm), internodes (1 - 1.5 cm long), leaves (sectioned to about 1 cm^2 pieces), and root segments (1 - 1.5 cm long) were harvested from actively growing plants and washed thoroughly in running tap water for 20 - 30 min followed by treatment with 5 % (v/v) *Teepol* for 5 - 10 min. The treated explants were washed with distilled water for 5 min, disinfected with 0.1 % (m/v) mercuric chloride for 3 - 5 min and rinsed 3 - 4 times with sterile distilled water under aseptic conditions inside the laminar hood.

The explants were cultured on full strength Murashige and Skoog (1962; MS) regeneration medium supplemented with 3 % (m/v) sucrose. The pH of the medium was adjusted to 5.8 before adding 0.8 % (m/v) agar (*Hi Media*, Mumbai, India). The medium (15 cm^3) was poured into culture tubes (*Borosil*, Mumbai, India) and autoclaved at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. Two explants were inoculated per tube. The cultures were incubated at temperature of 25 ± 2 °C, 16-h photoperiod, and irradiance of 50 - 60 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (*Philips*, India). MS medium enriched with 2,4-dichloro-phenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), 6-benzyl-aminopurine (BAP), and/or kinetin (KIN) at concentrations ranging from 0.5 to 3.0 µM was used for callus induction and indirect regeneration of shoots. Phloroglucinol (PG; 10 µM) was added in the medium to prevent tissue browning. The calli were subcultured regularly at an interval of four weeks.

Morphogenic calli from leaf explants which were fixed in formalin + acetic acid for 24 h were washed and stored in 70 % ethanol. The materials were then dehydrated in tertiary butanol (70, 80, 90, and 100 %) and were embedded in paraffin wax as in the procedure described by Johansen (1940). Thin sections (10 µm diameter) were cut with the help of a rotary microtome (*Sipcon*, India). These sections were affixed on glass slides using Haupt's adhesive solution. The slides were de-waxed using xylene and were stained using haematoxylin. The stained sections were covered with cover glass and observed under a microscope (*Nikon UFX-2*, Tokyo, Japan). Fifty slides were observed and those that showed initiation of shoot buds were photographed under a bright field using a camera (*Nikon FX 35WA*).

For production of artificial seeds, *in vitro* proliferated shoots (1.5 - 2 cm long) having 4 - 5 leaves were excised from the callus. The diameter of the shoots was approximately 1 mm. Nodal segments approximately 3 mm long were dissected aseptically and were washed in sterilized double distilled water for 5 - 10 min. Three different concentrations [2, 3, and 4 % (m/v)] of sodium alginate in distilled water or MS basal medium and 1 % (m/v) calcium chloride ($CaCl_2$) were steam-sterilized for 20 min under 1.2 kg cm^{-2} pressure at 120 °C after adjusting the pH of the solution to 5.8. Nodes were mixed with sodium alginate solution (at 25 ± 2 °C) and were subsequently dropped individually into calcium chloride solution using a wide mouthed glass dropper (inside diameter 4 mm). Calcium alginate beads were formed within 30 - 45 min on a gyratory shaker at 80 rpm. Sodium alginate (3 %) was the most suitable for the formation of firm, clear and isodiametric beads. Following encapsulation, the beads were recovered by decanting $CaCl_2$ solution and were washed thrice with sterilized double distilled water. The encapsulated nodal segments were kept in Petri dishes sealed with parafilm and stored in the laboratory fridge at 4 °C for 20 d before being transferred for regeneration.

The beads containing a single node were cultured in test tubes containing MS medium supplemented with various concentrations of BAP (1 to 3 µM) and KIN (0.5 to 1.5 µM) for regeneration of microshoots. The cultures were incubated at above mentioned conditions. The number of shoots produced per encapsulated node and the time taken for sprouting of the beads were recorded.

Regenerated shoots from callus and encapsulated nodes were transferred to rooting medium which consisted of MS medium supplemented with different concentrations of NAA (0.2 to 1 µM), IAA (0.2 to 0.5 µM), and KIN (0.2 to 2 µM). The cultures were kept

in the dark at 25 ± 2 °C.

Well-developed, healthy plantlets with approximately 6 cm long shoots (containing 6 - 10 leaves ranging from 0.5 - 1 cm) and approximately 13 cm long tap roots were removed from the culture flasks and washed thoroughly in running tap water to remove the adhering nutrient medium. The plantlets were then soaked in 1 % (m/v) 1H-benzimidazol-2-yl-carbamate (*Bavistin*) for 10 to 15 min and transferred to small plastic pots filled with various types of sterilized potting media as detailed in Table 6. Five plantlets were tested for every potting mixture and the growth rates of the plantlets were calculated after 30 d of hardening. During the hardening process, the plantlets were initially maintained under laboratory conditions at 25 ± 2 °C for 10 d. During this period, they were sprayed with liquid MS medium (quarter strength and without sucrose) daily and covered tightly with polyethylene bags. After 10 d, they were gradually exposed to the natural environmental conditions.

Leaf derived callus of *A. tagala* was subcultured on MS medium containing KIN (0.5 to 1 µM), IAA (0.5 to 1 µM), NAA (0.1 to 0.5 µM), and phloroglucinol (10 µM) to standardize a protocol for direct root organogenesis from callus cultures.

Biochemical parameters were measured in 4-week-old calli grown with and without PG in the medium. For preparation of enzyme extract, calli (450 mg) were homogenized with 9 cm^3 of cold 5 % (m/v) KCl. The homogenate was centrifuged at 0 ± 2 °C and 800 *g* for 10 min. The clear supernatant was used directly for the assay of enzyme activities and estimation of protein and sugars.

The activity of peroxidase was assayed according to the method of Malik and Singh (1980). The assay mixture containing 2.5 cm^3 of phosphate buffer (pH 6.5, 0.1 M), 0.2 cm^3 of suitably diluted enzyme extract, and 0.1 cm^3 of *o*-dianisidine (1 mg cm^{-3} of methanol) was incubated at 28 ºC in water bath for 2 min. The reaction was started by adding 0.2 cm^3 of H_2O_2. The change in absorbance was recorded at 430 nm at 30 s interval for 5 min.

The polyphenol oxidase activity was measured as in the method of Sarvesh and Reddy (1988). The assay mixture (3 cm^3) contained 2.0 cm^3 of 2 M carbonate-bicarbonate buffer (pH 10), 0.15 M *o*-catechol, and 0.2 cm^3 of suitably diluted enzyme extract. The assay mixture was incubated at 25 ºC for 2 min. The reaction was stopped by adding 0.5 cm^3 of 5 % (v/v) H_2SO_4. The change in absorbance was recorded at 420 nm for 2 min.

Catalase activity was measured as described by Beers and Sizer (1952). The enzyme was diluted in 0.05 M phosphate buffer (pH 7.0). To 0.1 cm^3 of diluted enzyme 0.1 cm^3 of H_2O_2 was added and incubated for 4 min. Decrease in absorbance was measured spectrophotometrically at 240 nm for 2 - 3 min.

Measurement of the total protein content was done using the Bradford's (1976) method. Quantitative estimation of reducing sugars was done by the Nelson (1944) method and total sugars were quantified by the Dubois (1956) method. Non-reducing sugars were calculated by subtracting the amount of reducing sugars from content of total sugars.

All the experiments were conducted with a minimum of 10 replicates per treatment. The experiments were repeated three times. The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at $P = 0.05$.

Results

In *A. tagala*, profuse callusing was observed only from the leaf explants. So, the callus derived from the leaf explants was used for further studies. Within a week of culture, the explants swelled and callus was initiated at the cut ends which later spread all over the surface. Best callusing (maximum dry mass of callus within minimum duration) was obtained when MS medium was fortified with 1 µM BAP, 0.5 µM NAA and 2 µM KIN (Table 1). Calli obtained from all the combinations of growth hormones tried were creamy and friable.

The leaf derived callus of *A. tagala* turned brown and did not become organogenic in the medium used for callus induction presumably due to accumulation of phenolics. When PG (10 µM) was added to MS medium supplemented with growth hormones, it evoked shoot bud regeneration from the callus (Fig. 1*A*). It was observed that a combination of 2 µM BAP, 1 µM NAA, and 10 µM PG induced the maximum number of shoots. Higher concentrations of BAP (3 µM) reduced the number of shoot buds. Addition of KIN instead of BAP also reduced the number of shoots per callus (Table 2). Histology showed asynchronous development of shoot buds (Fig. 1*B*).

A gelling matrix of 3.0 % sodium alginate and 1.0 % $CaCl_2$ was found most suitable for the bead formation (Fig. 1*C*) and subsequent conversion of encapsulated nodal segments into plantlets. Lower concentrations of sodium alginate (2 %) resulted in beads without a defined shape that were too soft to handle, whereas at higher concentrations of sodium alginate, the beads were hard enough to cause considerably delayed shoot emergence. Encapsulated nodes of *A. tagala* when cultured on MS basal hormone free medium produced only a single shoot. In the presence of BAP and KIN, multiple shoots were initiated from the beads (Fig. 1*D*). It was observed that the use of distilled water for preparing the gel matrix reduced shoot emergence as compared to liquid MS medium. The maximum number of multiple shoots was regenerated from the encapsulated nodes cultured on MS medium supplemented with 3 µM BAP and 0.5 µM KIN (Table 3).

The optimal medium for rooting of microshoots, contained 0.2 µM IAA, 0.2 µM KIN, and 1.0 µM NAA on which the regenerated shoots developed roots measuring 13.4 cm (Table 4, Fig. 1*E*). The compact leaf

derived callus when cultured in a medium containing varying concentrations of KIN, IAA, NAA, and PG showed the appearance of white spots on the surface that gradually became white nodular structures which developed into adventitious roots. A maximum root length of 3.96 cm was obtained in MS medium supplemented with 1 µM KIN, 0.5 µM IAA, 0.1 µM NAA, and 10 µM PG (Table 5).

Plantlets with six to ten fully expanded leaves and well-developed roots obtained from the rooted microshoots were successfully acclimatized in plastic pots containing various potting mixtures. The maximum percentage survival was 80 % (Table 6). They were successfully transplanted into field where they grew normally without any morphological variations (Fig. 1*G*).

Table 1. Effect of growth regulators on the percentage of shoot induction from the leaf derived callus of *A. tagala*, number of days necessary for shoot induction, and shoot fresh and dry mass determined after 30 d. Values are mean ± SE, $n = 10$. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

2,4-D [µM]	BAP [µM]	NAA [µM]	KIN [µM]	Callus induction [%]	Callus induction [d]	Fresh mass [g]	Dry mass [g]
1	-	-	-	50.04 ± 0.12^{c}	21.20 ± 0.23^{g}	0.87 ± 0.30^{a}	0.08 ± 0.13^{c}
2	-	-	-	64.46 ± 026^{d}	19.60 ± 0.31^{f}	0.91 ± 0.24^{b}	0.08 ± 0.11^{d}
3	-	-	-	40.22 ± 0.24^{b}	23.20 ± 0.42^{i}	0.88 ± 0.16^{a}	0.08 ± 0.23^{bc}
0.5	0.5	-	-	75.18 ± 0.38a	22.60 ± 0.26^{li}	1.33 ± 0.33^{f}	0.12 ± 0.35^{g}
1	0.5	-	-	85.20 ± 0.16^{g}	19.80 ± 0.43^{f}	1.13 ± 0.38^{e}	0.13 ± 0.27^{h}
2	0.5	-	-	80.02 ± 0.22^{f}	21.20 ± 0.47^{g}	1.04 ± 0.23^{d}	0.11 ± 0.26^{f}
0.5	-	-	-	90.50 ± 0.28^{l}	19.80 ± 0.29^{f}	2.16 ± 0.17^{j}	0.16 ± 0.32^{k}
1	-	-	-	96.42 ± 0.23^{ij}	18.80 ± 0.35^{ef}	2.16 ± 0.26^{j}	0.16 ± 0.24^{l}
2	-	-	-	85.48 ± 0.24^{g}	19.40 ± 0.41^{f}	2.34 ± 0.12^{k}	0.17 ± 0.10^{m}
0.5	2	-	-	98.60 ± 0.15^{kl}	13.20 ± 0.22^{a}	1.54 ± 0.11^{h}	0.15 ± 0.31^{i}
1	2	-	-	$97.78 \pm 0.18j^{k}$	18.20 ± 0.48^{d}	1.67 ± 0.35^{I}	0.15 ± 0.36^{j}
2	2	-	-	95.84 ± 0.27^{l}	21.60 ± 0.36^{gh}	1.46 ± 0.32^{g}	0.15 ± 0.15^{i}
-	1	-	-	98.00 ± 0.32^{kl}	17.40 ± 0.27^{d}	0.87 ± 0.29^{a}	0.08 ± 0.18^{ab}
-	3	-	-	99.20 ± 0.36^{kl}	14.20 ± 0.30^{ab}	0.89 ± 0.13^{b}	0.09 ± 0.22^{d}
-	1	-	1	84.82 ± 0.11^{g}	23.20 ± 0.43^{i}	2.54 ± 0.17^{l}	0.18 ± 0.27^{n}
-	2	-	1	99.22 ± 0.34^{kl}	18.20 ± 0.40^{de}	2.57 ± 0.25^{m}	0.18 ± 0.16^{n}
-	3	-	1	99.36 ± 0.17^{lm}	17.40 ± 0.34^{d}	2.69 ± 0.20^{1n}	0.18 ± 0.34^{n}
-	1	-	2	98.60 ± 0.14^{kl}	14.20 ± 0.40^{ab}	3.05 ± 0.14^{o}	0.20 ± 0.35^{o}
-	2	-	2	99.74 ± 0.32^{m}	15.40 ± 0.35^{c}	3.08 ± 0.13^{p}	0.21 ± 0.28^{p}
-	3	-	2	99.16 ± 0.29^{kl}	14.80 ± 0.40^{bc}	3.13 ± 0.24^{q}	0.25 ± 0.11^{q}
-	1	0.5	2	98.00 ± 0.25^{kl}	13.60 ± 0.33^{ab}	3.54 ± 0.15^{s}	0.28 ± 0.15^{s}
-	2	0.5	2	95.78 ± 0.13^{I}	19.60 ± 0.32^{f}	3.31 ± 0.23^{r}	0.27 ± 0.12^{r}
-	3	0.5	2	$98.60 \pm 0.16k^{l}$	21.00 ± 0.45^{g}	3.30 ± 0.10^{r}	0.26 ± 0.37^{r}

Table 2. Effect of auxins and cytokinins on the time necessary for shoot induction in callus derived from the leaf explants of *A. tagala* and number of shoots and their length measured after 30 d . Values are mean ± SE, $n = 10$. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

BAP [µM]	NAA [µM]	KIN [µM]	PG [µM]	Shoot induction [d]	Number of shoots [callus^{-1}]	Shoot length [cm]
0.50	0.50	-	10.00	31.31 ± 0.29^{a}	1.60 ± 0.73^{ab}	1.46 ± 0.15^{a}
1.00	0.50	-	10.00	30.40 ± 0.51^{ab}	2.00 ± 0.82^{bc}	2.02 ± 0.12^{bc}
1.50	0.50	-	10.00	32.66 ± 0.46^{b}	2.60 ± 0.86^{c}	2.40 ± 0.14^{c}
2.00	0.50	-	10.00	28.42 ± 0.44^{d}	9.00 ± 0.76^{f}	3.36 ± 0.10^{e}
0.50	1.00	-	10.00	26.81 ± 0.36^{bc}	1.80 ± 0.91^{ab}	1.28 ± 0.18^{a}
1.50	1.00	-	10.00	25.20 ± 0.42^{d}	6.80 ± 0.88^{e}	2.50 ± 0.12^{c}
2.00	1.00	-	10.00	20.40 ± 0.26^{d}	9.20 ± 0.71^{e}	3.04 ± 0.11^{d}
3.00	1.00	-	10.00	22.39 ± 0.31c	8.78 ± 0.95^{d}	3.08 ± 0.16^{d}
-	0.50	0.50	10.00	31.84 ± 0.35^{d}	0.79 ± 0.82^{ab}	1.26 ± 0.14^{a}
-	1.00	0.50	10.00	30.26 ± 0.37^{ab}	0.96 ± 0.89^{ab}	1.39 ± 0.17^{a}

Table 3. Effect of different encapsulation matrices on number of shoots, shoot length measured after 30 d, and time necessary for germination of *A. tagala* synthetic seeds on MS medium with different concentrations of cytokinins. Values are mean ± SE, $n = 10$. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

Cytokinins [μM]	Sodium alginate in distilled water			Sodium alginate in MS medium		
	number of shoots	shoot length [cm]	time [d]	number of shoots	shoot length [cm]	time [d]
-	1.23 ± 0.37^{a}	1.14 ± 0.10^{a}	30.31 ± 0.21^{a}	1.20 ± 0.31^{a}	1.38 ± 0.23^{a}	25.84 ± 0.32^{a}
BA (1.0) + KIN (1.0)	3.86 ± 0.46^{b}	1.64 ± 0.13^{b}	28.20 ± 0.24^{b}	5.00 ± 0.47^{b}	1.84 ± 0.18^{a}	25.29 ± 0.28^{b}
BA (1.0) + KIN (1.5)	5.00 ± 0.33^{b}	1.74 ± 0.25^{b}	28.30 ± 0.33^{c}	6.42 ± 0.39^{bc}	1.80 ± 0.13^{a}	20.40 ± 0.26^{a}
BA (2.0) + KIN (0.5)	6.60 ± 0.59^{c}	2.24 ± 0.12^{c}	25.64 ± 0.35^{b}	7.68 ± 0.51^{c}	2.32 ± 0.25^{b}	20.63 ± 0.35^{bc}
BA (2.0) + KIN (1.0)	7.23 ± 0.54^{cd}	2.26 ± 0.24^{c}	25.48 ± 0.28^{c}	8.20 ± 0.43^{cd}	2.60 ± 0.13^{b}	18.00 ± 0.39^{a}
BA (3.0) + KIN (0.5)	8.68 ± 0.45^{d}	2.46 ± 0.16^{c}	25.26 ± 0.37^{b}	9.82 ± 0.32^{d}	3.14 ± 0.18^{c}	18.00 ± 0.20^{c}

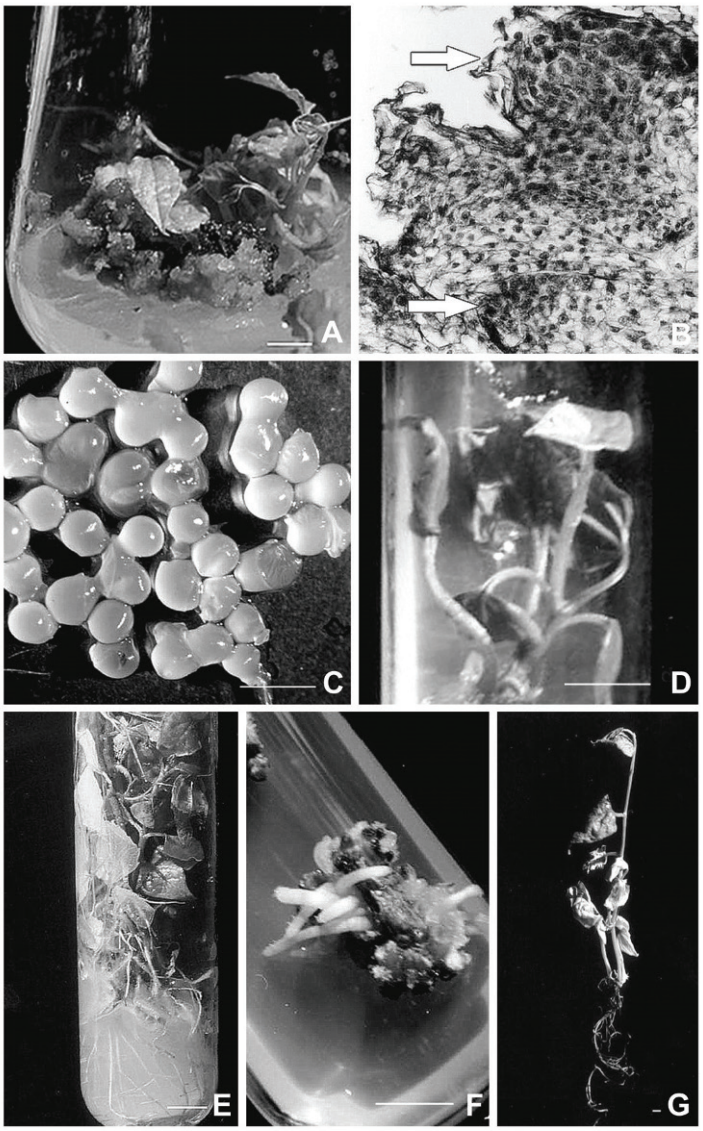

Fig. 1. *A* - Regenerating shoots from the leaf derived callus of *A. tagala; B* - histology of the morphogenic callus showing differentiation of shoot buds; *C* - artificial seeds; *D* - germination of an artificial seed; *E* - rooted shoot; *F* - adventitious roots from the callus; *G* - rooted regenerant. *Bar* = 1 cm.

It was observed that the calli grown in the presence of PG had higher amounts of antioxidant enzymes like peroxidase and catalase and lesser amount of polyphenol oxidase when compared to non regenerating calli. The amount of metabolites like proteins and sugars were also higher in the regenerating calli (Table 7).

Table 4. Effect of growth regulators in MS medium on the length of roos induced from the shoots of *A. tagala* measured after 30 d. Values are mean ± SE n = 10. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

KIN [μM]	IAA [μM]	NAA [μM]	Root length [cm]
0.50	0.50	1.00	9.36 ± 0.14^{bc}
1.00	0.50	0.10	7.96 ± 0.11^{c}
1.50	0.50	-	4.94 ± 0.18^{ab}
2.00	-	-	2.38 ± 0.18^{a}
0.20	0.20	1.00	13.46 ± 0.19^{b}
0.50	0.50	0.50	11.12 ± 0.16^{bc}
0.50	0.50	0.20	10.38 ± 0.15^{bc}

Table 5. Effect of growth regulators on the formation of adventitious roots from the leaf derived callus of *A.tagala.* Root length was measured after 30 d.Values are mean ± SE, n = 30. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

KIN [μM]	IAA [μM]	NAA [μM]	PG[μM]	Root length[cm]
0.5	0.5	0.1	10	3.96 ± 0.23^{ac}
0.5	0.5	0.5	10	3.54 ± 0.25^{c}
0.5	1.0	0.1	10	2.36 ± 0.18^{ab}
1.0	0.5	0.1	10	2.58 ± 0.21^{a}

Table 6. Effect of various potting mixtures on the hardening of *in vitro* regenerated plantlets of *A. tagala* lasting for 30 d. Values are mean ± SE, n = 10. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

Potting mixture	Shoot length [cm] initial	final	Root length [cm] initial	final	Survival [%]
Coir pith alone	6.36 ± 0.45^{ab}	6.96 ± 0.36^{a}	13.16 ± 0.47^{a}	13.66 ± 0.34^{a}	60.00
Coir pith:*Vermiculite* (1:1)	6.16 ± 0.39^{a}	7.52 ± 0.32^{a}	13.12 ± 0.43^{a}	14.34 ± 0.41^{a}	70.00
Coir pith:*Vermiculite*:Soil (1:1:1)	6.42 ± 0.37^{ab}	9.68 ± 0.48^{c}	13.44 ± 0.31^{a}	15.28 ± 0.46^{c}	80.00
Vermiculite:sand:*Sphagnum* moss (1:1:1)	6.26 ± 0.42^{ab}	7.28 ± 0.37^{a}	13.62 ± 0.46^{a}	14.26 ± 0.42^{a}	55.00
Vermiculite: charcoal:sand (1:1:1)	6.53 ± 0.44^{ab}	8.42 ± 0.35^{b}	13.50 ± 0.35^{a}	16.00 ± 0.42^{b}	70.00
Charcoal:sand (1:1)	6.80 ± 0.48^{b}	8.40 ± 0.31^{b}	13.60 ± 0.42^{a}	15.28 ± 0.47^{b}	70.00

Table 7. Biochemical parameters of the regenerating and non regenerating calli of *A. tagala.* Values are mean ± SE. In a column, means followed by a common letter are not significantly different at the 5 % level determined by DMRT.

Parameter	Regenerating calli	Non-regenerating calli
Peroxidase [(ΔA mg^{-1}(protein) min^{-1}]	9.16 ± 1.20^{a}	4.50 ± 1.61^{ab}
Polyphenol oxidase [μmol(quinine) mg^{-1}(protein) min^{-1}]	3.35 ± 2.26^{a}	37.42 ± 1.83^{b}
Catalase [μmol mg^{-1}(protein) min^{-1})	100.73 ± 1.18^{ab}	87.00 ± 2.64^{b}
Total proteins [μg mg^{-1}(d.m.)]	6.17 ± 2.19^{b}	4.25 ± 2.46^{bc}
Reducing sugars [μmol mg^{-1}(d.m.)]	10.00 ± 1.32^{a}	8.53 ± 1.76^{ac}
Non-reducing sugars [μmol mg^{-1}(d.m.)]	6.27 ± 2.30^{b}	4.00 ± 2.70^{ab}
Total sugars [μmol mg^{-1}(d.m.)]	16.47 ± 1.64^{ac}	11.30 ± 1.25^{a}

Discussion

In other species of *Aristolochiaceae*, *in vitro* differentiation of shoots and plantlets have been reported from a variety of explants such as leaves, shoot tips (Manjula *et al.* 1997, Soniya and Sujitha 2006), stems (Remeshree *et al.* 1997, Bliss *et al.* 2009) nodal segments (Manjula *et al.* 1997, Remeshree *et al.* 1994, Soniya and Sujitha 2006), and petiole sections (Bliss *et al.* 2009). Our preliminary experiments with explants from different types of tissues did not produce good amount of callus. The results of the study described here clearly demonstrate that callusing with highest success was obtained using the leaf explants of *A. tagala.*

Traditional approaches to plant regeneration from calli by manipulating the relative ratio of auxin to

cytokinin have been successfully used in the current investigation. A similar auxin-cytokinin effect was previously observed in the callus cultures of *A. indica* (Manjula *et al*. 1997, Soniya and Sujitha 2006) and *A. bracteolata* (Remeshree *et al.* 1994) . Su *et al.* (2011) have demonstrated that a proper ratio between auxin and cytokinin maintained the cell proliferation or stimulated cell differentiation to form new organs, such as shoots or roots. In our experiments, NAA was found to be more effective than 2,4-D in inducing the callus (Table 1). These results are in consonance with those reported in *Dorema ammoniacum* (Irwani *et al.* 2010).

With *A. tagala,* the most critical step was organogenesis. A combination of the phytohormones alone caused browning of the callus and was ineffective in inducing shoot buds. Also, leaching of polyphenols into the culture media was a major problem which influenced further development. This phenomenon could be substantially delayed or reduced by the use of phloroglucinol, thus oxidizing the phenolic substances exuded from the segment (Kim *et al.* 2007). Several authors have reported that the addition of PG to *in vitro* culture medium stimulates shoot development (Sarkar and Naik 2000, Sankar *et al.* 2008, Steephen *et al.* 2010). When this compound was used in the propagation phase of *A.tagala*, it evoked shoot bud regeneration from the callus and reduced leaching**.** These results obtained with PG are in agreement with those observed in *A. indica* by Manjula *et al.* (1997). PG apparently plays an auxin protector role and promotes xylem and chloroplast development (Németh 1986).

The artificial seed has opened up a new vista for future plant production programs and can be applied for germplasm conservation, storage, or a means to reduce the need for transplanting and subculturing during off-season periods (Ahmad and Anis 2010). In the present study, 3 % alginate was found to be the best for formation of ideal, good quality beads. Both sodium alginate and $CaCl_2$ play an important role in capsule quality (Singh *et al.* 2010). Similar reports of a maximum frequency of plantlet regeneration with 3 % alginate were given by Gozukirmizi (2003) in *Paulownia elongate*, Mohanraj *et al.* (2009) in *Coelogyne breviscapa, and* Ali *et al.* (2012) in *Stevia rebaudiana*. It was observed that the use of liquid MS medium for preparing the gel matrix was beneficial in increasing the shoot emergence. The reason for these results may be attributed to the presence of nutrients in the gel matrix which apparently served as a nutrient bed around the propagules, facilitated growth and survival, and allowed them to germinate (Redenbaugh *et al.* 1987, Ara *et al.* 1999). Matrix of synthetic seed mimics endosperm of natural seed. Artificial endosperm should contain nutrients to maintain survival of gerplasm (Antonietta *et al.* 1999). Addition of growth regulators in the germination medium enhanced the production of multiple shoots from encapsulated nodes of *A. tagala*. Similar results have been reported in banana (Ganapathi *et al.* 1992), pineapple (Soneji *et al.* 2002), and *C. breviscapa* (Mohanraj *et al.* 2009). An important feature of the encapsulated vegetative propagules is their capacity to retain viability after storage for a sufficient period required for exchange of germplasm between laboratories and extension centers (Rai *et al.* 2008).

The optimal medium for the rooting of microshoots contained 0.2 µM IAA, 0.2 µM KIN, and 1.0 µM NAA. Existing reports suggest that cytokinins at lower concentrations along with auxins have a critical role in rooting in several plants like *Catharanthus roseus* (Ataei-Azimi *et al.* 2008), *Anthocephalus cadamba* (Apurva and Thakur 2009), and *Erythrina variegata* (Shasthree *et al.* 2009). Cytokinin influences cell-to-cell auxin transport important for regulation of activity and size of the root meristem by modification of expression of several auxin transporters (Růžička *et al.* 2009). In the root meristem, auxin induces the cell division whereas cytokinin promotes the cell to switch from the meristematic to differentiated state (Su *et al.* 2011).

Roots were induced from the leaf derived callus on MS medium fortified with KIN, IAA, NAA, and PG. The positive role of PG in root formation from callus has also been reported by Te-chato and Lim (1999) in mangosteen. Macháčková *et al*. (2008) states that rooting may be stimulated by PG when added to rooting media together with auxins. The above findings are in consonance with the results obtained in the present study. It was noted that, when BAP was used instead of KIN, the calli did not produce adventitious roots. Therefore, it is suggested that KIN stimulated root formation from the callus of *A. tagala.* Such an effect of KIN was also observed by Abdelmajid and Annie (2001) in opium poppy. Since roots are the major component harvested from *A. tagala*, the standardization of a protocol for adventitious root formation from the callus provides avenues for the large scale production of roots *in vitro*.

Acclimatization is the final step in a successful micropropagation. During this stage plants have to adapt to the new environment of a greenhouse or field. The plantlets usually need several weeks of acclimation in shade with the gradual lowering of air humidity (Pospíšilová *et al.* 1998). Fully developed *A. tagala* plantlets with proper shoots and roots were removed from culture medium, transferred to plastic pots containing sterile potting mixture, and acclimatized by adopting the standard procedure. Considering the importance of this step of micropropagation, various combinations of potting mixtures were tried and the maximum survival rate (80 %) was obtained in the potting medium containing coir pith along with *Vermiculite* and soil. The acclimatized plants were transferred to field where they grew well. In contrast to the present findings, Saez *et al.* (2012) reported that in *Castanea sativa*, the *in vitro* grown plants exhibited very low survival rates because of reduced photosynthetic activity.

Analysis of biochemical parameters in the present study revealed an increment of antioxidant enzymes (peroxidase and catalase) and metabolites (sugars and proteins) in the calli grown in the the presence of PG. Reactive oxygen species (ROS) and their scavenging

enzymes participate in regulation of plant growth and development (Mitrović and Bogdanović 2008) as well as in differentiation and growth of plant cells during morphogenesis *in vitro*. The beneficial effects of peroxidase, catalase (Tang and Newton 2005, Rajeshwari and Paliwal 2008, Jana and Shekhawat 2011), and enhanced protein content (Rajeshwari and Paliwal 2008) in shoot induction has already been established. We found that there was no browning or tissue damage in the calli grown in the presence of PG. This may be due to low H_2O_2 accumulation due to increased activity of catalase and peroxidase.

An increase in protein and sugar content is consistent with the results of Patil *et al.* (2010) in sugarcane and Mitrovic *et al.* (2012) in *Tacitus bellus*. As the cells undergo organogenesis, nitrogen is assimilated and used for protein synthesis. Sugars, important constituents of the growing cells, accumulate in the initial regenerating phase and they are rapidly utilized in the organogenesis (Patil *et al.* 2010). Accumulation of polyphenols in brown recalcitrant calli was observed in the current study and this is in agreement with the results obtained by Tang and Newton (2004) in pine. A decrease in the amount of polyphenol oxidase in the regenerating calli of *A. tagala* suggests that PG plays an important role in reducing the polyphenol oxidase content and thereby prevents tissue browning. Also PG could have activated the basal metabolism of cells and consequently reduced secondary metabolism such as polyphenol production. This may be another reason for the reduction of tissue browning by the addition of PG.

In conclusion, the micropropagation method reported here was characterized by a rapid proliferation of shoots. This is highly advantageous for the conservation of this species, production of secondary metabolites, and possible genetic modification. This is the first protocol for indirect regeneration and synthetic seed production of *A. tagala*. Successful plant retrieval from encapsulated nodal segments indicates that this method could be potentially used to preserve elite genotypes of *A. tagala* and facilitate their transport to laboratories and extension centers of distant places.

References

Abdelmajid, K., Annie, J.: Somatic embryogenesis, rhizogenesis, and morphinan alkaloids production in two species of opium poppy. - J. Biomed. Biotechnol. **1**: 70-78, 2001.

Ahmad, N., Anis, M.: Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. - Biol. Plant. **54**: 748-752, 2010.

Ali, A., Gull, I., Majid, A., Saleem, A., Naz, S., Naveed, N.H.: *In vitro* conservation and production of vigorous and desiccation tolerant synthetic seeds in *Stevia rebaudiana.* - J. med. Plants Res. **6**: 1327-1333, 2012.

Antonietta, G.M., Emanuele, P., Alvaro, S.: Effects of encapsulation on *Citrus reticulata* Blanco. somatic embryo conversion. - Plant Cell Tissue Organ Cult. **55**: 235-237, 1999.

Apurva, P., Thakur, P.C.: Somatic embryogenesis and root proliferation from internode of *Anthocephalus cadamba in vitro.* - Asian J. exp. Sci. **23**: 99-102, 2009.

Ara, H., Jaiswal, U., Jaiswal, V.S.: Germination and plantlet regeneration from encapsulated somatic embryos of mango (*Mangifera indica* L.). - Plant Cell Rep. **19**: 166-170, 1999.

Ataei-Azimi, A., Hashemloian, B.D., Ebrahimzadeh, H., Majd, A.: High *in vitro* production of anti-canceric indole alkaloids from periwinkle (*Catharanthus roseus*) tissue culture. - African J. Biotechnol. **7**: 2834-2839, 2008.

Beers, R.F.,Jr., Sizer, I.W.: A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. - J. biol. Chem. **195**: 133-140, 1952.

Biswas, A., Bari, M.A., Mohashweta, R., Bhadra, S.K.: *In vitro* regeneration of *Aristolochia tagala* Champ. a rare medicinal plant of Chittagong hill tracts. - J. biol. Sci. **15**: 63-67, 2007.

Bliss, B.J., Landherr, L., DePamphilis, C.W., Ma, H., Hu, Y., Maximova, S.N.: Regeneration and plantlet development from somatic tissues of *Aristolochia fimbriata*. - Plant Cell Tissue Organ Cult. **98**: 105-114, 2009.

Bradford, M.: A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Castillo, J.A., Jordan, M.: *In vitro* regeneration of *Minthostachya andina* (Brett) Epling – a Bolivian native species with aromatic and medicinal properties. - Plant Cell Tissue Organ Cult. **49**: 157-160, 1997.

Dubois, M.K.A., Gilles, J.K., Hamilton, P.K., Repers, F.: Colorimetric method for determination of sugars and related substances. - Anal. Chem. **28**: 350-356, 1956.

Ganapathi, T.R., Suprasanna, P., Bapat, V.A., Rao, P.S.: Propagation of banana through encapsulated shoot tips. - Plant Cell Rep. **11**: 571-575, 1992.

Gozukirmizi, Z.I.N.: Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. - Plant Cell Rep. **22**: 16-24, 2003.

Irwani, N., Solouki, M., Omidi, M., Zare, A.R., Shahnazi, S.: Callus induction and plant regeneration in *Dorema ammoniacum*, an endangered medicinal plant. - Plant Cell Tissue Organ Cult. **100**: 293–299, 2010.

Jana, S., Shekhawat, G.S.: *In vitro* regeneration of *Anethum graveolens*, antioxidative enzymes during organogenesis and RAPD analysis for clonal fidelity. - Biol. Plant. **56**: 9-14, 2011.

Johansen, D.A. (ed.): Plant Microtechnique. - McGraw-Hill, New York 1940.

Kim, K.M., Kim, M.Y., Yun, P.Y., Chandrasekhar, T., Lee, H.Y., Song, P.S.: Production of multiple shoots and plant regeneration from leaf segments of fig tree (*Ficus carica* L.). - J. Plant Biol. **50**: 440-446, 2007.

Macháčková, I., Zažímalová, E., George, E.F.: Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. - In: George, E.F., Hall, M.A., De Klerk, G.-J. (ed.): Plant Propagation by Tissue Culture. Vol. 1. The Background. Pp.175-204. Springer, Dordrecht 2008.

Malik, C.P., Singh, M.B. (ed.): Plant Enzymology and Histo-enzymology. - Kalyani Publisher, New Delhi 1980.

Manjula, S., Thomas, A., Daniel, B., Nair, G.M.: *In vitro* plant regeneration of *Aristolochia indica* through axillary shoot multiplication and organogenesis. - Plant Cell Tissue Organ Cult. **51**:145-148, 1997.

Mitrović, A., Bogdanović, J.: Activities of antioxidative enzymes during *Chenopodium rubrum* L. ontogenesis *in vitro*. - Arch. biol. Sci. **60**: 223-231, 2008.

Mitrović, A., Janošević, D., Budimir, S., Bogdanović Pristov, J.: Changes in antioxidative enzymes activities during *Tacitus bellus* direct shoot organogenesis. - Biol. Plant. **56**: 357-361, 2012.

Mohanraj, R., Ananthan, R., Bai, V.N.: Production and storage of *Coelogyne breviscapa* Lindl. - Asian J. Biotech. **1:** 124-128, 2009.

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

Murugan, R., Shivanna, K.R., Rao, R.R.: Pollination biology of *Aristolochia tagala*, a rare species of medicinal importance. - Curr. Sci. **91**: 795-798, 2006.

Nelson, N.: A photometric adaptation of the Somogyi method for the determination of glucose. - J. biol. Chem. **153**: 375-380, 1944.

Németh, G.: Induction of rooting. - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 1. Pp. 49-64. Springer-Verlag, Berlin 1986.

Novak, F.J.: *Musa* (bananas and plantains). - In: Hammerschlag, F.A., Litz, R.E. (ed.). Biotechnology of Perennial Fruit Crops. Pp. 449-488. CAB International, Wallingford 1992.

Patil, C.S., Gangawane, A.K., Anjali, R., Naik, G.R.: Organic constituents in the different stages of regenerated callus of sugarcane Var Co-740. - Int. J. mol. Biol. **1**: 15-20, 2010.

Pospíšilová, J., Wilhelmová, N., Synková, H., Čatský, J., Krebs, D., Tichá, I., Hanáčková, B., Snopek, J.: Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. - J. exp. Bot. **49**: 863-869, 1998.

Rai, M.K., Jaiswal, V.S., Jaiswal, U.: Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. - Sci. Hort. **118**: 33-38, 2008.

Rajeshwari, V., Paliwal, K.: Peroxidase and catalase changes during *in vitro* adventitious shoot organogenesis from hypocotyls of *Albizia odoratissima* L.f. (Benth). - Acta Physiol. Plant. **30**: 825-832, 2008.

Ravikumar, K., Ved, D.K. (ed.): 100 Red Listed Medicinal Plants of Conservation Concern in Southern India. - Foundation for Revitalization of Local Health Traditions, Bangalore 2000.

Redenbaugh, K., Slade, D., Viss, P., Fujii, J.A.: Encapsulation of somatic embryos in synthetic seed coats. - Hort. Sci. **22**: 803-809, 1987.

Remeshree, A.B., Hariharan, M., Unnikrishnan, K.: *In vitro* organogenesis in *Aristolochia indica.* - Phytomorphology **47**: 161-165, 1997.

Remeshree, A.B., Hariharan, M., Unnikrishanan, K.: Micropropagation and callus induction of *Aristolochia bracteolata* Lam. a medicinal plant. - Phytomorphology **44**: 247-252, 1994.

Růžička, K., Simaskova, M., Duclercq, J., Petrasek, J., Zazimalova, E., Simon, S., Friml, J., Van Montagu M.C.E., Benková, E.: Cytokinin regulates root meristem activity via modulation of the polar auxin transport. - Proc. nat. Acad. Sci. USA **106**: 4284-4289, 2009.

Sáez, P.L., Bravo, L.A., Sáez, K.L., Sánchez-Olate, M., Latsague, M.I., Ríos, D.G.: Photosynthetic and leaf anatomical characteristics of *Castanea sativa*: a comparison between *in vitro* and nursery plants. - Biol. Plant. **56**: 15-24, 2012.

Sankar, A., Libin, M.S., Vijayakumar, A., Karthi Rani, R., Raja Selvam, J., Kohila, R., Liby, I., Vadivukarasi, S., Ganesh, D.: Phloroglucinol enhances shoot proliferation in nodal explants of *Vanilla planifoloia* Andr. - J. Plantation Crops **36**: 127-129, 2008.

Sarkar, D., Naik, P.S.: Phloroglucinol enhances growth and rate of axillary shoot proliferation in potato shoot tip cultures *in vitro*. - Plant Cell Tissue Organ Cult. **60**: 139-149, 2000.

Sarvesh, A., Reddy, T.P.: Peroxidase, polyphenol oxidase, acid phosphatase and alkaline inorganic pyrophosphatase activities during leaf senescence in varieties of castor (*Ricinus communis* L.). - Indian J. exp. Biol. **26**: 133-136, 1988.

Shasthree, T., Imran, M.A., Mallaiah, B.: *In vitro* rooting from callus cultures derived from seedling explants of *Erythrina variegate* L. – Curr. Trends Biotechnol. Pharmacol. **3**: 447-452, 2009.

Singh, S.K., Manoj, K., Pooja A., Sahoo, L.: Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). - Acta Physiol. Plant. **32**: 607-610, 2010.

Soneji, J.R., Rao, P.S., Mhatre, M.: Germination of synthetic seeds of pineapple (*Ananas comosus* L. Merr.). - Plant Cell Rep. **20**: 891-894, 2002.

Soniya, E.V., Sujitha, M.: An efficient *in vitro* propagation of *Aristolochia indica*. - Biol. Plant. **50**: 272-274, 2006.

Steephen, M., Nagarajan, S., Ganesh, D.: Phloroglucinol and silver nitrate enhances axillary shoot proliferation in nodal explants of *Vitex negundo* L. – an aromatic medicinal plant. - Iranian J. Biotechnol. **8**: 82-89, 2010.

Su, Y.H., Liu, Y.B., Zhang, X.S.: Auxin-cytokinin interaction regulates meristem development. - Mol. Plant. **4**: 1-11, 2011.

Tang, W., Newton, R.J.: Regulated gene expression by glucocorticoids in cultured Virginia pine (*Pinus virginiana* Mill.) cells. - J. exp. Bot. **55**: 1499-1508, 2004.

Tang, W., Newton, R.J.: Peroxidase and catalase activities are involved in direct adventitious shoot formation induced by thidiazuron in eastern white pine (*Pinus strobus* L.) zygotic embryos. - Plant Physiol. Biochem. **43**: 760-769, 2005.

Te-Chato, S., Lim, M.: Plant regeneration of mangosteen via nodular callus formation. - Plant Cell Tissue Organ Cult. **59**: 89-93, 1999.