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In vitro regeneration through adventitious buds in *Wattakaka volubilis*, a rare medicinal plant

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High frequency shoot regeneration from *in vitro* derived leaf explants of *Wattakaka volubilis* (L.f.) Stapf was achieved through callus mediated organogenesis. Organogenic calli were induced from 20 day old aseptic seedling explants on Murashige and Skoog medium fortified with various concentrations and combinations of plant growth regulators, benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), indole 3-butyric acid (IBA) and gibberellic acid (GA_3). A mean of 8.6 shoots developed from organogenic callus induced from a 2 x 2 cm leaf explants on MS medium with 3% sucrose having 5.37µM NAA in combination with 2.22 µM BAP with 60% induction capacity. Further development of adventitious shoots could be achieved by sub culturing the callus to the same medium with 4.40 µM BAP and 0.288 µM GA_3. Organogenesis could not be achieved from the calli of *ex vitro* derived leaf explants. The developed shoots were rooted on half-strength MS medium with 1% sucrose, 4.90 µM IBA and 0.93 µM kinetin at a frequency of 85%. Well rooted plantlets were then transplanted to vermiculite soil (3:1) mixture in polythene covered pots kept under culture room conditions. Approximately 60% plantlets survived and grew into whole plants.

Key words: Adventitious shoots, caulogenesis, organogenic callus-histology.

INTRODUCTION

Wattakaka volubilis (L.f.) Stapf (Syn.: *Dregea volubilis* (L.f.) Benth. ex Hook.f.) (Asclepiadaceae) is an important monotypic woody twiner distributed in India, Sri Lanka, and Java. Stem and leaf explants of this medicinal plant have been reported to have anti-cancerous activity against sarcoma 180 in mice and the alcoholic extract of the total plant is used widely in India as a traditional medicine to cure boils and abscesses (Kirtikar and Basu, 1935). An ointment preparation with trade name "Hemajeevanthi" from leaves of this plant is commonly used for treatment of pyoderma, tineapedis, scabies, plantar psoriasis, as well as cuts and wounds (Thomas et al., 1996). Stems of this woody twiner were exploited commercially as a substitute for ropes. Moreover, this plant is an excellent source of strong fiber. Due to its medicinal and economic significance, the anthropogenic interference has restricted its distribution to highly protected areas such as thorny bushes and has become vulnerable in the Eastern Ghats region. Traditional propagation of *W. volubilis* is rather difficult, limited to rooting of cuttings, with seed propagation hindered by long duration (one year) of seed dormancy and low seed viability. Hence, there is a strong need for an alternative method of mass propagation for best utilization of germplasm of this plant for pharmaceutical needs. Though several parts of *W. volubilis* have been thoroughly investigated for their chemical consti-

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Abbreviations: BAP, Benzyl-6-aminopurine; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog (1962) medium; **NAA,** α-naphthaleneacetic acid; **TDZ,** 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron); **FAA,** formaldehyde acetic acid alcohol; **GA_3,** gibberellic acid; **IAA,** indoacetic acid; **Kn,** kinetin; **TBA,** tertiary butyl alcohol.

tuents (Reddy et al., 2002; Sahu et al., 2002; Yoshimura et al., 1983) only limited progress has been made on *in vitro* regeneration. In previous report, we have described the effect of explants source on axillary shoot multiplication of *W. volubilis* using nodal explants (Chakradhar and Pullaiah, 2006). Other regeneration protocols also reported nodal explants in *W. volubilis* (Yogananth et al., 2011; Vinothkumar et al., 2011). However, there has been no report on regeneration of *W. volubilis* through organogenesis and the present study describes a reproducible, efficient protocol of regeneration system by adventitious shoots using immature leaf explants. Tissue culture techniques have been reported for conservation and multiplication of several medicinal plants of Asclepiadaceae (Kumar et al., 2002; Arya et al., 2003). Adventitious shoots, which arise directly from the non-meristematic tissues of the explant, can provide a reliable method to get true-to-type plants, where as the adventitious shoots arise through caulogenesis has the advantage of creating useful somaclonal variations and in gene transfer technology. Micropropagation through adventtious shoots has been reported as an appropriate method of multiplication in many Asclepiadaceous plants (Prasad et al., 2004; Saha et al., 2003; Faisal and Anis, 2003).

MATERIALS AND METHODS

Stock and explant selection

Mature dry follicles of *W. volubilis* were collected from an identified superior plant growing in the dry deciduous forest areas of Nallamalais in the Kurnool district of Andhra Pradesh, India. Following fruit dehiscence, seeds were collected, thoroughly washed with 1% labolene, (Qualigens, Mumbai) and soaked for 24 h in sterile distilled water containing 144 µM gibberellic acid (GA_3)

Establishment of aseptic culture

Seeds were then surface sterilized with 30% H_2O_2 for 5 min followed by three rinses with sterilized double-distilled water, and grown on half-strength MS medium (Murashige and Skoog, 1962) solidified with 0.6% (w/v) agar (sd fine, Mumbai, India). The MS medium used for all experiments except rooting media consisted of MS basal salts supplemented with 2% sucrose (sd fine, Mumbai) and 0.8% agar. For rooting medium, ½ MS medium with 1% sucrose was used. Culture tubes (150 × 25 mm) containing 15 ml of the medium were autoclaved for 15-20 min at 15 psi and 121°C. Young leaves (2×2 cm) from 60 day old aseptic seedlings (*in vitro*) and two months old field grown plants (*ex vitro*) were used as source of explants. Other explants of aseptic seedlings like internodes and hypocotyls were also tested for organogenic capacity (data not shown).

Callus and soot initiation and proliferation

To induce organogenic callus, the leaf explants were cultured on MS medium fortified with different concentrations of auxins 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), indoacetic acid (IAA) and cytokinins kinetin (Kn), benzyl-6-amino-purine (BAP), 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron) (TDZ), 2-iP. For obtaining adventitious shoots, MS medium was supplemented with various concentrations of cytokinins, BAP (4.40 – 22.20 µM), TDZ (0.0454 – 4.54 µM), Kn (4.62 – 23.20 µM) either individually or in combination with NAA (0.54-22.8 µM) and 2,4-D (0.45-20.66 µM). Sub-culturing of the callus was done for every 30 days once on the respective medium. Cultures were incubated in the culture room at 25±2°C with 36 µ mol $m^{-2}s^{-1}$ spectral flux photon (SFP) and a 16/8 photoperiod. Length and number of differentiated shoots were recorded in all cultures after four weeks of inoculations.

Rooting

For rooting, the *in vitro* produced shoots measuring 3 – 4 cm were individually excised and cultured on half-strength MS medium alone and also with various concentrations of auxins including IAA at 2.28 to 11.42 µM, NAA at 2.69 to 10.74 µM and indole-3-butyric acid (IBA) at 2.46 to 9.80 µM concentrations. 0.93 µM Kn was also used in combination with auxins.

Plantlet transplantation and acclimatization

After 30 days, plants with well developed roots were removed from the culture tube and roots were washed gently in running tap water to remove solid agar. The plantlets with well-proportioned shoots and roots were transferred from *in vitro* conditions to vermiculite-soil (3:1) containing pots which were covered with polythene bags and kept in the culture room conditions. During this initial period quarter strength MS salts (liquid) without sucrose was supplied to the plants. After two weeks, the hardened plants were transferred to plastic pots and kept in shade.

A standard histological procedure (Berlyn and Miksche, 1976) was followed for fixation in formaldehyde acetic acid alcohol (FAA; 0.5:0.5:9.0, v/v/v), embedding in paraffin and sectioning. Microtome sections (10 µm) were collected on glass slides smeared with egg albumin, warmed up by passing over the spirit lamp and placed in xylene for 1 h to dissolve the wax. The wax free slides were reverse processed through dehydration series leaving 1 min in each solution to bring the tissue to the aqueous medium. Sections were stained with safranin for 5 min and passed through the dehydrating solution to dehydrate the tissue, leaving 2 min each solution. Then the slides were placed in tertiary butyl alcohol (TBA) and xylene mixture (1:1) for 5 min and mounted with Permount (Fisher scientific, Fair Lawn, NJ). All the experiments were repeated thrice. The effects of different treatments were quantified and the data was analyzed and expressed as the mean ± standard deviation of three experiments.

RESULTS AND DISCUSSION

Callus formation was observed from both immature (*in vitro* derived) and mature (*ex vitro*) leaf explants on MS medium with auxins NAA, 2,4-D and cytokinins BAP, Kn and TDZ either alone or in combination. Hormone free MS medium failed in inducing callus in the present study as opposite to the results of Martin (2002) in *Holostemma ada-kodien* a related member of the family Asclepiadaceae. Among different auxins used, 2,4-D at 2.26 µM to 18.09 µM and NAA at 5.37 µM to 10.74 µM induced brown, compact callus which is a pre requisite for obtaining adventitious shoot buds (Table 1). Other auxins picloram at 2.07 to 8.28 µM and IAA between 11.42 to 28.54 µM yielded white callus with varied texture in both kinds of explants (data not shown). NAA at 5.37 µM has induced brown, nodular callus from immature leaf explants which

Table 1. Effect of different concentrations and combinations of 2,4 D, NAA, BAP and TDZ on callus induction from immature (aseptic) and mature (wild) leaf explants of *W. volubilis* (L.f.) Stapf.

Plant growth regulator	Concentrations of PGRs (µM)	Immature leaves			Mature leaves		
		Percentage of callus formation (%)	Intensity of callus formed	Nature of response	Percentage of callus formation (%)	Intensity of callus formed	Nature of response
2,4-D	2.26	75	+++	LBC	40	+	LBC
	4.52	80	+++	LBC	60	+	LBC
	9.05	85	++ +++	LBE	55	++	BC
	18.09	80		DBF	50	++	BC
NAA	2.69	60	+++	LGF	50	-	LGC
	5.37	65	+++	BC	55	++	LGF
	10.74	65	++	DBC	53	+	WC
	21.48	70	+	WC	52	++	WC
BAP	2.22	60	+++	LGC	41	-	WC
	4.4	55	+++	LGC	49	+	WC
	8.9	65	++	WC	56	+	WC
	17.6	65	++	WC	53	++	WC
TDZ	0.91	NP	+++	NP	48	+	BC
	1.36	60	++	BC	43	++	BC
	1.82	65	+	DBC	44	+	LGC
	2.27	60	++	DBC	45	++	WC
2,4-D+BAP	2.26+0.44	73	+++	BC	65	++	WC
	2.26+0.44	75	++	BC	58	++	WC
	4.52+0.44	58	+	LGC	63	+	LBC
	4.52+2.22	67	++	OC	66	++	BC
NAA+BAP	2.69+0.44	74	+++	BC	58	+	WC
	2.69+2.22	70	++	LGC	57	++	BC
	5.37+0.44	68	+	LGC	63	+	BC
	5.37+2.22	66	++	OC	66	++	LGC

Data were recorded after 4 weeks of culture. LBC, Light brown compact; LBE, light brown embryogenic; DBF, dark brown friable; DBC, dark brown compact BC, brown compact; LGF, yellowish green with friable; LYF, light yellow friable; WC, white compact; LGC, light green compact, OC, organogenic callus; +++, profuse; ++, medium; +, scanty; -, no response. Observations made after 4 weeks of inoculation.

can be used for organogenic callus production with the combination of cytokinins (Figure 1a). Among the cytokinins tested individually for their ability in inducing callogenesis, BAP exhibited positive response with production of green, compact callus from leaf explants at 17.6 µM. The other cytokinin TDZ exhibited poor response with single shoot bud developed from brown, compact callus from immature leaf explants at 2.27 µM in 40% of the cultures. The mature leaf explants proved non-morphogenic in the present study in spite of various combinations and concentrations of plant growth regulators tested. Kn failed in inducing callus at all concentrations with both explants tested. Further development of organogenic callus into shoots could not be achieved with either BAP or NAA alone in this study. Among various combinations of BAP and NAA the leaf explants at 5.37 µM NAA + 2.22 µM BAP induced light brown coloured, compact organogenic calli with green, monopolar nodules on its surface (Figure 1b). The callus developed on 5.37 µM NAA with 0.44 µM BAP was also nodular, pale yellow but without green buds on its surface. Occurrence of green spots which are considered meristematic centers can be a predictor of the capacity of the callus to produce shoots (Nabors et al., 1982; Ishii, 1982). Organogenic callus with friable, translucent and watery nature was also found with 2,4-D at 4.52 µM in combination with 2.22 µM BAP. But shoot bud development could not be achieved with this organogenic callus even on sub culturing to different media with less/no concentration of 2,4-D. This result is contradictory to the statement that translucent, watery callus is seldom morphogenic where as nodular callus frequently is (George, 1996). The green nodules developed on 2.22 µM BAP+ 5.37 µM NAA turned into clear shoot buds on sub culturing to MS medium fortified with 4.40 µM BAP without NAA (Figure 1c). Further elongation of shoot buds could be achieved by sub culturing the callus having adventitious shoot buds on to the medium with 0.18 µM to GA_3 (Figure 1d). The elongated

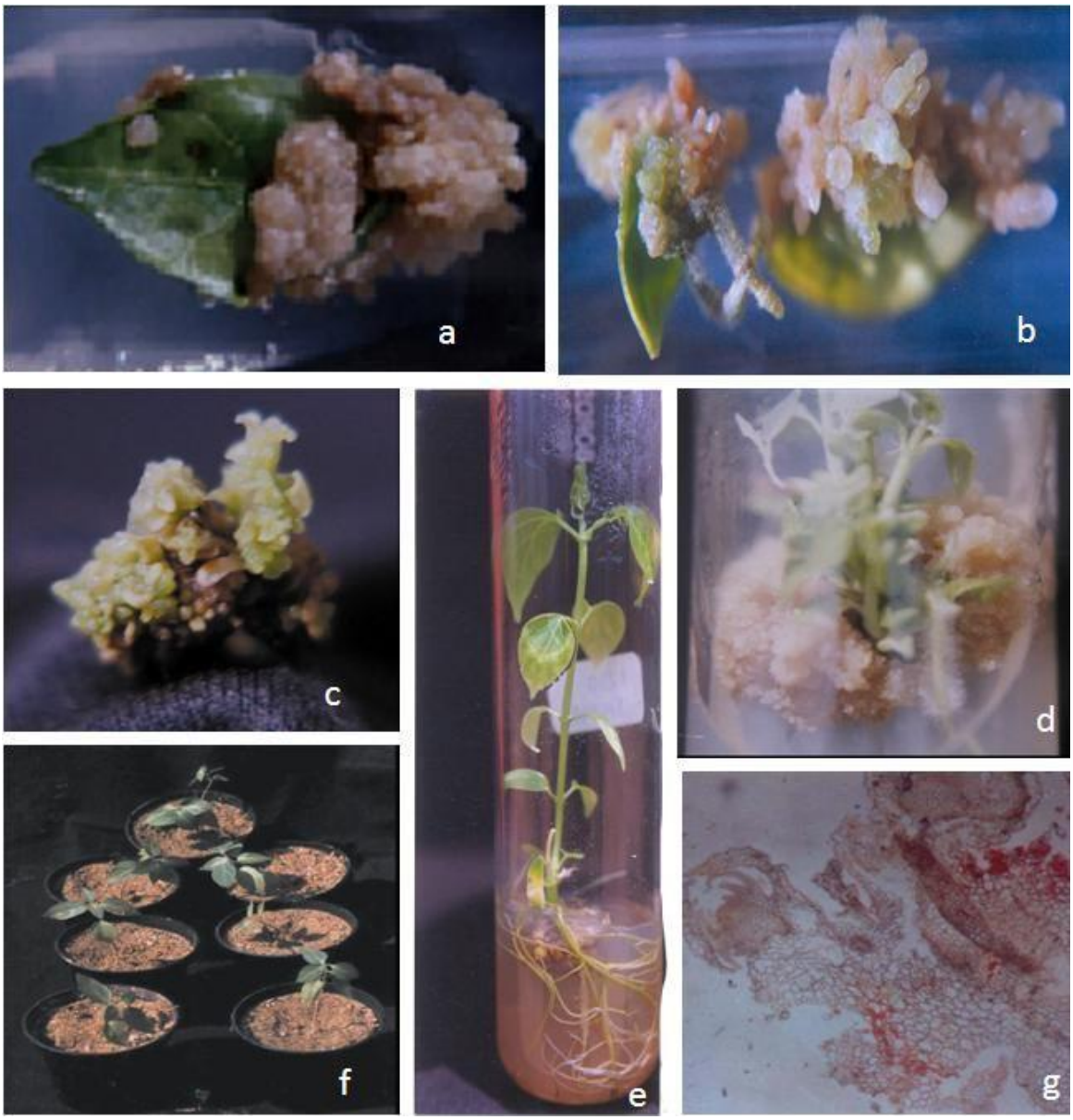

Figure 1. a, Brown, compact callus induced; **b,** initiation of adventitious shoots from green nodular callus; **c,** proliferation and development of adventitious shoots; **d,** elongation of adventitious shoots; **e,** *in vitro* rooting of excised shoots; **f,** aclimatised plants in pots in garden; **g,** longitudinal section of organogenic callus derived from *in vitro* leaf explants. Arrow indicates emerging shoot primordial.

shoots attained an average height of 6.3 cm within 3 weeks with optimum shoot number of 12.56 at 0.288 µM GA_3 (Figure 2). GA_3 at higher concentrations reduced shoot number with abnormalities like hyperhydricity (Figure 2). GA_3 induced shoot bud elongation was earlier observed by Sahoo et al. (1997) and Vasanth et al. (2002). Generally GA_3, which is necessary for shoot bud development, was found inhibitory at bud induction stage due to its inhibitory action on meristamoid formation (Rubluo et al., 1984). NAA was found inhibitory for development of shoots once after their differentiation. Reddy et al. (1998) stated that the presence of NAA or 2,4-D in the medium tends to loose the chlorophyll in the green callus, and suppresses shoot buds once after their initiation, which supports the present study. Formation of adventitious shoots from the surface of the callus with BAP and NAA combination has also been reported in *H. ada-kodien* and *Tylophora indica*, both belonging to Asclepiadaceae (Martin, 2002; Manjula et al., 2000).

The differentiated shoots from the above cultures failed to root in either half strength or full strength MS medium, free of plant growth regulators. Among the three auxins tested, IBA was most effective in inducing roots. Fifteen (15) days after inoculation, root formation with varied

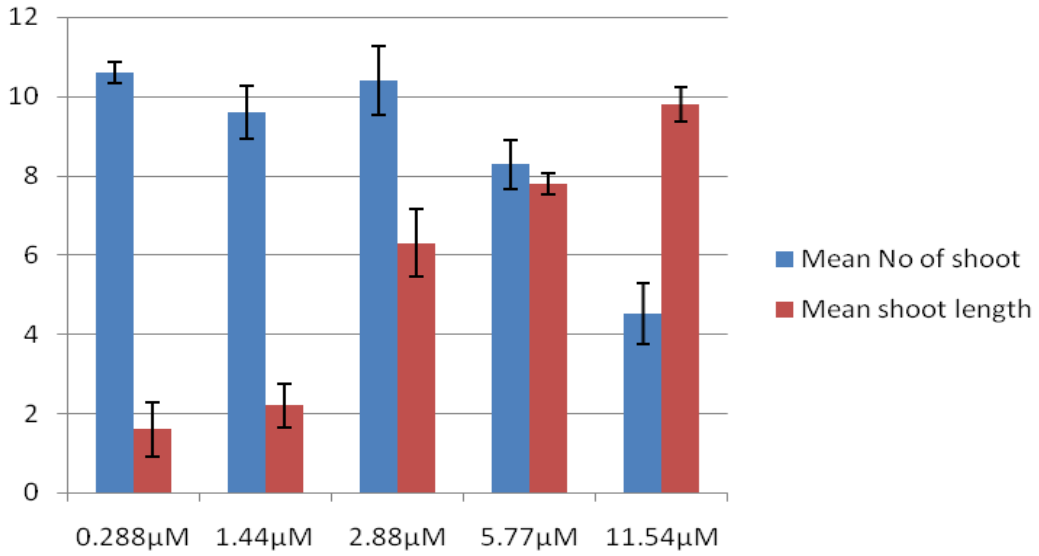

Figure 2. Effect of different concentrations of GA_3 in combination with 4.40 µM BAP on shoot elongation from regenerated shoots on MS medium. The results are expressed as the mean±SD of three replicates per treatment. The data were recorded after 3 weeks of culture.

Table 2. *In vitro* rooting of *Wattakaka volubilis* on half strength MS medium fortified with different concentrations of auxins with Kn.

Growth regulators (µM)			Percentage	Number of roots/shoot (mean±S.D)	Average shoot length (mean±S.D)
IBA	NAA	Kn			
2.46			55	6.70±0.76	9.00±0.00
4.92			90	8.40±0.34	12.30±0.42
9.84			85	10.60±0.92	10.40±0.45
	0.53		50	1.70±0.80	5.40±0.64
	2.68		60	2.40±0.63	4.50±0.43
	5.37		70	1.83±0.84	2.80±1.23
4.64		0.92	85	12.40±0.51	12.20±0.45
4.64		2.32	83	11.35±0.84	11.00±0.00

Data of 10 replicates repeated thrice. Values taken after 3 weeks.

morphology was observed from basal cut portion of the shoots on half strength MS medium containing 1% sucrose with IBA ranging from 2.46 to 9.80 µM. Reduction of MS salts to half strength enhanced root formation in shootlets. It was also observed that reduction of sucrose concentration in rooting media enhances plant let survival rate at later stage. About 90% of excised shoots developed woody root system on half strength MS medium containing IBA at 4.90 µM with maximum mean root length of 12.3 cm (Table 2). A profuse root system from the cut end of the shoots, having root hairs with 85% of response, was obtained when 0.93 µM Kn was added to the above medium (Figure 1e). The other auxin, NAA, at lower concentrations (2.69 µM) induced stout 1-2 unbranched roots without root hairs, whereas at higher concentrations (>2.69 µM) inhibited rooting with increased basal callusing. IAA at 2.85 – 11.42 µM induced long, weak roots which are unable to establish the shoots in soil. The effectiveness of IBA in root induction has been reported in other Asclepiadaceae members, *Hemidesmus indicus* (Sreekumar et al., 2000), and *H. ada-kodien* (Martin, 2002). The positive effect of Kn for best rooting was well supported by Raghuramulu et al. (2002) in *Cynanchum callialatum* another member of Asclepiadaceae. The plantlets with well-proportioned shoots and roots were transferred from *in vitro* conditions to vermiculite-soil (3:1) containing pots which were covered with polythene bags and kept in the culture room conditions. During this initial period quarter strength MS salts (liquid) without sucrose was supplied to the plants.

After two weeks, the hardened plants were transferred to plastic pots and kept in shade, with maximum of 50% survival rate (Figure 1E).

Microtome sections of 3 week old organogenic callus derived from 5.37 µM BAP+2.22 µM NAA cultures revealed meristematic activity at the sub-epidermal region. The meristematic cells were distinguishable from other cells by their small size and thick stained cytoplasm. These meristematic cells are delimited distally by a single layer cells to form shoot primordia which led to the organization of shoot meritem and a pair of leaf primordia (Figure 1g). These meristemoids produced adventitious soot buds in later stage which subsequently developed into shoots.

Conclusion

The present work describes a reproducible and efficient protocol for *in vitro* mass propagation of rare, valuable medicinal plant *W. volubilis* through indirect organogenesis with successful acclimatization of *in vitro* rooted plants. The explant source and the growth regulators inducing the callus exhibited a significant influence on *in vitro* organogenesis in the present study.

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