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ESTABLISHMENT OF AN IMPROVED, EFFICIENT AND ECO-FRIENDLY MICROPROPAGATION SYSTEM IN *SALACIA CHINENSIS* L. AN ENDANGERED ANTI-DIABETIC MEDICINAL PLANT

SUMMARY

An efficient micropropagation system via indirect organogenesis was developed in *Salacia chinensis* L., an endangered anti-diabetic medicinal plant. Accurate investigation of the various plant sources (Leaf, node, shoot tip) and plant growth regulators (PGRs) impacts were accomplished in this study. Maximum rate of callus induction (93.43 ± 2.75%) was achieved from nodes inoculated on MS medium fortified with 1.0 mg/l NAA+ 2.0 mg/l BAP. Maximum shoot induction (93.33 ± 2.02%), number of shoots/explant (5.12 ± 0.09) and shoot length (3.17 ± 0.00 cm) were obtained from nodal explants inoculated on MS medium with 1.5 mg/l BAP + 1.0 mg/l NAA. IBA (2.0 mg/l) in ½ MS medium was observed to be the best rooting treatment, which promoted the highest frequency of rooting (91.66 ± 2.33%). The results suggested an efficient regeneration system for conservation and large scale production for pharmaceutical industry demands.

Keywords: *Salacia chinensis*, Anti-diabetic, Medicinal plant, Indirect organogenesis, *In vitro* conservation.

INTRODUCTION

Salacia chinensis L. (Celastraceae family) broadly dispersed in tropical and subtropical regions of the world, particularly in Indian subcontinent, China and Southeast Asian countries (Muraoka et al., 2010). Various parts of the plant have been extensively used in various traditional medicinal systems especially as a unique Ayurvedic medicine to treat a broad range of ailments (Singh et al., 2010; Sikarwar and Patil 2012). Phytoconstituents such as mangiferin, salacinol, kotalanol, phenolic glycosides and triterpenes have been isolated from the plant which showed different therapeutic properties (Yoshikawa et al., 2003). Although the plant is renowned for its anti-diabetic property but other activities such as anti-inflammatory, anti-cancer, anti-mutagen**,** anti-oxidant and cardioprotective are also been well documented (Guha et al., 1996; Yoshimi et al., 2001; Kishino et al., 2009).

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The plant habitat destruction, excessive and unpredictable collection for supplementation of worldwide demands particularly obvious anti-diabetic property placed *S. chinensis* on the endangered species category (Sharma et al., 2011). There has been an increased interest using plant tissue culture techniques in mass propagation of medicinal plants for recuperation of endangered species, thus reducing the risk of extinction (Nadeem et al., 2000; Phulwaria et al., 2013**)**.

During last two years, there are only two protocols reported for the *in vitro* micropropagation of this endangered anti-diabetic medicinal plant (Chavan et al., 2015; Majid et al., 2016). Both the studies were limited to only direct regeneration system which is not applicable for further studies such as establishment of cell suspension culture in this plant. Establishment of an improved, efficient and eco-friendly propagation system using different plant growth regulators (PGRs) and various explant types for conservation and large scale production of *S. chinensis* for industrial utilization was the main goal of the present study.

MATERIALS AND METHODS

Indirect organogenesis

Plant material, surface sterilization and culture conditions

Young and healthy shoot tips, nodal segments and leaves collected from field grown *S. chinensis* were washed twice in running tap water, pre-washed with concentrated dishwasher gel (3-4 drops/100 ml double-distilled water (ddH_2O) (Vim, India) for 5 min. The explants were submerged in 70% (v/v) ethanol for 2 min, sterilized with 1.0% (w/v) sodium hypochlorite (NaOCl) plus 2-3 drops Tween 20 (HiMedia, India) for 15 min (Majid et al., 2014). The explants were washed thoroughly with ddH_2O four times and cut into suitable sizes (1.0 - 2.0 cm).The explants were inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar (HiMedia, India). Before autoclaving at 121^{o}C for 20 min, the medium pH was adjusted to 5.7. Cultures were kept at temperature of 25 ± 2^{o}C for 16-h photoperiod with 40 µmolm^{-2} s^{-1} provided by cool-white florescent lamps (Philips, India).

Callus induction

To induce callus, the explants were first cultured on MS medium fortified by various auxins (NAA, 2,4-D, IBA) (0.5- 2.0 mg /l) individually to achieve the optimum concentration. The explants were then inoculated on MS medium supplemented with optimized auxin concentration in combination with various concentrations (0.5-2.0 mg/l) of BAP or Kin.

Shoot regeneration

Callus from various explants that gave the optimum level was cut into a number of segments and sub-cultured on MS shoot regeneration medium supplemented with different concentrations of BAP or Kin (1.0- 4.0 mg /l) individually or in combination with NAA, IAA and IBA (0.5-2.0 mg/l). Subcultures were carried out every 4 weeks. The shoot induction percentage,

number of shoots/explant and the mean length of shoots were recorded after 8 weeks.

Rooting and acclimatization of regenerants

Elongated shoots (3-5 cm in height) were cultured on full and ½ MS medium fortified with NAA, IAA or IBA (0.5- 2.5 mg/l) for root formation. The rooting percentage, root number and length were recorded after 6 weeks of inoculation. Well-regenerated plantlets were removed from the medium, washed gently by rinsing in running tap water and transplanted to pots containing organic fertilizer, sand and peat (2:2:1) and covered by transparent plastic bags in culture room for 30 days. Transferring of the plantlets to the pots containing garden soil and maintaining in the glasshouse for another 2 weeks was the final step.

Statistical analysis

All the tissue culture experiments of callus induction, shoot proliferation and root formation were repeated three times with 12 replicates per treatment. One-way analysis of variance (ANOVA) was used for data analyzing in the statistical package of SPSS (Version 20.0. Armonk, NY, USA: IBM Corp.) The significant differences between means were scored using Duncan's Multiple Range Test ($P = 0.05$).

RESULTS AND DISCUSSION

Callus induction

Successful explant surface sterilization without using highly toxic and environmental-enemy chemical $HgCl_2$ was established in this study. Explants on MS medium in the absence of PGRs failed to initiate callus even 4 weeks after inoculation, but using various auxins (NAA, 2,4-D, IBA) at different concentrations (0.5-2.0 mg/l) initiate the callus induction and showed variation in percentage. Similar results were reported in callus induction in various tissue culture studies of medicinal plants (Raha and Roy, 2003; Simmons et al., 2008; Wei et al., 2015). Callus was initiated after 14, 21 and 28 days after inoculation from the leaf, shoot and nodal explants respectively. All cultures were sub-cultured on the same medium after 4 weeks. According to data among various kind and concentration of auxins individually tested, the maximum callus (51.80 ± 0.70%) was induced on MS medium fortified by 1.0 mg/l NAA in nodal explants (Table 1) after 8 weeks. Variation in callus production and regenerated plantlets may depend on the genotype, source and physiological status of the explants (Nair and Seeni, 2001). Combination of optimum auxin (1.0 ml NAA) with cytokinins markedly improved the callus induction rate. The same influence was reported in previous studies (Jayaraman et al., 2014). Among the two cytokinins (BAP and Kin), BAP was most effective and maximum callus induction percentage (93.43 ± 2.75%) was obtained from nodal explants inoculated on MS medium fortified with 1.0 mg/l NAA and 2.0 mg/l BAP after 8 weeks (Table 1; Fig. 1B). Positive effect of auxin-cytokinin combination is well-documented in medicinal plant tissue culture studies (Rao et al., 2011; Brijwal et al., 2015).

Table 1. The Effect of auxins (NAA, 2,4-D, IBA) individually or with cytokinins (BAP, Kin) on callus induction from various explants of *S. chinensis*.

PGRs	Conc.(mg/l)	Callus induction (%) in each explant		
		Node	Leaf	Shoot tip
NAA	0.5	37.74 ± 3.93^{f}	00.00 ± 0.00^{a}	9.66 ± 1.76^{bcd}
	1.0	51.80 ± 0.70^{g}	13.33 ± 0.88^{cd}	17.66 ± 1.20^{d}
	2.0	23.49 ± 2.16^{de}	5.66 ± 1.76^{ab}	00.00 ± 0.00^{a}
2,4-D	0.5	16.25 ± 3.76^{cd}	7.33 ± 2.02^{bc}	00.00 ± 0.00^{a}
	1.0	27.94 ± 3.60^{e}	10.66 ± 1.76^{bc}	13.33 ± 0.88^{cd}
	2.0	14.36 ± 1.65^{bc}	24.33 ± 1.45^{e}	19.33 ± 1.45^{d}
IBA	0.5	0.00 ± 0.00^{a}	5.66 ± 1.20^{ab}	2.66 ± 1.76^{ab}
	1.0	12.22 ± 2.75^{bc}	35.00 ± 2.30^{f}	14.66 ± 2.33^{cd}
	2.0	5.79 ± 0.24^{ab}	20.00 ± 3.75^{e}	6.33 ± 3.75^{abc}
NAA + BAP	1.0 + 0.5	64.13 ± 0.28^{h}	19.33 ± 4.33^{de}	14.33 ± 4.91^{cd}
	1.0 + 1.0	77.98 ± 3.39^{i}	32.33 ± 2.60^{f}	31.33 ± 3.17^{e}
	1.0 + 1.5	81.66 ± 1.86^{i}	41.66 ± 0.33^{g}	42.66 ± 1.20^{f}
	1.0 + 2.0	93.43 ± 2.75^{j}	57.33 ± 3.17^{h}	55.33 ± 0.88^{g}
NAA + Kin	1.0 + 0.5	37.21 ± 1.48^{f}	11.33 ± 0.88^{bc}	16.66 ± 5.78^{d}
	1.0 + 1.0	54.90 ± 5.02^{g}	25.33 ± 1.45^{e}	43.66 ± 4.63^{f}
	1.0 + 1.5	76.39 ± 1.37^{i}	33.33 ± 2.60^{f}	51.33 ± 3.17^{fg}
	1.0 + 2.0	79.19 ± 5.30^{i}	52.33 ± 0.88^{h}	32.66 ± 1.20^{e}

Mean ± SE of 12 replicates (in triplicate) per treatment. Means followed by same letter within each column does not differ statistically according to DMR test ($p = 0.05$).

Shoot induction

Shoot induction on MS medium supplemented only with cytokinins was poor and improved when the medium was fortified with optimized level of cytokinin in combination with different concentration of auxins (0.5-2 mg/l). The concomitant use influence of auxins and cytokinins is well documented (Huang et al., 1994; Martin, 2002). BAP was found most suitable than Kin for shoot initiation in all treatments. BAP is structurally stable and the plant cells ability to its easy assimilation makes this kind of cytokinin stand out among the others (Ahmad et al., 2013). BAP strong promotive effects on shoot regeneration were reported previously (Rao and Purohit, 2006; Mozafari et al., 2015). Suitable individual concentration of BAP was 1.5 mg/l for shoot induction in node, while this rate was 2.0 mg/l for Kin. The effective interaction of auxins in low concentration with BAP or Kin was shown in this study. The similar finding in different medicinal plants was also reported (Thomas and Yoichiro, 2010). Maximum shoot induction percentage (93.33 ± 2.02%) with 5.12 ± 0.09 shoot per explant and 3.17 ± 0.00 cm length were obtained from nodal explants inoculated on MS medium supplemented with 1.5 mg/l BAP + 1.0 mg/l NAA (Table 2; Fig. 1D). The same effects were demonstrated in many tissue culture studies (Taylor and Van Staden, 2001; Sahai and Shahzad, 2013; Rawat et al., 2013).

Table 2. Effect of cytokinins (BAP, Kin) individually or with auxins (NAA, IAA, IBA) on shoot regeneration from nodal explants of *S. chinensis*.

PGRs	Conc. (mg/l)	Shoot induction (%)	Number of shoot/explant	Shoot length (cm)
BAP	1.0	38.33 ± 3.75^{defg}	1.13 ± 0.02^{b}	0.72 ± 0.05^{a}
	1.5	67.33 ± 2.02^{kl}	1.93 ± 0.01^{f}	1.55 ± 0.01^{f}
	2.0	52.66 ± 2.90^{i}	1.78 ± 0.05^{e}	1.33 ± 0.02^{e}
	2.5	50.66 ± 4.63^{hi}	1.38 ± 0.06^{c}	1.16 ± 0.01^{d}
	3.0	35.33 ± 2.60^{cde}	1.16 ± 0.02^{b}	0.95 ± 0.03^{bc}
	4.0	11.33 ± 4.33^{a}	1.00 ± 0.03^{a}	0.65 ± 0.06^{a}
Kin	1.0	21.33 ± 1.45^{b}	1.18 ± 0.03^{b}	0.62 ± 0.04^{a}
	1.5	45.66 ± 5.20^{fghi}	1.36 ± 0.10^{c}	0.87 ± 0.05^{b}
	2.0	53.33 ± 2.60^{ij}	1.66 ± 0.00^{e}	1.17 ± 0.02^{d}
	2.5	42.66 ± 0.66^{efgh}	1.50 ± 0.02^{d}	1.03 ± 0.06^{c}
	3.0	29.66 ± 1.76^{bcd}	1.19 ± 0.08^{b}	0.71 ± 0.02^{a}
	4.0	00.00 ± 0.00^{p}	0.00 ± 0.00^{q}	0.00 ± 0.00^{m}
BAP + NAA	1.5 + 0. 5	47.66 ± 5.87^{ghi}	2.17 ± 0.04^{g}	2.59 ± 0.02^{j}
	″ + 1.0	93.33 ± 2.02^{o}	5.12 ± 0.09^{p}	3.17 ± 0.00^{l}
	″ + 1.5	83.66 ± 3.52^{n}	4.33 ± 0.02^{o}	2.92 ± 0.01^{k}
	″ + 2.0	77.33 ± 2.60^{mn}	3.28 ± 0.03^{k}	2.69 ± 0.06^{j}
BAP + IBA	1.5 + 0. 5	27.33 ± 4.33^{bc}	2.00 ± 0.07^{f}	1.72 ± 0.03^{g}
	″ + 1.0	$72.33 \pm 2.02l^{m}$	3.83 ± 0.04^{m}	2.17 ± 0.00^{hi}
	″ + 1.5	58.33 ± 3.17^{jk}	3.25 ± 0.01^{k}	2.11 ± 0.02^{h}
	″ + 2.0	36.33 ± 1.45^{cdef}	2.77 ± 0.02^{i}	1.29 ± 0.07^{e}
BAP+ IAA	1.5 + 0. 5	33.33 ± 1.45^{cde}	2.34 ± 0.02^{h}	2.25 ± 0.03^{i}
	″ + 1.0	84.33 ± 3.17^{n}	3.58 ± 0.02^{l}	2.66 ± 0.08^{j}
	″ + 1.5	74.33 ± 2.60^{lm}	3.96 ± 0.03^{n}	2.66 ± 0.01^{j}
	″ + 2.0	67.33 ± 3.17^{kl}	2.95 ± 0.05^{j}	2.21 ± 0.03^{hi}

Mean ± SE of 12 replicates (in triplicate) per treatment. Means followed by same letter within each column does not differ statistically according to DMR test ($p = 0.05$).

Plantlet root formation and acclimatization

No rooting was noted on PGRs free MS medium, while low response was initiated upon transfer to full strength MS medium fortified with auxins. ½ MS medium fortified with auxins (NAA, IBA and IAA) showed better effects on rooting (Table 3). The same effects of low strength MS medium led to higher rooting were demonstrated in species such as *R. officinalis* (Misra and Chaturvedi, 1993) and *A. mearnsii* (Huang et al., 1994). This result approved the requirement of the rooting to lower nitrogen ions than shooting once more (Driver and Suttle, 1987).

Table 3. Effect of various auxins (NAA, IBA, IAA) in full and ½ MS medium on plantlet rooting from *S. chinensis* shoots.

PGRs (mg/l)	Rooting (%)	Root number/shoot	Root length (cm)
Full-strength MS + PGRs			
0.5 NAA	00.00^{a}	0.00^{a}	0.00^{a}
1.0 NAA	00.00^{a}	0.00^{a}	0.00^{a}
1.5 NAA	04.66 ± 1.20^{a}	1.08 ± 0.28^{b}	0.72 ± 0.01^{cde}
2.0 NAA	22.33 ± 2.33^{cd}	1.11 ± 0.02^{bc}	0.66 ± 0.01^{bcd}
2.5 NAA	00.00^{a}	0.00^{a}	0.00^{a}
0.5 IAA	07.33 ± 1.85^{ab}	1.03 ± 0.07^{b}	0.60 ± 0.02^{b}
1.0 IAA	14.33 ± 4.05^{bc}	1.02 ± 0.11^{b}	0.80 ± 0.02^{ef}
1.5 IAA	00.00^{a}	0.00^{a}	0.00^{a}
2.0 IAA	38.66 ± 2.02^{fg}	1.18 ± 0.02^{bcd}	0.80 ± 0.02^{ef}
2.5 IAA	28.66 ± 4.25^{de}	1.26 ± 0.02^{cde}	0.62 ± 0.04^{bc}
0.5 IBA	19.33 ± 1.45^{cd}	1.41± 0.01^{ef}	0.88 ± 0.02^{fg}
1.0 IBA	40.33 ± 1.85^{fgh}	1.13 ± 0.04^{bc}	0.75 ± 0.01^{de}
1.5 IBA	60.66 ± 0.88^{kl}	1.32 ± 0.02^{de}	0.79 ± 0.04^{ef}
2.0 IBA	67.00 ± 4.01^{lm}	1.59 ± 0.03^{g}	0.97 ± 0.03^{gh}
2.5 IBA	43.33 ± 3.48^{gh}	1.48 ± 0.03^{fg}	0.88 ± 0.01f^{fg}
½ MS + PGRs			
0.5 NAA	28.66 ± 0.33^{de}	2.01 ± 0.03^{h}	0.71 ± 0.02^{cde}
1.0 NAA	18.33 ± 1.85^{cd}	2.23 ± 0.05^{i}	0.82 ± 0.02^{ef}
1.5 NAA	33.33 ± 2.02^{ef}	2.47 ± 0.17^{j}	1.01 ± 0.06^{h}
2.0 NAA	46.66 ± 3.52^{ij}	2.88 ± 0.01^{k}	1.11 ± 0.02^{i}
2.5 NAA	38.33 ± 1.45^{fg}	2.79 ± 0.01^{k}	1.17 ± 0.04^{i}
0.5 IAA	51.66 ± 3.38^{jk}	2.84 ± 0.02^{k}	1.17 ± 0.04^{i}
1.0 IAA	58.66 ± 5.45^{jk}	3.16 ± 0.04^{l}	1.31 ± 0.06^{j}
1.5 IAA	53.33 ± 3.17^{jk}	3.24 ± 0.03^{lm}	1.38 ± 0.03^{jk}
2.0 IAA	70.33 ± 2.18^{m}	3.44 ± 0.06^{n}	1.56 ± 0.04^{lm}
2.5 IAA	37.33 ± 3.17^{fg}	3.16 ± 0.05^{l}	1.46 ± 0.01^{kl}
0.5 IBA	36.66 ± 2.60^{fg}	3.35 ± 0.05^{mn}	1.51 ± 0.04^{l}
1.0 IBA	55.53 ± 1.85^{jk}	3.49 ± 0.03^{n}	1.65 ± 0.03^{m}
1.5 IBA	67.00 ± 1.52^{lm}	3.97 ± 0.04^{o}	1.82 ± 0.05^{n}
2.0 IBA	91.66 ±2.33^{n}	4.38 ± 0.05^{p}	2.52 ± 0.03^{p}
2.5 IBA	72.33 ± 2.90^{m}	3.21 ± 0.05^{lm}	2.18 ± 0.02^{o}

Mean ± SE of 12 replicates (in triplicate) per treatment. Means followed by same letter within each column does not differ statistically according to DMR test ($p = 0.05$).

Of the auxins tested, IBA was most effective on rooting in both MS mediums. The higher stability of IBA to chemical degradation during autoclaving and at room temperature than the other auxins could be convincing (Cuenca et al., 1999). The best root formation (91.66 ± 2.33%) highest number of roots per shoot (4.38 ± 0.05) with a mean length of 2.52 ± 0.03 cm was obtained in ½ MS

with 2.0 mg/l IBA (Table 3; Fig. 1E-F). The IBA stimulatory effect on plantlet rooting were reported in other medicinal plant species (Fracaro and Echeverrigary, 2001; Chandra et al., 2006; Mir et al., 2014).

Hardening of the plantlets was done at room temperature on plastic pots filled with sand, peat and organic fertilizer. Further growth was obtained after transferring into the pots containing garden soil in the glasshouse for more 14 days, led to subsequent shifting to the field. Above 91% survivability in field condition was recorded and no morphological variation was observed from mother plants. The results showed improvement comparing to the previous, survival rates of 87% recorded Majid et al., (2016).

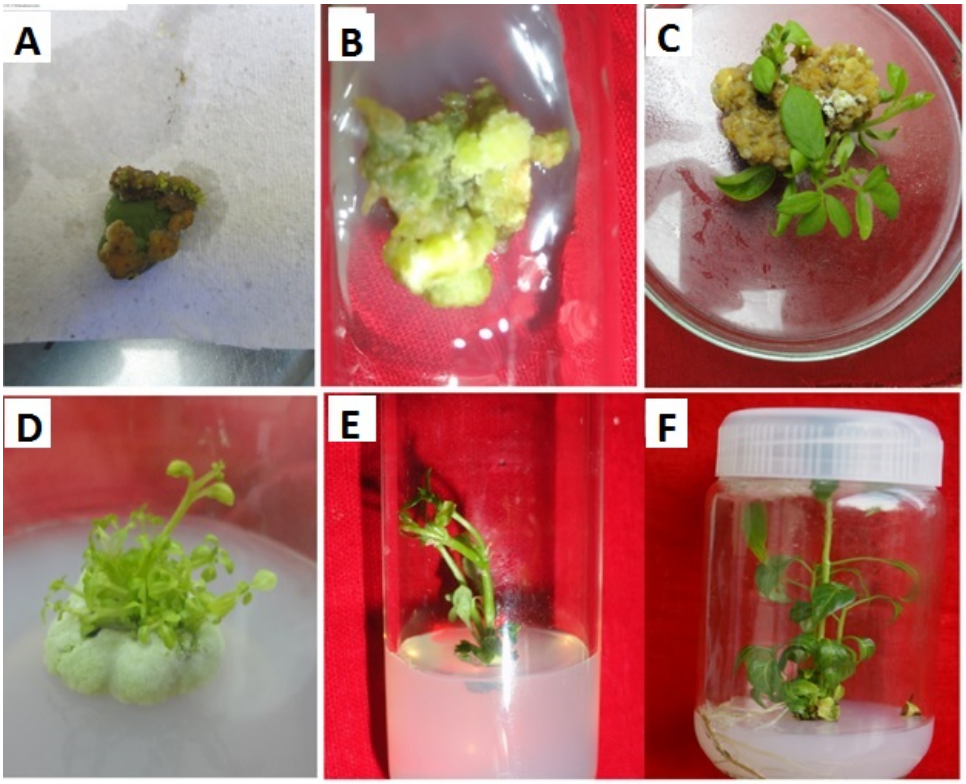

Figure 1. ***In vitro*** **regeneration of** ***S. chinensis.*** **A-B** Induced callus from leaf and nodal explants (MS + 1.0 mg/l NAA + 2.0 mg/l BAP). **C-D** Shoot induction from leaf and nodal explants (MS + 1.5 mg/l BAP + 1.0 mg/l NAA). **E** Pre-culture for rooting of the elongated shoots. **F** Shoot rooting (½MS + 2.0 mg/l IBA).

In this study, we developed an efficient and eco-friendly propagation system via accurate investigation of the various explants (Leaf, node, shoot tip) and PGRs impacts in *S. chinensis*. Overcoming the constraints of the two previous reports (Chavan et al., 2015; Majid et al., 2016) was achieved successfully. Evaluation of the various explant types and NaClO as a disinfectant

instead of using limited type of explants (one type) and highly toxic non eco-friendly surfactant $HgCl_2$ for surface sterilization are the main advantages.

Although Majid et al., (2016) developed a proper micropropagation system but the study was limited to only direct regeneration system which is not applicable for further studies such as establishment of cell suspension culture in this plant, was overcome in this report via indirect regeneration protocol. Above 91% survivability in field conditions showed improvement comparing to the previous survival rates.

CONCLUSIONS

According to the obtained results, this simple, rapid and eco-friendly established protocol could be successfully applied for conservation and large-scale production of *S. chinensis* in order to supply the daily-growing demand of pharmaceutical industry. Strategies to develop the use of established micropropagation system for this plant as renewable source of raw material for industrial utilization should be focus of the future studies.

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