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Rapid Mass Propagation of *Salacia Chinensis* L., an Endangered Valuable Medicinal Plant through Direct Organogenesis

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Abstract

Background/Objectives: *Salacia chinensis* L., a high valuable anti-diabetic medicinal plant of Hippocrateaceae family, possess various medicinal properties. Its anti-inflammatory, hepatoprotective and anticancer activities originated from huge number of isolated phytochemicals such as mangiferin and salacinol. Excessive and indiscriminate collection for supplement of global demands has caused classified this plant as endangered species. Establishment of an effective regeneration system for *S. chinensis* via direct organogenesis as a most reliable method was the main goal of this study. **Methods:** The explants include leaf, node and shoot tips were inoculated on Murashige and Skoog (MS) medium fortified with different concentrations and combinations of Plant Growth Regulators (PGRs) including 6-Benzylaminopurine (BAP), Kinetin (Kin), 1-Naphthalene Acetic Acid (NAA) and Indole-3-Acetic Acid (IAA) for direct regeneration by shoot formation. **Findings:** The most efficient shoot regeneration in terms of the proliferation percentage (87.81 ± 3.22%), number (5.37 ± 0.02) and length of shoots (3.22 ± 0.04 cm) was obtained on MS basal medium supplemented by BAP (1.0 mg/l) and NAA (0.5 mg/l). The highest frequency in vitro root formation (91.33 ± 2.02%) with maximum number of 4.35 ± 0.03 roots with a length of 2.47 ± 0.03 cm was successfully achieved in half strength MS medium supplemented with 2.0 mg/l IBA. **Application:** The established system has this potential to be successfully employed in order to conservation and mass-propagation of *S. chinensis* for commercial utilization.

Keywords: Antidiabetic, Auxin, Conservation, Cytokinin, Nodal Explants, Shoot Induction

1. Introduction

Salacia chinensis L. (Syn. *S. prinoides DC.*) family of *Hippocrateaceae* commonly known as Saptarangi in Hindi is a woody climbing shrub, widely distributed in tropical and subtropical areas of the world, especially in India, Sri Lanka, China and Southeast Asian countries such as Thailand, Indonesia and also in a torrid zone area such as Brazil[1-3]. The plant different parts have been extensively used to treat a variety of ailments[4,5]. Biologically active compounds such as salacinol[6,7], kotalanol, neokotalanol, neosalacinol, salaprinol[3,8], mangiferin[9], Phenolic glycosides[10,11] and triterpenes[12,13] have been isolated from the plant. Different organs of the plant have shown number of activities like anti-diabetic[14-16], anti-inflammatory[17], antitumor[18,19], cardiac disorders[20], antioxidant[16], tonic[9], blood purifier[21] and recently anticancer property of this plant had been identified[22].

Due to the lack of proper cultivation practices, destruction of plant habitats, excessive and indiscriminate collection for supplement of global demands, especially

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obvious anti-diabetic property many medicinal plant such as *S. chinensis* are severely threatened and categorized as endangered species[23-25].

Plant tissue culture technique as one of the great achievement of biotechnology offers many valuable applications especially in easy regeneration of hardly plant species propagated by traditional system or mass propagation of healthy and pathogen-free plants[26,27]. Establishment of an effective regeneration system for conservation and mass propagation of *S. chinensis* was the main goal of the present study. Reproducible system for plant regeneration via direct organogenesis as the most reliable method for true-to-type and rapid micropropagation[28] was developed in *S. chinensis*. The impact of different Plant Growth Regulators (PGRs), various explant type in regeneration capacity was investigated properly.

2. Materials and Methods

2.1 Explant Source and Surface Sterilization

Young and healthy leaves shoot tips and nodal segments as explant sources were collected from three-year-old *S. chinensis* plants maintained in the Botanical Garden of DOS in Biotechnology, University of Mysore, Mysore. The undamaged collected explants were washed twice in running tap water and were then pre-washed with concentrated dishwasher gel (3-4 drops/100 ml double-distilled water (ddH_2O) (Vim, India) for 5 min followed by rinsing five times in ddH_2O. Thereafter, the explants were submerged in 70% (v/v) ethanol for 2 min. Nodal segments and shoot tips were surface sterilized with 0.1% (w/v) of mercuric chloride ($HgCl_2$) (SD Fine-Chem. Ltd, India) for 5 min, while leaf explants were sterilized by 1.0% (w/v) of Sodium hypochlorite (NaOCI) (HiMedia, India) plus Tween 20 (2-3 drops/100 ml ddH_2O) for 15min separately[29]. The solutions were removed and surface sterilized explants were washed thoroughly with ddH_2O for four times under aseptic conditions and placed on a sterilized petridish covered with autoclaved filter paper to remove excess moisture.

2.2 Medium Composition and Culture Conditions

Surface sterilized explants were inoculated on Murashige and Skoog's medium (MS)[30] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (HiMedia, India). The pH of the medium was adjusted to 5.6-5.8 before autoclaving at 121°C at 1.06 kg cm^{-2} for 20 minutes. The explants were cut into suitably-sized pieces (1.0 cm^2 for leaf, 1.5 and 2.0 cm for nodal segment and shoot tips respectively) and inoculated on pre-sterilized medium in test tubes or suitable containers carefully. The petridishes and culture bottles were sealed by parafilm and culture tubes were plugged by autoclave sterilized non-absorbent cotton plugs. Cultures were kept at temperature of 25 ± 2°C, air humidity of 55% and a 16-h photoperiod with irradiance of 125 $\mu molm^{-2} s^{-1}$ supplied by cool-white florescent tubes (Philips, India).

2.3 Preparation of PGRs

The PGRs (auxins, cytokinins) stocks were prepared at a concentration of 1.0 mg/ml. The desired PGRs stock solutions were prepared by weighing 13 mg of desired PGR dissolving in 13 ml of ddH_2O with the help of respected solvents (6-Benzylaminopurine (BAP), Kinetin (Kin) and Indole-3-Acetic Acid (IAA) with 95% ethanol; Indole-3-Butyric Acid (IBA) and 1-Napthalene-Acetic Acid (NAA) with 1N NaOH). The stocks were poured in micro-centrifuge tubes each contain 2 ml and stored at suitable temperature (At 4°C for several months). IAA was stored in amber bottles in order to photodecomposition prevention.

2.4 Shoot Induction and Multiplication

2.4.1 Effect of Cytokinins

The explants include leaf, node and shoot tips were inoculated on MS basal medium fortified with different concentrations (0.5- 3.0 mg /l) of BAP or Kin individually for direct regeneration by shoot formation. The cultures on culture bottles containing 40 ml medium with 4 explants (or Petri dishes containing 20 ml medium with 6 explants, or culture tubes containing 15 ml medium with 1.0 explant) were set in tissue culture chamber. The cultures were then sub-cultured in the same medium for more 4 weeks to determination of suitable cytokinin type and its optimum concentration. Data on shoot proliferation percentage shoots number per explant and shoot length were recorded after two months of inoculation.

2.4.2 Effect of Auxins in Combination with Selected Cytokinin

According to the results obtained from last step, well-suited types of explants were inoculated on MS medium supplemented with appropriate cytokinin in combination with (0.1- 2.0 mg/l) IAA or NAA. The clumps with

adventitious buds were then divided into smaller clumps and sub-cultured every four weeks on the same media. Data on shoot initiation percentage, number of shoots induced per explant and the mean length of shoot were assessed.

2.5 Rooting of Regenerated Shoots

Healthy elongated shoots (3-5 cm in length) were excised and transferred to full and half-strength MS basal medium supplemented with NAA, IAA, IBA (0.5- 2.5 mg/l) for induction of root. Data on the rooting percentage, the mean number of roots per shoot and root length were recorded after one months of culture.

2.6 Acclimatization

The complete rooted plantlets with 3–5 leaves were removed from the media and the agar on the roots was washed gently and removed by rinsing in running tap water. The plantlets were transplanted into plastic pots with sterile compost in the ratio of 2:2:1 (v:v:v) organic fertilizer: sand: peat and covered by transparent plastic bags with the temperature from 18-28^{0}C and relative humidity 75% to 90%. The pots were irrigated with water every 3 days for 20 days and sprayed with fungicide (Bavistin 0.05%). The relative humidity was reduced gradually first by rolling up at the nighttime and keeping partly closed at daytime and then by full removing. 30 days later, the plantlets were transferred into the pots containing garden soil and kept in the green house for another 2 weeks for further growth and development and subsequently transferred to field.

2.7 Statisti cal Analysis

All the tissue culture experiments of initial 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).

3. Results and Discussions

3.1 Shoot Induction and Multiplication

3.1.1 Effect of Cytokinins

Efficiency of various type and concentrations of cytokinins on shoot proliferation from leaf, nodal and shoot tip explants are summarized in Tables 1—3**.** Explants cultured on MS medium without PGRs failed to produce shoots even after 4 weeks of inoculation, but the explants when cultured on MS medium supplemented with different cytokinins (BAP, Kin) at different concentrations (0.5-3.0 mg/l) showed variation in the proliferation percentage, number and length of shoots produced. Among the two cytokinins (BAP, Kin) individually tested, the best response was obtained in the presence of 1.0 mg/l BAP in all explant types. The maximum proliferation percentage (82.36 ± 0.78%), highest number of shoots per explant (4.97 ± 0.18) and maximum shoot length (2.93 ± 0.03 cm) was obtained from nodal explants following by leaf and shoot tip explants in the medium containing 1.0 mg/l of BAP (Table 1). Thus these two (nodal explants and 1.0 mg/l BAP) were selected to further investigation by combination with different type and concentration of auxins in next step. In the BAP concentrations higher than 1.0 mg/l, all the measured items were reduced (Table 1, Table 2 and Table 3). Reduction in the number of shoots propagated per explant at BAP concentration higher than the optimal level was demonstrated in several medicinal plants[31,32]. Explants cultured on MS medium with different concentrations of Kin showed lower induction of shoot proliferation. Of the two cytokinin (BA, Kin) tested, BAP was most effective for shoot initiation. Overcome the apical dominance, releasing lateral buds from dormancy and promotion of the shoot formation is the main reported effects of BAP[33]. The prominent effect of BAP on multiple shoot induction has been approved earlier in many medicinal plant including *Talinum triangulare*, *Mentha arvensis* L., *Portulaca oleracea* L. and *Ocimum basilicum* L.[34-37]. Rate of cell division might be increased in the axillary and terminal meristematic zone of explant tissues at the presence of BAP. Large number of shoots is produced because of faster pace division of cells in this zone.

3.1.2 Effect of Auxins in Combination with Selected Cytokinin

Although the utilization of BAP or Kin individually with MS medium can initiate the shoots, but addition of an auxin can improve the shoot induction capacity. This study reported the first successful *in vitro* direct organogenesis with above 87% shoot proliferation from nodal explants of *S. chinensis*. The interactions of the optimal concentration of BAP (1.0 mg/l) in combination with various concentrations (0.1-2.0 mg/l) of auxins (NAA

Table 1. Effect of cytokinins (BAP or Kin) on multiple shoot induction from nodal explants of *S. chinensis*

Growth regulators (mg/l)		Shoot Proliferation (%)	Number of shoots/ explant	Length of shoots (cm)
Control	0.00	0.00^{k}	0.00^{h}	0.00^{g}
BAP	Kin			
0.5	-	66.30 ± 0.63^{h}	2.13 ± 0.15^{ab}	1.17 ± 0.02^{b}
1.0	-	82.36 ± 0.78^{j}	4.97 ± 0.18^{g}	2.93 ± 0.03^{f}
1.5	-	74.02 ± 0.63^{i}	3.55 ± 0.20^{f}	2.33 ± 0.06^{e}
2.0	-	53.88 ± 0.58^{f}	2.66 ± 0.11^{cd}	2.41 ± 0.03^{e}
2.5	-	45.63 ± 0.57^{e}	2.44 ± 0.11^{bc}	2.33 ± 0.05^{e}
3.0	-	33.11 ± 0.42^{b}	1.72 ± 0.10^{a}	1.19 ± 0.02^{b}
	0.5	43.80 ± 0.57^{d}	1.69 ± 0.11^{a}	0.83 ± 0.03^{a}
	1.0	53.44 ± 0.48^{f}	2.13 ± 0.14^{ab}	1.25 ± 0.05^{b}
	1.5	62.50 ± 0.52^{g}	2.88 ± 0.16^{cde}	1.25 ± 0.03^{b}
	2.0	66.47 ± 0.65^{h}	3.22 ± 0.18^{ef}	1.75 ± 0.04^{d}
	2.5	35.80 ± 1.13^{c}	2.97 ± 0.18^{de}	1.61 ± 0.02^{c}
	3.0	24.36 ± 0.54^{a}	2.47 ± 0.16^{bc}	1.22 ± 0.03^{b}

Mean ± S.E. of 12 replicates (in triplicate) per treatment. Means followed by same does not differ significantly when compared by Duncan Multiple Range Test (p = 0.05).

Table 2. Effect of cytokinins (BAP or Kin) on multiple shoot induction from leaf explants of *S. chinensis*

Growth regulators (mg/l)		Shoot Proliferation (%)	Number of shoots/ explant	Length of shoots (cm)
Control	0.00	0.00^{j}	0.00^{h}	0.00
BAP	Kin			
0.5	-	24.30 ± 0.22^{ef}	1.19 ± 0.03^{a}	0.71 ± 0.01^{b}
1.0	-	43.20 ± 0.13^{i}	2.74 ± 0.08^{g}	1.62 ± 0.02^{ef}
1.5	-	32.26 ± 0.22^{h}	2.39 ± 0.07^{f}	1.55 ± 0.04^{e}
2.0	-	29.34 ± 1.00^{gh}	1.81 ± 0.04^{d}	1.54 ± 0.04^{e}
2.5	-	21.26 ± 1.70^{de}	1.84 ± 0.03^{d}	0.91 ± 0.04^{c}
3.0	-	14.52 ± 1.63^{bc}	1.15 ± 0.04^{a}	0.52 ± 0.01^{a}
	0.5	17.58 ± 3.01^{cd}	1.14 ± 0.01^{a}	0.59 ± 0.03^{a}
	1.0	31.74 ± 2.71^{gh}	1.51 ± 0.01^{c}	1.14 ± 0.05^{d}
	1.5	27.44 ± 0.45^{fg}	1.36 ± 0.01^{b}	1.20 ± 0.02^{d}
	2.0	18.84 ± 2.08^{cd}	2.20 ± 0.01^{e}	1.66 ± 0.03^{f}
	2.5	11.29 ± 0.85^{ab}	1.52 ± 0.02^{c}	1.17 ± 0.04^{d}
	3.0	7.66 ± 0.47^{a}	1.16 ± 0.02^{a}	0.71 ± 0.01^{b}

Mean ± S.E. of 12 replicates (in triplicate) per treatment. Means followed by same does not differ significantly when compared by Duncan Multiple Range Test (p = 0.05).

Table 3. Effect of cytokinins (BAP or Kin) on multiple shoot induction from shoot tip explants of *S. chinensis*

Growth regulators (mg/l)		Shoot Proliferation (%)	Number of shoots/ explant	Length of shoots (cm)
Control	0.00	0.00^{f}	0.00^{f}	0.00^{h}
BAP	Kin			
0.5	-	32.06 ± 2.44^{de}	1.15 ± 0.04^{a}	0.91 ± 0.03^{c}
1.0	-	37.26 ± 4.64^{e}	1.96 ± 0.08^{e}	1.48 ± 0.03^{g}
1.5	-	23.30 ± 3.33^{cd}	1.34 ± 0.03^{b}	1.23 ± 0.04^{de}
2.0	-	23.19 ± 1.32^{cd}	1.32 ± 0.02^{b}	1.19 ± 0.01^{d}
2.5	-	17.77 ± 2.42^{bc}	1.15 ± 0.04^{a}	0.84 ± 0.01^{bc}
3.0	-	9.68 ± 1.57^{ab}	1.07 ± 0.02^{a}	0.70 ± 0.03^{a}
	0.5	17.93 ± 5.25^{bc}	1.16 ± 0.00^{a}	0.67 ± 0.02^{a}
	1.0	22.93 ± 6.95^{cd}	1.35 ± 0.02^{b}	1.32 ± 0.02^{ef}
	1.5	21.17 ± 0.86^{cd}	1.49 ± 0.01^{c}	1.37 ± 0.03^{fg}
	2.0	23.57 ± 0.89^{cd}	1.76 ± 0.06^{d}	1.44 ± 0.02^{g}
	2.5	15.12 ± 4.51^{bc}	1.43 ± 0.04^{bc}	1.19 ± 0.04^{d}
	3.0	3.95 ± 0.78^{a}	1.13 ± 0.02^{a}	0.76 ± 0.09^{ab}

Mean ± S.E. of 12 replicates (in triplicate) per treatment. Means followed by same does not differ significantly when compared by Duncan Multiple Range Test (p = 0.05).

and IAA) on nodal explants summarized in Table 4. The supplementation of NAA resulted in a higher induction rate than IAA. Higher efficiency in shoot regeneration in terms of the shoot proliferation percentage, number and length of shoots (87.81 ± 3.22%, 5.37 ± 0.02, 3.22 ± 0.04 cm respectively) was obtained with the combination of BAP (1.0 mg/l) with NAA (0.5 mg/l) (Table 4, Figures 1a-1b). The results indicate that BAP has higher potential to promote micropropagation in *S. chinensis* than Kin and supplementation of NAA was more advantageous than IAA. Similar results were reported with *Gymnema sylvestre* and *Ocimum gratissimum*[38,39]. A gradual decrease in propagation frequency and the number of shoots regenerated from each explant was observed upon increasing the concentration of NAA and IAA up to 2.0 mg/l. Among the various concentration of IAA, 0.5 mg/l in combination with 1.0 mg/l BAP, resulted in highest shoot regeneration frequency (73.89 ± 0.70%), number of shoots per explants (3.24 ± 0.05) along with the maximum shoot length (1.85 ± 0.01 cm) (Table 4). Enhancement the concentration of auxins in combination with higher concentration of cytokinins led to callus initiation at cut end of nodal explants may due to auxins accumulation which promote the cell division in the presence of

cytokinins[40]. The same findings were demonstrated in medicinal plants such as *Holostemma annulare*[41]and *Tylophora indica*[42]. These differential responses revealed that the type and concentration of PGRs and explant sources are the critical factors influencing organogenesis and different species respond differently to PGRs, in terms of combination and concentration. This is a demonstrated fact that the required amount of exogenous PGRs mainly depends on the endogenous level of the plant tissue which is vary with organ, genotype, and the phase of the plant growth[43].

Table 4. Effect of auxins (NAA or IAA) with the optimal concentration of BAP on shoot regeneration from nodal explants of *S. chinensis*

Growth regulators (mg/l)			Shoot Proliferation (%)	Number of shoots/ explant	Length of shoots (cm)
BAP	NAA	IAA			
1.0	0.1	-	79.04 ± 3.74^{ef}	4.23 ± 0.06^{g}	2.61 ± 0.01^{g}
1.0	0.5	-	87.81 ± 3.22^{f}	5.37 ± 0.02^{h}	3.22 ± 0.04^{h}
1.0	1.0	-	76.62 ± 0.85^{de}	3.75 ± 0.04^{f}	2.13 ± 0.05^{f}
1.0	1.5	-	68.86 ± 6.26^{bcde}	3.37 ± 0.01^{e}	1.77 ± 0.02^{cd}
1.0	2.0	-	63.14 ± 1.94^{bc}	2.46 ± 0.03^{c}	1.71 ± 0.03^{bc}
1.0	-	0.1	67.12 ± 2.70^{bcd}	3.43 ± 0.02^{e}	1.65 ± 0.02^{b}
1.0	-	0.5	73.89 ± 0.70^{cde}	3.24 ± 0.05^{d}	1.85 ± 0.01^{de}
1.0	-	1.0	66.44 ± 2.45^{bcd}	2.44 ± 0.01^{c}	1.94 ± 0.05^{e}
1.0	-	1.5	59.88 ± 1.06^{ab}	2.17 ± 0.07^{b}	1.81 ± 0.01^{cd}
1.0	-	2.0	51.96 ± 5.48^{a}	1.97 ± 0.02^{a}	1.27 ± 0.03^{a}

Mean ± S.E. of 12 replicates (in triplicate) per treatment. Means followed by same does not differ significantly when compared by Duncan Multiple Range Test (p = 0.05).

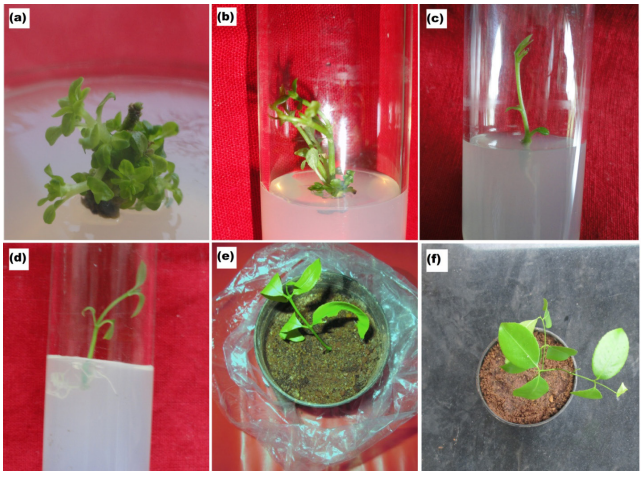

Figure 1. Micropropagation of *S. chinensis* L. **(a-b)** Direct shoot induction and proliferation from nodal explants (MS + 1.0 mg/l BAP + 0.5 mg/l NAA), **(c-d)** Rooting of a regenerated shoot (½MS + 2.0 mg/l IBA), **(e)** Hardened rooted plantlets and **(f)** Acclimatized plants.

3.2 Rooting of Regenerated Shoots

No rooting was observed in shoots who were cultured in the absence of PGRs in (Control) MS basal medium. Very poor response in rooting was observed on Full strength MS medium containing auxins, but half strength MS medium supplemented with NAA, IBA & IAA was most effective to achieve well developed roots. The same results by higher rooting frequency on low strength MS medium were reported in many species such as *Lavandula vera*[44] and *Crataeva nurvala*[45]. The main reason behind the favorable effect of reduced macronutrient concentration can be that the root formation requires lower nitrogen ions than shoot initiation and growth[46]. Compare to other two types of tested auxins (IAA and NAA) IBA was found to be more effective for rooting in both half and full strength MS medium. The higher stability of IBA to chemical degradation in tissue culture media, during autoclaving and at room temperature than IAA and NAA[47] can be a compelling reason. Similar findings were reported in other species[48,49]. The highest frequency *in vitro* root formation (91.33 ± 2.02%) was successfully achieved in half strength MS medium supplemented with 2.0 mg/l IBA, although in all treatments the 2.0 mg/l concentration gave the better results than other concentrations regardless of the kind of Ms medium or auxin type (Table 5). The maximum number of 4.35 ± 0.03 roots with a length of 2.47 ± 0.03 cm were achieved from the same medium (Table 5, Figures 1c-d). This results show vitality of auxins optimal concentration for better rooting response. Early dedifferentiation of xylem with subsequent root development could be affected noticeably by optimum concentration of IBA. Stimulation of the individual quiescent cells in the pericycle to differentiate and proliferate to form roots primodium could be affected by this PGR as well[50,51].

Further enhancement in the auxin concentration to 2.5 mg/l reduced the rooting percentage and length of roots. Addition of exogenous auxin to the shoots that contain high level of endogenous auxins can caused the inhibition of root development[52]. Induction of higher level of degradative metabolites in tissue and subsequent blocking of the regeneration process[53] or ethylene production, a root growth inhibitor[54] might be the other reason behind root length inhibition. It has been reported

that use of semi solid medium and addition of auxins can promote root development in medicinal plants[55]. The IBA stimulating and positive effect on root initiation was reported in many medicinal plant species such as *Cunila galioides*[56], *Adhatoda vasica*[57] and *Tylophora indica*[58].

Table 5. Effect of NAA, IBA and IAA in full and half-strength MS medium on root induction from regenerated shoots of *S. chinensis*

PGRs (mg/l)	Rooting (%)	No of roots/ shoot	Root length (cm)
MS basal medium (Control)	00.00^{a}	0.00^{a}	0.00^{a}
MS full strength + PGR			
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	00.00^{a}	0.00^{a}	0.00^{a}
2.0 NAA	17.66 ± 2.33^{b}	1.16 ± 0.01^{b}	0.65 ± 0.00^{b}
2.5 NAA	00.00^{a}	0.00^{a}	0.00^{a}
0.5 IAA	00.00^{a}	0.00^{a}	0.00^{a}
1.0 IAA	00.00^{a}	0.00^{a}	0.00^{a}
1.5 IAA	22.66 ± 1.20^{bc}	1.25 ± 0.08^{bc}	0.83 ± 0.04^{def}
2.0 IAA	35.66 ± 1.76^{de}	1.27 ± 0.02^{bc}	0.86 ± 0.03^{efg}
2.5 IAA	28.33 ± 4.91^{cd}	1.25 ± 0.03^{bc}	0.69 ± 0.02^{bc}
0.5 IBA	00.00^{a}	0.00^{a}	0.00^{a}
1.0 IBA	38.66 ± 4.63^{ef}	1.33 ± 0.01^{c}	0.74 ± 0.02^{bcd}
1.5 IBA	55.33 ± 2.02^{ijk}	1.33 ± 0.01^{c}	0.77 ± 0.02^{cde}
2.0 IBA	64.00 ± 5.19^{kl}	1.56 ± 0.02^{d}	0.95 ± 0.04^{g}
2.5 IBA	51.66 ± 5.78^{hij}	1.47 ± 0.03^{d}	0.92 ± 0.02f^{g}
MS half- strength + PGR			
0.5 NAA	27.33 ± 1.45^{cd}	1.94 ± 0.05^{e}	0.74 ± 0.00^{bcd}
1.0 NAA	35.33 ± 1.45^{de}	2.25 ± 0.04^{f}	0.82 ± 0.02^{def}
1.5 NAA	41.33 ± 2.60^{efg}	2.63 ± 0.04^{g}	0.96 ± 0.01^{g}
2.0 NAA	49.33 ± 3.75^{ghi}	2.91 ± 0.02^{h}	1.17 ± 0.02^{h}
2.5 NAA	45.33 ± 0.88^{fgh}	2.82 ± 0.02^{h}	1.14 ± 0.02^{h}
0.5 IAA	48.66 ± 2.33^{ghi}	2.88 ± 0.03^{h}	1.15 ± 0.05^{h}
1.0 IAA	53.33 ± 7.79^{hij}	3.12 ± 0.01^{i}	1.27 ± 0.07^{i}
1.5 IAA	59.33 ± 1.45^{jkl}	3.28 ± 0.06^{j}	1.38 ± 0.03^{j}
2.0 IAA	68.66 ± 1.76^{lmn}	3.42 ± 0.04^{kl}	1.52 ± 0.06^{kl}
2.5 IAA	63.00 ± 4.61^{kl}	3.31 ± 0.03^{jk}	1.43 ± 0.03^{jk}
0.5 IBA	65.33 ± 2.02^{lm}	3.39 ± 0.04^{kl}	1.56 ± 0.07^{lm}
1.0 IBA	73.66 ± 2.33^{mno}	3.47 ± 0.05^{l}	1.62 ± 0.00^{m}
1.5 IBA	77.33 ± 3.75^{no}	3.95 ± 0.06^{n}	1.85 ± 0.03^{n}
2.0 IBA	91.33±2.02^{p}	4.35 ± 0.03^{o}	2.47 ± 0.03^{p}
2.5 IBA	81.33 ± 1.45^{o}	3.77 ± 0.06^{m}	2.23 ± 0.00^{o}

Mean ± S.E. of 12 replicates (in triplicate) per treatment. Means followed by same does not differ significantly when compared by Duncan Multiple Range Test (p = 0.05).

3.3 Acclimatization

The complete rooted plantlets were removed from the media, after washing the agar on the roots, were transplanted into plastic pots filled with organic fertilizer, sand and peat covered by transparent plastic bags. Hardening successfully done at the 18- 28^{0}C room temperature with 75% to 90% relative humidity. Transferring of the plantlets into the pots containing garden soil in the green house for another 2 weeks resulted into further growth and development, provided the suitable status to subsequent transferring to the field. Above 87% survivability in field condition was recorded and transferred plantlets showed normal morphological appearance (Figures 1e-f).

4. Conclusion

In conclusion, this study comprehensively investigated the effects of various kinds and concentration of PGRs and different plant sources on *S. chinensis* micropropagation. Nodal explants are the most suitable plant source than other materials for regeneration. BAP among the different cytokinins have the higher potential for both shoot and root induction and NAA for shoot and IBA for root initiation among the auxins. The present *in vitro* regeneration system that was developed via direct organogenesis has this potential to be used easily as an effective method for large-scale production for commercial utilization and conservation of this medicinally important plant.

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