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In Vitro Propagation Through Nodal Explants in *Helicteres isora* L., a Medicinally Important Plant

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ABSTRACT *Helicteres isora* is medicinally important plant effective against asthma, diabetes, hypolipidemia, HIV, besides a good source of diosgenin. Seed dormancy and low rate of natural fruit production make this plant a perfect candidate for developing an in vitro method useful for its clonal propagation and further biotechnological developments. This is the first report on in vitro production of this plant. Nodal explants obtained from aseptically germinated seedlings were cultured on MS medium (Murashige and Skoog 1962) fortified with indole-3-acetic acid (IAA) (0.57-22.83 μM), indole-3-butyric acid (IBA) (0.41-16.58 μM), 6-benzylaminopurine (BA) (0.44-17.75 μM) and kinetin (Kin) (0.46-13.94 μM) either singly or in combinations of IAA + BA, IAA + Kin and BA + Kin. Combinations of cytokinins (BA and Kin) were most suitable for multiple shoot induction and 13.94 μM Kin + 13.31 μM BA was optimum (79% frequency) associated with high number of microshoots (7.1 shoots per explant) after 20 days of culture. Maximum shoot elongation and proliferation (10 shoots per explant with 4.8 cm average height) was achieved on MS media containing 2.32 μM Kin + 2.22 μM BA + 2.85 μM IAA. High rooting frequency (70%) was achieved on MS medium (½ basal strength) fortified with 4.14 μM IBA, while activated charcoal showed inhibitory effects on rooting. Hardening was done with 76% survival rate and these plants were growing without any visual defects and morphologically mimicking the naturally growing plants.

Introduction

Helicteres isora L. (Sterculiaceae) commonly known as East Indian screw tree, is a medicinally important sub-deciduous shrub or small tree requires rich humus soil to grow. The plant is used in Ayurveda for curing various diseases like asthma, diarrhea, vulnerary haemostatic and against snake bite (Anonymous 1995). The plant possesses anti-HIV (Otake et al. 1995), antispasmodic (Pohocha and Grampurohit 2001), hypolipidemic, hypoglycemic and hepatoprotective properties (Chakrabarti et al. 2002, Venkatesh et al. 2003, Kumar et al. 2006) and showed inhibitory effects on reverse transcriptase of a RNA tumor virus-I and on avian myeloblastosis virus (Kusumoto et al. 1992). The plant is a good source of diosgenin, an antifertility agent (Barik et al. 1981) besides a good source of fiber (Joshy et al. 2006).

H. isora is exploited heavily from wild conditions by Ayurvedic practitioners and local tribes in India for its medicinal uses. The seed dormancy due its hard seed coat is a major constraint in its natural regeneration (Badave and Jadhav 1998) in addition to very low rate of natural fruit production due to the inadequacy of pollinators (Atluri et al. 2000). Therefore, an efficient and reproducible procedure for in vitro propagation may provide an effective means for its mass proliferation and ex situ germplasm conservation. Even though, *H. isora* is a medicinally and economically important plant

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species, till date no attempt for in vitro cultures in this plant has been taken. Considering the varied medicinal values of this plant, this study may also be useful for further biotechnological advances in this plant species and for optimum production of bioactive compounds in vitro. Therefore, this study describes a simple and reproducible method for micropropagation of *H. isora* using nodal explants.

Material and Methods

Plant Material

Seeds from mature pods from healthy plants grown in wild conditions in the Satara district in Western Ghats region (Maharashtra, India) were collected and used for this study.

Seed Sterilization, Germination and Establishment of Stock Cultures

The seeds were washed in running tap water and then treated with concentrated sulfuric acid for 1 min to break the seed coat and to overcome seed dormancy. Seeds were washed with 1.0% Tween-20 for 5 min followed by 3 rinses with sterile distilled water (SDW) and then surface disinfected with 0.1% (w/v) mercuric chloride for 10 min and finally rinsed 5 times with SDW. Seeds were germinated on half basal (major and minor) strength MS media (containing full strength of vitamins) designated as ½ MS media, supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India) at pH 5.8. Initially, seed cultures were maintained at 25 ± 2°C in dark for 4 days for germination and then shifted to 16 h day^{-1} photoperiod, with 25μmol $m^{-2}s^{-1}$ light intensity and 80-85% relative humidity. After 3 weeks, nodes excised from young shoots of 8-9 cm (seedlings) were used as the source for shoot induction and micropropagation.

Culture Conditions

Nodal explants were inoculated on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar and fortified with various auxins such as IAA (0.57 μM to 22.83 μM), IBA (0.41 μM to 16.58 μM) and cytokinins like BA (0.44 μM to 17.75 μM) and Kin (0.46 μM to 13.94 μM) either alone or in combinations of IAA plus BA, IAA plus Kin and BA plus Kin. The cultures were maintained for 20 days on a 16 h day^{-1} photoperiod, with 25 μmol $m^{-2}s^{-1}$ light intensity and 80-85% relative humidity. These culture conditions were used in all the experiments mentioned below unless otherwise stated. After 20 days of inoculation, the multiple shoot buds obtained were transferred to shoot elongation media (SEM) comprising MS basal medium supplemented with either Kin (0.46 to 4.64 μM) alone or in combination of Kin and BA (0.44 to 4.44 μM) and Kin plus BA plus IAA (1.14 to 5.71 μM). Four media compositions (SEM 1 to 4) were carried forward for shoot elongation (Table 3).

Rooting of Shoots in Vitro

Elongated shoots (4 to 5 cm) were excised and cultured on root induction media (RIM) comprising full and/or ½ MS medium supplemented with auxins, IAA (5.71 and 11.41 μM) and IBA (4.14 and 8.28 μM) along with or without activated charcoal (AC, 0.5%). The cultures showing root induction were transferred to fresh media after three weeks; basal calluses were removed prior to subculturing. Observations were recorded after 35 days of inoculation in RIM.

Hardening of Regenerated Plants

Rooted plantlets were removed from RIM and washed in SDW to remove the agar and callus and then transferred to plastic pots containing garden soil mixed with vermiculite and sand (1:1:1). The plastic pots were temporarily covered with transparent plastic covers to maintain high humidity and were maintained at 25 ± 2°C with light intensity of 25 μmol m^{-2} s^{-1} on 16 h day^{-1} photoperiod for two weeks in the culture room and the pots were then transferred to shade (60 μmol m^{-2} s^{-1}) in the third week. The exposure period of plants to full sunlight was gradually increased (4 h in 4th, 6 h in 5th and 8 h in 6th week). After acclimatization, the plantlets were transferred to green house at the Department of Botany, University of Pune.

Statistical analysis

All the experiments were set up in a completely randomized design, and repeated three times with minimum ten explants per treatment before conducting the statistical analyses. The data was subjected to analysis of variance (ANOVA) to detect significant difference between means. Means differing significantly were compared using Duncan's multiple range test (DMRT) at $P \leq 0.05$. All the statistical analyses were done by using MSTATC statistical software package.

Results and Discussion

Influence of Plant Growth Regulators on Shoot Induction and Multiplication

Results clearly indicated that cytokinins were found suitable for multiple shoot induction in *H. isora* (Table 1), while both the auxins used singly failed to induce multiple shoots. IAA could induce only single shoot up to 2.85 μM while no shoot was observed beyond this concentration (data not shown). Similar results were obtained on IBA and only single shoot was observed associated with root induction on all the concentrations from 0.41 to 4.14 μM.

At lower concentration of BA (2.22 μM and 4.44 μM), multiple shoot induction was observed. Maximum numbers of shoots (3.8 per explant) were observed at 4.44 μM BA, indicating this as optimum concentration of BA for multiple shoot induction. Beyond this concentration, no shoot induction took place (data not shown). Kin could induce multiple shoots at 4.64 and 6.97 μM (2.13 and 4.43 shoots per explant respectively) indicating that the later was optimum concentration of Kin for multiple shoot induction. At lower concentration (0.46 μM to 2.32 μM) of Kin, however, single shoot was induced with vigorous growth. Amongst the various combinations of auxin and cytokinins used, BA plus Kin were best suited for multiple shoot induction (Table 2). IAA and BA in combination favored multiple shoot induction (4.3 shoots per explant) at lower concentrations of BA and IAA (2.22 μM and 0.57 μM respectively). However, the shoot induction frequency was low (44.7%). No sign of multiple shoot induction was observed on all the combinations of IAA and Kin used in this experiment, indicating this combination not suitable for multiple shoot induction, in spite, elongated shoot (5.1 cm height) were observed at 2.85 μM IAA + 4.64 μM Kin (data not shown). The best combination for multiple shoot induction was

Table 1. Effect of various PGRs on nodal explants of *H. isora* singly after 20 days of culture

Plant Growth Regulators (μM)		Cultures producing shoots (%)	Number of shoots per explant	Height of shoots (cm)
Control	0.00	55.5 ± 2.5^{c}	0.5 ± 0.02^{a}	0.37 ± 0.02^{a}
IAA	0.57	45.5 ± 1.8^{b}	1^{b}	3.0 ± 0.15^{c}
	2.85	25.9 ± 1.3^{a}	1^{b}	2.3 ± 0.06^{b}
IBA	0.41	48.8 ± 1.3^{b}	1^{b}	1.5 ± 0.03^{b}
	2.07	40.2 ± 1.9^{b}	1^{b}	1.6 ± 0.03^{b}
	4.14	22.0 ± 1.1^{a}	1^{b}	1.7 ± 0.02^{b}
BA	0.44	52.9 ± 2.4^{c}	1.3 ± 0.1^{b}	0.51 ± 0.06^{a}
	2.22	60.2 ± 2.1^{cd}	2.3 ± 0.3^{c}	0.87 ± 0.05^{a}
	4.44	65.5 ± 1.3^{d}	3.8 ± 0.3^{d}	1.07 ± 0.20^{ab}
Kin	0.46	49.0 ± 1.9^{bc}	1.16 ± 0.1^{b}	1.1 ± 0.10^{a}
	2.32	53.9 ± 2.0^{c}	1.63 ± 0.1^{bc}	1.6 ± 0.15^{ab}
	4.64	57.7 ± 2.1^{c}	2.13 ± 0.2^{c}	3.2 ± 0.40^{c}
	6.97	60.9 ± 2.5^{cd}	4.43 ± 0.2^{e}	0.7 ± 0.50^{a}
	9.29	50.4 ± 1.8^{c}	1.20 ± 0.1^{b}	0.6 ± 0.05^{a}

Shown are treatments that gave significant results, all others were omitted. Results are means of three replicates (10 X 3) S.D. Means within columns followed by different letters are significantly different from each other at P = 0.05 according to Duncan's Multiple Range Test.

Table 2 Effect of various PGRs in combination on nodal explants of H. isora after 20 days of culture

Plant Growth Regulators (μM)			Cultures producing shoots (%)	Number of shoots per explant	Height of shoots (cm)
IAA	BA	Kin			
0.57	0.44	-	25.2 ± 1.5[a]	0.9 ± 0.04[b]	0.7 ± 0.04[a]
0.57	2.22	-	44.7 ± 0.9[c]	4.3 ± 0.15[f]	1.2 ± 0.05[b]
0.57	4.44	-	35.0 ± 1.0[b]	1.2 ± 0.15[b]	0.9 ± 0.02[ab]
0.57	8.88	-	31.2 ± 1.4[b]	1.0 ± 0.20[b]	0.7 ± 0.01[a]
2.85	0.44	-	20.7 ± 0.5[a]	0.6 ± 0.05[a]	0.5 ± 0.01[a]
2.85	2.22	-	32.3 ± 1.9[b]	1.9 ± 0.18[c]	0.6 ± 0.05[a]
2.85	4.44	-	41.2 ± 1.8[c]	3.1 ± 0.20[d]	0.6 ± 0.02[a]
2.85	8.88	-	26.1 ± 1.0[a]	0.7 ± 0.05[a]	0.4 ± 0.01[a]
-	4.44	0.46	51.5 ± 1.2[d]	1.2 ± 0.15[b]	0.8 ± 0.03[a]
-	8.88	0.46	61.5 ± 2.1[e]	2.6 ± 0.15[cd]	0.7 ± 0.03[a]
-	13.32	0.46	65.5 ± 2.5[e]	3.9 ± 0.03[ef]	0.5 ± 0.03[a]
-	4.44	2.32	54.5 ± 1.5[d]	2.1 ± 0.10[c]	0.6 ± 0.07[a]
-	8.88	2.32	59.8 ± 2.3[de]	2.3 ± 0.02[c]	0.8 ± 0.02[a]
-	13.32	2.32	45.5 ± 1.5[c]	1.4 ± 0.01[b]	0.4 ± 0.02[a]
-	4.44	4.64	59.5 ± 2.3[de]	2.8 ± 0.03[cd]	0.7 ± 0.03[a]
-	8.88	4.64	70.9 ± 2.1[f]	4.9 ± 0.04[f]	2.4 ± 0.02[c]
-	13.32	4.64	61.5 ± 1.8[e]	2.6 ± 0.02[cd]	0.6 ± 0.03[a]
-	4.44	9.29	55.2 ± 1.5[d]	1.9 ± 0.01[c]	0.5 ± 0.02[a]
-	8.88	9.29	59.0 ± 2.3[de]	2.2 ± 0.05[c]	1.3 ± 0.02[b]
-	13.32	9.29	60.5 ± 1.5[e]	2.0 ± 0.10[c]	0.6 ± 0.02[a]
-	4.44	13.94	63.7 ± 1.5[e]	2.1 ± 0.05[c]	4.2 ± 0.10[e]
-	8.88	13.94	74.8 ± 2.0[f]	4.2 ± 0.01[f]	3.1 ± 0.20[d]
-	13.32	13.94	79.0 ± 1.1[f]	7.1 ± 0.20[g]	0.6 ± 0.05[a]

Shown are treatments that gave significant results, all others were omitted. Results are means of three replicates (10 X 3) ± S.D. Means within columns followed by different letters are significantly different from each other at $P \leq 0.05$ according to Duncan's Multiple Range Test.

BA and Kin, and optimum shoot induction was achieved with 79% frequency and 7.1 shoots per explant at 13.94 μM Kin + 13.32 μM BA (Table 2, Figure 1a). Similar results have been reported earlier in *Acacia catechu* (Kaur and Kant 2000), in *Citrus aurantifolia* (Al-Khayri and Al-Bahrany 2001) and in *Piper longum* (Soniya and Das 2002) and found MS media supplemented with BA and Kin in combination suitable for multiple shoot induction. Results of the present investigation clearly suggested that the combination of Kin and BA favored multiple shoot induction; however, these shoots were stunted when subcultured on the same media, which demanded two separate media for multiple shoot induction and their elongation. Therefore, the shoots obtained on optimum media (13.94 μM Kin + 13.32 μM BA) for multiple shoots induction were transferred on 4 different SEM media (Table 3).

Influence of plant growth regulators on shoot elongation

When multiple shoots were transferred on all the combinations of SEMs (data not shown), four media referred as SEM 1 to 4 showed better results than other SEMs and SIMs and hence were carry forward for shoot elongation (Table 3). When the shoots obtained on 13.94 μM Kin plus 13.32 μM BA were transferred on SEM-4, 10 shoots per explant were observed with 4.8 cm height, indicating SEM-4 as optimum for elongation as well as proliferation of induced shoots (Table 3,

Table 3. Effect of different PGR combinations on multiple shoot elongation of *H. isora* after 20 days of culture

Shoot induction media (SIM)		Multiple shoots subcultured on shoot elongation media (SEMs)							
		Number of shoots				Height of shoots (cm)			
Kin (μM)	BA (μM)	SEM 1	SEM 2	SEM 3	SEM 4	SEM 1	SEM 2	SEM 3	SEM 4
0.46	8.88	1.0 ± 0.4^{a}	2.0 ± 0.4^{a}	2.2 ± 0.3^{a}	2.0 ± 0.6^{a}	1.2 ± 0.2^{a}	1.0 ± 0.5^{b}	1.7 ± 0.9^{a}	4.5 ± 0.5^{c}
0.46	13.32	1.0 ± 0.2^{a}	3.0 ± 0.5^{b}	2.0 ± 0.4^{a}	3.2 ± 0.3^{b}	2.1 ± 0.4^{b}	0.6 ± 0.8^{a}	3.8 ± 0.1^{b}	5.7 ± 0.8^{d}
4.64	4.44	1.0 ± 0.2^{a}	2.0 ± 0.3^{a}	2.0 ± 0.2^{a}	2.2 ± 0.3^{a}	3.9 ± 0.5^{c}	1.4 ± 0.4^{b}	7.4 ± 0.3^{e}	2.6 ± 0.4^{a}
4.64	8.88	2.0 ± 0.5^{b}	3.0 ± 0.3^{b}	2.4 ± 0.3^{a}	4.5 ± 1.1^{c}	4.2 ± 0.7^{c}	2.0 ± 0.8^{c}	5.8 ± 0.5^{c}	4.5 ± 1.6^{c}
9.29	8.88	1.0 ± 0.3^{a}	2.0 ± 0.5^{a}	2.1 ± 0.2^{a}	4.0 ± 0.9^{c}	7.5 ± 0.2^{d}	1.5 ± 0.1^{b}	3.5 ± 1.6^{b}	3.3 ± 0.8^{b}
13.94	8.88	2.0 ± 0.5^{b}	2.4 ± 0.3^{a}	3.5 ± 0.7^{b}	6.1 ± 1.5^{d}	4.3 ± 0.3^{c}	3.7 ± 0.5^{d}	3.4 ± 0.7^{b}	5.2 ± 1.4^{d}
13.94	13.32	2.0 ± 0.6^{b}	3.4 ± 0.2^{b}	5.5 ± 0.4^{c}	10.0 ± 1.8^{e}	4.5 ± 0.4^{c}	1.2 ± 0.1^{b}	6.6 ± 4.7^{d}	4.8 ± 1.2^{d}

Results are means of three replicates (10 X 3) μ S.D. SEM-1: 2.32 μM Kin, SEM-2: 2.22 μM BA, SEM-3: 2.32 μM Kin + 2.22 μM BA, SEM-4: 2.32 μM Kin + 2.22 μM BA + 2.85 μM IAA. All the other SEMs, which did not show significant results, were omitted. Means within columns followed by different letters are significantly different from each other at $P \leq 0.05$ according to Duncan's Multiple Range Test.

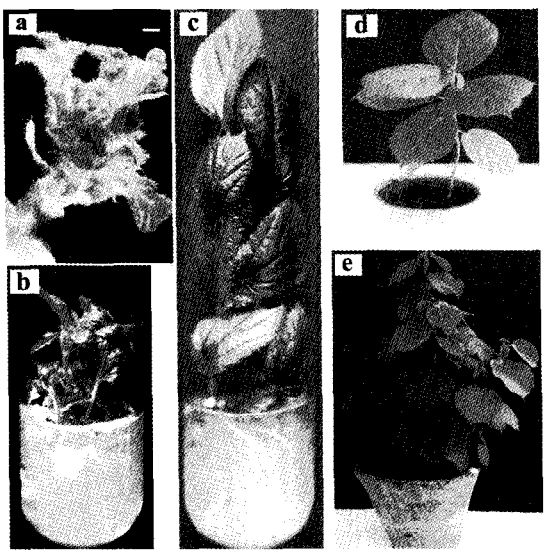

Figure 1 Micropropagation of *H. isora* using nodal explants derived from in vitro germinated seedlings, a) Microphotograph of microshoots on MS + 13.32 μM BA + 13.94 μM Kin after 10 days of culture, bar represents 1 mm b) Elongated shoots obtained on shoot elongation media (SEM)-4 after 20 days of culture, c) Rooting of elongated shoot on ½ MS + 4.14 μM IBA after 35 days of culture, d) Hardened plant in plastic pot, e) Micropropagated plants growing in the pot in garden.

Figure 1b). From these results it is clear that cytokinins at higher level favor multiple shoot induction while lower level of cytokinins results in to shoot proliferation and elongation. SEM-4 was followed by SEM-3 with 6 shoots per explant and 5.2 cm average height. Results clearly indicated that SEM-1 and SEM-2 were comparably less suited media for shoot elongation and proliferation. SEM-2 could neither elongated nor proliferated the shoots while SEM-1 did not improve number of proliferating shoots but height of the shoots increased considerably and the shoots obtained were healthy. (Martin et al. 2003) in *Wedelia chinensis* and (Meng-ling et al. 2005) in *Aquilaria agallocha* have reported similar results and suggested the need of separate media for multiple shoot induction and their elongation.

Rooting of Shoots in Vitro

Root induction was initiated after 15 days of transferring the shoots to RIM. Half MS medium fortified with 4.14 μM IBA was most effective for root induction and elongation (5.1 roots per shoot with 4.2 cm length), while IAA showed weak roots with basal callusing. Activated charcoal inhibited the root induction in both the media. About 70% cultures were observed with well-developed roots after 35 days of inoculation on ½ MS fortified with 4.14 μM IBA (Table 4, Figure 1c). Similar results have been reported in a number of plants and these reports emphasized on the use of low salt MS medium for in vitro rooting of shoots such as in *Philodendron* species (Maene and Debregh 1985), *Sterculia urens* (Purohit and Dave 1996), and *Gymnema sylvestre* (Komalavalli and Rao 2000). Results of the present investigation are on similar lines. IBA was found to be the most effective phytohormone for root induction

Table 4 Rooting of *H. isora* plantlets in vitro

Media used for rooting	Average no. of roots	Average length of roots (cm)	Average plants rooted (%)
½ MS + 5.71 μM IAA	2.9 ± 0.8^{d}	3.3 ± 0.6^{e}	43 ± 1.6^{d}
MS + 5.71 μM IAA	2.1 ± 0.9^{c}	2.9 ± 0.6^{cd}	39 ± 2.5^{cd}
½ MS + 5.71 μM IAA + AC	1.6 ± 0.8^{b}	1.8 ± 0.5^{b}	23 ± 3.2^{ab}
MS + 5.71 μM IAA + AC	1.0 ± 0.9^{a}	1.7 ± 0.7^{b}	25 ± 1.9^{ab}
½ MS + 11.41 μM IAA	2.1 ± 0.8^{c**}	2.3 ± 0.6^{c}	34 ± 2.6^{c}
MS + 11.41 μM IAA	1.5 ± 0.5^{ab**}	1.8 ± 0.6^{b}	28 ± 2.8^{ab}
½ MS + 11.41 μM IAA + AC	1.1 ± 0.3^{a**}	1.4 ± 0.8^{ab}	16 ± 1.2^{a}
MS + 11.41 μM IAA + AC	0.9 ± 0.2^{a**}	1.2 ± 0.3^{ab}	12 ± 1.8^{a}
½ MS + 4.14 μM IBA	5.1 ± 1.0^{f*}	4.2 ± 0.9^{fg}	70 ± 2.3^{f}
MS + 4.14 μM IBA	4.2 ± 0.9^{e*}	4.0 ± 0.8^{f}	40 ± 3.1^{d}
½ MS + 4.14 μM IBA + AC	2.8 ± 0.8^{cd*}	2.7 ± 0.5^{cd}	25 ± 2.8^{ab}
MS + 4.14 μM IBA + AC	2.4 ± 0.7^{c*}	1.9 ± 0.2^{b}	17 ± 1.7^{ab}
½ MS + 8.28 μM IBA	2.7 ± 0.5^{cd}	2.8 ± 0.6^{cd}	54 ± 3.1^{e}
MS + 8.28 μM IBA	2.1 ± 0.5^{c}	2.5 ± 0.5^{cd}	49 ± 3.4^{cde}
½ MS + 8.28 μM IBA + AC	1.8 ± 0.3^{bc}	1.9 ± 0.7^{b}	32 ± 1.9^{c}
MS + 8.28 μM IBA + AC	0.9 ± 0.4^{a}	0.6 ± 0.1^{a}	23 ± 2.5^{ab}

Results are mean of three replicates (10 X 3) S.D. AC- Activated charcoal, *Basal callusing, ** Excess basal callusing. Means within columns followed by different letters are significantly different from each other at P = 0.05 according to Duncan's Multiple Range Test.

by a number of researchers such as Soniya and Das (2002) in *Piper longum* and Makunga et al. (2003) in *Thapsia garganica*.

Hardening of Regenerated Plants

Out of 50 plantlets with well-developed roots, 38 were survived with a survival rate of 76% on transfer to plastic pots containing a mixture of garden soil, vermiculite and sand in the ratio of 1:1:1 (Figure 1d). The same composition of soil, sand and vermicompost was used for hardening by Komalvalli and Rao (2000). The regenerated plants did not show detectable variation in morphological or growth characteristics compared with the parent plant. These acclimatized plantlets were successfully transferred to green house conditions at the Botanical garden, University of Pune; with 100% survival rate (Figure 1e). This protocol can be useful for further tissue culture and biotechnological advances and optimized secondary metabolite production in vitro by keeping in view the pharmaceutical importance of this plant.

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