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ORIGINAL PAPER

Direct adventitious shoot induction and plant regeneration of *Embelia ribes* Burm F.

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Abstract An efficient micropropagation system via direct shoot organogenesis from hypocotyl segments of *Embelia ribes* Burm F. was developed. A high frequency (84%) of adventitious shoot induction was obtained on Murashige and Skoog (MS) medium supplemented with additives (283.85 μM ascorbic acid [AA], 118.96 μM citric acid [CA], 142.33 μM cysteine, and 684.22 μM glutamine) and 1.13 μM of thidiazuron (TDZ) after 4 weeks following culture. Further development of shoot primordia into well-grown shoots of 4–5 cm in length was achieved by sub-culturing explants along with shoot primordia on MS medium supplemented with 0.44 μM benzyl adenine (BA) and 0.49 μM indole butyric acid (IBA) for three sub-culture periods with an interval of 15 days between them. The highest shoot multiplication was obtained when explants were incubated on MS medium supplemented with 2.2 μM BA and 0.49 μM IBA in 4 weeks. All in vitro developed shoots, 3–4 cm in length, rooted when grown on half-strength MS basal medium along with 2.47 μM IBA within 4 weeks. Moreover, 100% of shoots developed roots when these were treated with 4.93 μM IBA for 20 min and then transferred to pots containing soilrite mix and grown in the greenhouse. In vitro and ex vitro rooted plants showed a survival of 85 and 95% respectively, during hardening in the greenhouse for a 6-week period.

Keywords *Embelia ribes* · Ex vitro · In vitro · Hypocotyl · Organogenesis · Vidanga

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Abbreviations

AA	Ascorbic acid
ANOVA	Analysis of variance
BA	Benzyl adenine
CA	Citric acid
GA_3	Gibberellic acid
HF	Hormone-free
IBA	Indolebutyric acid
Kn	6 furfuryl amino purine
NAA	α-naphthalene acetic acid
NOA	Naphthoxyacetic acid
MS	Murashige and Skoog medium
PGR	Plant growth regulator
TDZ	Thidiazuron (N-phenyl-N′-1, 2, 3-thidiazol-5-urea)

Introduction

Embelia ribes Burm F., a medicinal woody climber, belongs to the Myrsinaceae family. It is also commonly known as false black pepper or vidanga. This species is reported to be vulnerable in the Western Ghats of Tamil Nadu and Karnataka states of India and at a lower risk in Kerala state of peninsular India (Ravikumar and Ved 2000). *E. ribes* grows in semi-evergreen and deciduous forests at an altitude of 1,500 m, throughout India. It is considered to be vulnerable due to excessive harvesting, because of its many uses (it is used in 75 ayurvedic preparations). *E. ribes* is a highly valuable medicinal plant with anthelmintic, carminative, antibacterial, antibiotic, hypoglycemic, and antifertility properties (Mitra 1995; Anon 2002).

Natural regeneration of *E. ribes* is poor due to over-harvesting and exploitation, more fragmented populations

resulting in inbreeding, development of abortive embryos, and the slow germination of fertile seeds that are small in size (Anon 1990). On the other hand, artificial regeneration of this species is difficult due to its poor seed viability, low rate of germination, and poor rooting from stem cuttings (Ved et al. 2003). *E. ribes* is one of the 32 medicinal plant species identified by the Medicinal Board, Govt. of India, New Delhi, as being important for large-scale cultivation because of its commercial use (Anon 2008). Unfortunately, traditional methods of propagation are not successful in the large-scale production of this species.

Direct organogenesis using hypocotyl segments as explants has been reported in many other species (Kim et al. 1997; Rai 2002; Cui et al. 2004; Başalma et al. 2008; Makunga and van Staden 2008; Ghnaya et al. 2008). The type of explants used in direct organogenesis is highly important in establishing an efficient regeneration system (Babaoglu and Yorgancilar 2000; Koroch et al. 2002; Cui et al. 2004; Uranbey et al. 2005). Raghu et al. (2006) carried out preliminary studies on direct shoot induction from seedling-derived leaf explant in *E. ribes*. Attempts were made for the direct organogenesis of *E. ribes* by using another type of explant, i.e., hypocotyl for a rapid and efficient regeneration system.

Materials and methods

In vitro seed germination

Wild fruits of *E. ribes* (Fig. 1a, b) were collected from natural populations in Sringeri, Karnataka, during July 2007. After depulping the fruits, the seeds were dried under shade for 24 h. The seeds were decoated manually by cracking them with pestle, giving slight pressure, and these decoated seeds (Fig. 1c) were used in this study. Seeds were surface-sterilized with 70% (v/v) ethanol for 15 s, followed by 3–4 washes with distilled water. The seeds were further surface-sterilized with 0.075% (w/v) mercuric chloride for 3.5 min and then washed with sterile distilled water 5–6 times. These sterilized seeds were pretreated by soaking them in filter-sterilized 1.45 μM GA_3 solution for 4 h and then inoculated on MS (Murashige and Skoog 1962) medium containing 5.78 μM GA_3 in test tubes. The tubes were maintained at 25 ± 2°C, for 10 days in the dark and transferred to light for a 12-h photoperiod with a light intensity of 27 μE m^{-2} s^{-1} in a culture room for another 30 days.

Culture medium and culture conditions

Murashige and Skoog (1962) medium supplemented with sucrose (3%), vitamins (nicotinic acid, pyridoxine HCl, and thiamine-HCl, 0.5 mg/l each), and 2 mg/l glycine were used in all experiments related to shoot induction, elongation, and multiplication. In order to minimize the problem of leaching of secondary metabolites, thereby browning of the medium, and to improve shoot growth, additives like 283.85 μM ascorbic acid (AA), 118.96 μM citric acid (CA), 142.33 μM cysteine, and 684.22 μM glutamine were also added to MS medium. For in vitro rooting experiments, half (MS/2) and quarter (MS/4) strength macro and micro nutrient salts of MS media were used. Agar (Hi-Media, India) was added to the MS media at 0.6% (w/v). The pH of the medium along with additives was adjusted to 6.4 before autoclaving. The cultures were maintained at 25 ± 2°C under a 12-h photoperiod with a light intensity of 27 μE m^{-2} s^{-1} (Philips India, Mumbai, India).

Adventitious shoot induction

Hypocotyl segments (1.0–1.5 cm in length) were used as an explant in these studies. Explants were inoculated on MS medium supplemented with different concentrations of benzyl adenine (BA; 4.44, 11.1, and 22.2 μM), 6 furfuryl amino purine (Kn; 11.63 and 23.25 μM), and thidiazuron (TDZ; 0.45, 1.13, and 2.27 μM), and with and without α-naphthalene acetic acid (NAA; 1.34 μM), or pulse treatment in 15 different combinations (Table 1). Hypocotyl segments were inoculated in both horizontal and vertical positions.

Shoot elongation

For shoot elongation, shoot primordia produced were sub-cultured on MS medium with various concentrations of GA_3 (2.89 and 5.78 μM) and BA (0.44 and 1.11 μM), either alone or in combination with indolebutyric acid (IBA; 0.49 μM) and hormone-free (HF) medium in eight different combinations for three sub-cultures, with each passage of 15-day intervals (Table 2).

Multiplication of shoots

Shoots produced in vitro were sub-cultured on MS medium with additives and 0.49 μM IBA as described above, and further supplemented with different cytokinins such as BA (2.22–22.2 μM), Kn (4.65–23.25 μM), and TDZ (0.23–1.13 μM), either alone or in combination, giving rise to 14 different treatments for shoot multiplication and elongation (Table 3). In order to maintain vigorous growth, shoot cultures were maintained for 2 years by further sub-culturing every 4 weeks on fresh medium.

Fig. 1 **a–i** Various progressive stages of in vitro propagation through direct adventitious shoot induction from hypocotyl segments of *Embelia ribes* Burm F. **a** Branch of *E. ribes* with fruits. **b** Fruits of *E. ribes*. **c** Seeds with and without seed coating. **d** Shoot induction after 4 weeks. **e** Shoot elongation after 8 weeks following culture initiation. **f** Shoot elongation after 10 weeks following culture initiation. **g** Shoot multiplication. **h** In vitro rooted plantlets. **i** Ex vitro rooted plantlets. **j** Hardened plants after 3 months raised in 270-cc block-type root trainers

Rooting of shoots

After five sub-cultures, with an interval of 15 days between each, on shoot multiplication media, in vitro-produced shoots were used further for in vitro and ex vitro rooting. In order to induce high frequency of in vitro rooting, shoots of 3–4 cm in length were used to assess the effects caused by: (1) different auxins like IAA, IBA, NAA, and NOA (5.71,

Table 1 Effect of plant growth regulators (PGRs) on direct adventitious shoot induction from hypocotyls of *Embelia ribes* on Murashige and Skoog (MS) medium with additives

PGRs (μM)				% response (after 4 weeks from inoculation)	Mean no. of shoots/explant (after 10 weeks from inoculation)
TDZ	0.45			81.33 (64.38) b	50.67 b
TDZ	0.45	NAA	1.34	93.75 (75.52) a	51.8 b
TDZ	1.13			84.11 (66.50) ab	66.8 a
TDZ	1.13	NAA	1.34	91.33 (72.84) a	64.8 a
TDZ	2.27	NAA	1.34	41.7 (40.22) c	23.17 c
TDZ	0.45	Pulse treatment for 6 h		41.7 (40.22) c	5.0 d
LSD				10.0	2.62

Values in parentheses are arc sine transformations

LSD least significant difference at $\alpha = 0.01$

Values represented with the same letter were not significantly different at $\alpha = 0.01$

4.93, 5.38, and 4.95 μM, respectively) in MS/4 medium and (2) different concentrations of IBA (2.47–4.93 μM) in MS/2 and MS/4 media (Tables 4 and 5).

In order to reduce a step in in vitro rooting, and to improve the survival rate of plantlets, various auxins were used for ex vitro rooting (Table 6). In vitro raised shoots of 3–4 cm in length were treated with different auxins, such as IAA, IBA, NAA, and NOA at 5.71, 4.93, 5.38, and 4.95 μM concentrations, respectively, for 30 min and then planted in transparent plastic trays containing soilrite medium (Keltech Energies, Perlite Division, Bangalore, India) that was moistened with 0.1% (w/v) fungicide (Bavistin) solution. The trays were kept in a polytunnel in the greenhouse. Inside the polytunnel, humidity was maintained with a fogging system (Sil Biotech, India) at 85–90% for 3 weeks and then gradually reduced to 70%.

Hardening of plants

Block-type black color root trainers (Narain Enterprises, Delhi, India), with each block consisting of 12 cells with each cell having a volume of 270 cc (with five ridges), were used for this purpose. Both in vitro- and ex vitro-raised plants were transferred to root trainers containing potting mix of sand, soil, compost, charcoal, and small brick pieces in a 30:10:50:5:5 ratio, respectively. Plants were kept for 3–4 weeks in the greenhouse at 28 ± 5°C with 80% relative humidity (RH), followed by transferring them to an agro shade net with 50% light (S.K. Enterprises, Bangalore, India) for 2 weeks, prior to finally keeping them out in the open nursery.

Field planting

Plantlets of height of ≥30 cm, after hardening for 3 months, were used for field planting. Planting was done after the onset of monsoon season at Agumbe, Karnataka, and the data on survival was recorded after 4 months of planting.

Experimental design, data collection, and statistical analysis

For the studies involving adventitious shoot induction, we used four hypocotyl segments in each culture tube (dimensions 25 × 150 mm). This was done in 12 replicates for each treatment. Responses recorded as a percentage were based on the number of hypocotyls showing shoot induction (more than 50% of the explant area) after

Table 2 Effect of different PGR in MS medium with additives on shoot elongation in *E. ribes* after 6 weeks of culture with three sub-cultures at 2-week intervals

PGRs (μM)						Mean shoot length (cm)	Mean collar diameter (mm)	Remarks
HF							–	Deterioration of cultures
BA	1.11	IBA	0.49			3.88 b	1.67 a	Cultures healthy
BA	0.44	IBA	0.49			4.62 a	1.53 a	Cultures healthy
BA	1.11	GA_3	7.23			4.92 a	0.90 d	Cultures healthy
BA	1.11	GA_3	2.89			4.77 a	1.07 c	Cultures healthy
GA_3	7.23					–	–	Deterioration of cultures
GA_3	2.89					–	–	Deterioration of cultures
BA	1.11	IBA	0.49	GA_3	2.89	2.88 c	1.25 b	Cultures healthy
		LSD				0.42	0.16	

– No response; *LSD* least significant difference at $\alpha = 0.01$

Values represented with the same letter were not significantly different at $\alpha = 0.01$

Table 3 Effect of different PGRs along with indolebutyric acid (IBA; 0.49 μM) on shoot multiplication from the shoot segment on MS medium with additives in *E. ribes* after 4 weeks of incubation

PGRs (μM)				Mean no. of shoots/segment	Mean shoot length (cm)
HF				0.00 j	–
Kn	4.65			0.33 i	0.22 c
Kn	11.63			1.67 h	1.45 b
Kn	23.25			3.67 g	2.63 a
BA	2.22			5.67 f	3.60 a
BA	4.44			5.50 f	2.68 a
BA	11.10			6.17 e	–
BA	22.20			8.17 d	–
TDZ	0.23			9.19 c	–
TDZ	0.45			10.83 b	–
TDZ	1.13			15.67 a	–
Kn	4.65	BA	4.44	5.33 f	2.60 a
Kn	4.65	TDZ	0.23	8.20 d	–
BA	4.44	TDZ	0.23	11.60 b	–
		LSD		1.01	0.16

– Shoots were less than 0.2 cm; *LSD* least significant difference at $\alpha = 0.01$

Values represented with the same letter were not significantly different at $\alpha = 0.01$

Table 4 Effect of different auxins in quarter (MS/4) strength basal salts medium on rooting from in vitro multiplied shoots of *E. ribes* after 4 weeks

PGRs (μM)		% rooting	Mean no. of roots	Mean root length (cm)	Mean shoot length (cm)
HF		13.30 (21.39) c	1.00 c	2.67 c	4.35 ab
IBA	4.93	100.00 (90.00) a	12.33 a	5.28 a	4.43 ab
IAA	5.71	34.20 (35.79) b	2.83 b	3.13 b	4.68 a
NAA	5.38	0.00	–	–	3.12 b
NOA	4.95	0.00	–	–	3.20 b
LSD		7.89	1.53	0.49	1.02

Values in parentheses are arc sine transformations

– No response; *LSD* least significant difference at $\alpha = 0.01$

Values represented with the same letter were not significantly different at $\alpha = 0.01$

Table 5 Effect of modification of basal salts of MS medium and different concentrations of IBA on rooting from in vitro shoots of *E. ribes* after 4 weeks

PGRs (μM)			% rooting	Mean no. of roots	Mean root length (cm)	Mean shoot length (cm)
MS/4		HF	10.0 (18.44)	5.83	1.15	4.33
		2.47	86.70 (68.61)	8.17	2.77	4.63
	IBA	3.69	100.0 (90.00)	16.67	2.72	5.08
		4.93	100.0 (68.61)	22.00	2.70	3.63
MS/2			35.0 (36.77)	6.33	1.80	4.43
		2.47	100.0 (90.00)	10.67	2.97	6.57
	IBA	3.69	100.0 (90.00)	16.33	2.98	5.80
		4.93	100.0 (90.00)	24.50	3.47	3.68
LSD	Media		–	–	0.15	0.32
	Auxin		5.18	1.67	0.21	0.45
	Media × auxin		7.33	–	–	0.63

Values in parentheses are arc sine transformations

LSD least significant difference at $\alpha = 0.01$

Table 6 Effect of different auxins on ex vitro rooting of *E. ribes* afer 4 weeks

Auxin used (μM)		% rooting	Mean no. of roots/shoots	Mean root length (cm)	Mean shoot length (cm)
IAA	5.71	32.00 b	1.54 b	2.14 b	4.73 b
IBA	4.93	95.20 a	5.11 a	5.22 a	6.46 a
NAA	5.38	–	–	–	–
NOA	4.95	–	–	–	–
LSD		5.90	0.55	0.24	0.35

– No response; *LSD* least significant difference at $\alpha = 0.01$

Values represented with the same letter were not significantly different at $\alpha = 0.01$

4 weeks. For assessing the elongation of shoots, four shoot clumps were placed in one culture bottle of 300 ml capacity and this was done in eight replicates for each treatment. We recorded shoot length and collar diameter after a 6-week culture period. Shoot length was measured with a ruler and the collar diameter was measured with electronic vernier calipers for all of the shoots at the base. For shoot multiplication and rooting experiments, four shoots were used in each culture bottle, with six replicates for each treatment. After a 4-week culture period, the number of shoots multiplied per explant and the shoot lengths were recorded. For the rooting experiment, the percentage of rooting, root length, number of roots, and color of the roots were recorded after 4 weeks. For ex vitro rooting, 24 shoots were used for each treatment with four replications. All of the experiments were repeated at least three times. The one-way analysis of variance (ANOVA) was carried out using MS Office, Excel 2003, for all experiments and the means were compared using the critical difference (CD) at a 1% level of significance (Panse and Sukhatme 1978). Analysis was done after the transformation of percentages for percentage response and percent rooting.

Results and discussion

In vitro seed germination

Decoated seeds (Fig. 1c) pre-treated with GA_3 (1.45 μM) for 4 h resulted in 80–90% germination in 30–35 days. Exogenous application of GA_3 (1.45 μM) has significantly improved germination in *Santalum album* L. seeds (Nagaveni and Srimathi 1981; Fox et al. 1994). Inoculating the seeds on MS medium supplemented with GA_3 (5.78 μM) resulted in a quick growth of the seedlings within 15–20 days (data not shown). Seedlings of a two-leaf stage were obtained in 45–50 days and these were used for shoot induction with hypocotyl as an explant.

Adventitious shoot induction

It was observed that the orientation of explant had a significant effect on morphogenic response and the induction of shoot primordia. Shoot regeneration was highest when hypocotyls segments were placed in a horizontal position on the medium. Hypocotyl segments inoculated in vertical positions showed poor response but remained healthy, even after 4 weeks. This might be because hypocotyl segments in a horizontal position had a greater surface area in contact with the medium. The results discussed below were based on hypocotyl segments placed in a horizontal position only.

In this study, the effect of different PGRs (Kn, BA, TDZ, and NAA) on the percentage of adventitious shoot induction and the number of shoots per explant was assessed. It was observed that MS medium supplemented with additives (283.85 μM AA, 118.96 μM CA, 142.33 μM cysteine, and 684.22 μM glutamine) and 1.13 μM TDZ resulted in a high frequency (84%) of multiple shoots (66.8 shoots per explant) induction in a 10-week period (Table 1; Fig. 1d, e, f). The added effect of NAA could be seen with TDZ at 0.45 μM with respect to the percentage response on shoot induction only. A higher (1.13 μM) concentration of TDZ with NAA did not result in any improved effect on adventitious shoot induction. It was interesting to note that there was no response on the adventitious shoot induction on the medium supplemented with BA or Kn, even at substantially higher concentrations of both of them (22.2 and 23.25 μM, respectively).

Similar to our findings, the necessity of TDZ for adventitious shoot regeneration has been reported in *Nothapodytes foetida*, a medicinal tree (Rai 2002), and in *Astragalus cicer* (Başalma et al. 2008) using hypocotyl as an explant. In the present study, we achieved 66.8 shoots/explant from hypocotyl as an explant, whereas Raghu et al. (2006) reported only 16–18 shoots/explant, using a seedling-derived leaf of *E. ribes* as an explant, by culturing on MS medium supplemented with 0.272 μM TDZ and higher concentrations of TDZ resulted in callusing of the tissues.

These results emphasize that the type of explant used is highly important in establishing an efficient regeneration system, as demonstrated by Babaoglu and Yorgancilar (2000), Koroch et al. (2002), and Uranbey et al. (2005).

Elongation of shoots

MS medium supplemented with 0.49 μM IBA and 0.44 μM BA resulted in more robust elongated and sturdy shoots (Fig. 1f). Medium supplemented with only GA_3, although it helped in shoot elongation, showed deterioration of the shoot clump in subsequent sub-cultures. Medium supplemented with both IBA and GA_3 did not show any synergistic effect on shoot elongation (Table 2).

Similar to our results, in *N. foetida*, it was shown that the inhibition of shoot elongation by TDZ was overcome by transferring shoot cultures to MS medium supplemented with BA (2.2 μM) for over three sub-culture periods to produce healthy shoots (Rai 2002). Combined use of lower concentrations of BA with NAA has been reported for the elongation of shoots in *Eucalyptus grandis* (Barrueto Cid et al. 1999) and in *Phellodendron amurense* (Azad et al. 2005). Cultures of *Rauvolfia tetraphylla* L. grown continuously on TDZ-containing medium resulted in fasciated and distorted shoots (Faisal et al. 2005a).

Shoot multiplication

In order to assess further multiplication of the adventitiously induced elongated shoots, shoot segments with 2–3 nodes were used. MS medium supplemented with 1.13 μM TDZ produced the most (12–15) shoots from a shoot segment, but the shoots were dwarf and not suitable for further multiplication and rooting. On the other hand, MS medium supplemented with additives, 2.22 μM BA and 0.49 μM IBA, resulted in the production of only 4–6 shoots. But these shoots were found to be healthy and highly suitable for further shoot multiplication and rooting with a shoot length of 3–4 cm (Fig. 1g). Moreover, higher concentrations of BA resulted in an increased number of shoots, but the shoots were again stunted and were not suitable for shoot multiplication and rooting (Table 3). A five-fold shoot multiplication rate with good shoot length (4–5 cm) was observed consistently on MS medium supplemented with additives of 2.22 μM BA and 0.49 μM IBA. Subculturing of elongated shoots on fresh shoot multiplication medium within 3–4 weeks was found to be essential to avoid deterioration of shoots. Shoot cultures could be maintained for 2 years by sub-culturing on fresh shoot multiplication medium without loss of growth and vigor. In earlier studies on the adventitious shoot induction from leaf explants of *E. ribes*, there were no attempts made to study the further multiplication of shoots (Raghu et al. 2006).

In line with our findings, lower concentrations of BA favored high rates of shoot multiplication and better shoot elongation in *Cinnamomum camphora* (Huang et al. 1998) and *Holarrhena antidysenterica* (Kumar et al. 2005), whereas higher concentrations of BA (>20 μM) resulted in dwarf shoots. Sharma and Ramamurthy (2000) reported a profuse callusing with *Eucalyptus tereticornis* on MS medium supplemented with TDZ (2.27 and 4.54 μM) and NAA (0.54 μM), but the production of 4–8 healthy shoots per explant on medium with BA and NAA (0.44 and 0.54 μM, respectively).

Rooting of the shoots

Of the various auxins used in MS/4 basal salts medium, IBA was found to be the best in terms of yielding a high percentage of rooting without callusing from the shoot base. An increase in concentration of IBA from 1.23 to 4.93 μM enhanced the rooting percentage. MS/4 basal salts medium supplemented with both IBA 4.93 and 9.86 μM exhibited 100% rooting within a 4-week period. Medium containing IAA proved to be less effective in rooting in *E. ribes*, whereas NAA and NOA exhibited callusing from the shoot base (Table 4).

In addition, of the eight combinations of MS/2 and MS/4 basal salts medium with various concentrations of IBA, MS/2 medium containing 2.47 μM IBA proved to be the best and exhibited 100% rooting, with an average of 10.67 roots/shoot and a 6.57 cm maximum shoot length in 4 weeks (Table 5; Fig. 1h). An increase in the concentration of IBA in the medium increased the number of roots, but undesired callusing was observed from the shoot base. Though MS/4 basal salts medium with IBA also exhibited a high rate of rooting, the quality of the roots was not good, and blackening of the roots was observed. In earlier reports on *E. ribes*, profuse rooting was observed in MS/2 medium with a higher concentration (4.93 μM) of IBA (Raghu et al. 2006).

Similar to our observation, MS/2 medium supplemented with IBA (4.93 μM) resulted in a maximum average number of roots per shoot (4.47) in *Dalbergia sissoo* (Joshi et al. 2003), whereas MS/4 medium with IBA (14.79 μM) and 1.5% sucrose favored high rooting percentage in *Acacia catechu* (Kaur et al. 1998). In *P. amurense*, better root formation was observed on MS medium compared to MS/2 and MS/4 (Azad et al. 2005).

Ex vitro rooting of shoots

Out of the various auxins (IAA, IBA, NAA, and NOA) used for ex vitro rooting, shoots treated with IBA (4.93 μM) solution for 20 min exhibited the highest (95.2%) rooting, with a maximum shoot length of 6.46 cm without callus formation, within 4 weeks (Table 6; Fig 1i). Shoots treated

with NAA and NOA showed no rooting, but only callus formation from the shoot base, whereas shoots treated with IAA showed a much lower rate of root induction.

Ex vitro rooting has also been reported in other medicinal plant species, by the treatment of the in vitro shoots, with 2.46 μM IBA for 10 days in *Eupatorium triplinerve* (Martin 2003), with 150 μM IBA for 30 min in *Tylophora indica* (Faisal et al. 2005b), and 2.46 μM IBA for 7 days in *Wedelia chinensis* (Martin et al. 2003).

Hardening of plantlets

For the hardening of in vitro rooted plantlets, we found that it is essential to maintain them in the greenhouse for 4 weeks. The covering of containerized plants for the first 2 weeks with plastic sheets on an iron frame (polytunnel) in the greenhouse proved to be most effective for a high rate of survival (>85%). Later on, plants could be left for two more weeks without a plastic sheet in the greenhouse with no deleterious effects on their survival.

After transferring the ex vitro rooted plantlets to containers, they were kept in the greenhouse for another 2 weeks without plastic sheet, yielding at least 95% survival. During the hardening phase, new root and shoot growth was observed in both ex vitro and in vitro rooted plantlets (Fig. 1j). Also, keeping plants in 50% shade for 2 weeks before shifting to an open nursery was found to be essential for 100% survival.

Field planting

Plantlets of height of ≥30 cm, after hardening for 3 months, were used for field planting at Agumbe, Karnataka. The survival rate of plants after 4 months of field planting was 95%, and new shoot growth also was observed in these plants.

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