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RESEARCH ARTICLE

Organogenesis from leaf and internode explants of *Ophiorrhiza prostrata*, an anticancer drug (camptothecin) producing plant

A. Shahanaz Beegum
Department of Botany
University of Calicut
Kerala 673 635, India
Tel: 91 494 2401144 Ext. 407
E-mail: marttinet@rediffmail.com

Kottackal Poulose Martin*
Department of Biotechnology
University of Calicut
Kerala 673 635, India
Tel: 91 494 2401972
Fax: 91 4942 400269
E-mail: marttinet@rediffmail.com

Chun-Lai Zhang
Norman Borlaug Institute for Plant Science Research
Faculty of Health and Life Sciences
De Montfort University
The Gateway, Leicester LE1 9BH, UK
Tel: 44 116 2577719
Fax: 44 116 2577290
E-mail: clzhang@dmu.ac.uk

I. K. Nishitha
Department of Botany
University of Calicut
Kerala 673 635, India
Tel: 91 494 2401144 Ext. 407
E-mail: iknishitha@rediffmail.com

Ligimol
Department of Botany
University of Calicut
Kerala 673 635, India
Tel: 91 494 2401144 Ext. 407
E-mail: marttinet@rediffmail.com

Adrian Slater
Norman Borlaug Institute for Plant Science Research
Faculty of Health and Life Sciences
De Montfort University
The Gateway, Leicester LE1 9BH, UK
Tel: 44 116 2577843
Fax: 44 116 2577290
E-mail: ads@dmu.ac.uk

P. V. Madhusoodanan
Department of Botany
University of Calicut
Kerala 673 635, India
Tel: 91 494 2401144 Ext. 407
Fax: 91 494 2400269
E-mail: madhuspv@sify.com

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*Corresponding author

Abbreviations: BA: N^6-Benzyladenine
CPT: camptothecin
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
Kn: Kinetin
MS: Murashige and Skoog
NAA: α -Naphthaleneacetic acid
PGRs: plant growth regulators

Camptothecin (CPT) analogues and derivatives serve as a novel class of effective anticancer agents that exert their action against DNA topoisomerase I. This paper presents procedures for the rapid, high frequency regeneration of a camptothecin producing plant, *Ophiorrhiza prostrata* D. Don from leaf and internode explants *via* shoot organogenesis. The concentrations of plant growth regulators and explant types exhibited discrete roles in the efficacy of shoot induction. N^6-benzyladenine (BA) was the most effective cytokinin for the induction of shoots. Murashige and Skoog (MS) medium with 8.87 µM BA and 2.46 µM indole-3-butyric acid (IBA) yielded the highest number of shoots from leaf and internode explants (76.0 and 90.8 shoots respectively). In the case of leaf explants, explants from the proximal end produced a higher number of shoots than those from the mid and distal end. Leaf and internode explants cultured on MS medium supplemented with α-naphthaleneacetic acid (NAA) and BA developed shoots, calli and roots. Calli subcultured onto medium supplemented with 8.87 µM BA and 2.46 µM IBA developed a mean of 20.1 shoots within 40 days. Excision and culture of internode and proximal leaf explants from the established cultures on MS basal medium significantly enhanced the number of shoots and yielded a mean of 18.3 and 13.7 shoots respectively within 40 days. Histological examination of leaf explants showed that the shoots were of sub-epidermal origin, confined to the sub-epidermal cells above the vascular traces. Shoots cultured on half-strength MS basal medium with 10.74 µM NAA and 2.32 µM Kn produced a mean of 48.2 roots per shoot. Direct transfer of rootless healthy shoots showed a 50% survival rate, whilst it was 100 percent in the case of *in vitro* rooted shoots.

Camptothecin (CPT), isolated and characterized for the first time by Wall et al. (1966), is a monoterpene indole alkaloid originally derived from *Camptotheca acuminata* (Nyssaceae), a native of North China. Members of the Icacinaceae, Olacaceae, Rubiaceae, and Apocynaceae families are also reported to produce camptothecin. CPT analogues and derivatives are a novel class of effective anticancer agents that exert their action against DNA topoisomerase (topo) I (Redinbo et al. 1998). The worldwide market size of camptothecin derivatives (*e.g.* topotecan and irinotecan) reached 1.5 billion US dollars in 2002 (Lorence and Nessler, 2004). Due to the cytotoxicity of camptothecin itself, the CPT derivatives, irinotecan and topotecan, are used throughout the world for the treatment of various cancers, and over a dozen or more CPT analogues are currently at various stages of clinical development (Lorence et al. 2004). However, they are synthesized from natural camptothecin which is extracted from plants. The use of CPTs as inhibitors of replication, transcription, and packing of double stranded DNA-containing adenoviruses, papovaviruses, and the single-stranded DNA-containing autonomous parvoviruses has been studied (Pantazis et al. 1999). It appears that CPTs could be powerful antiviral drugs for several DNA viruses, which are causative agents for a large number of diseases. Since 1994, CPT has been in use clinically in Japan for the treatment of lung, ovarian and uterine cancer (Japan Pharmaceutical Information Center, 1995). It has also proved useful as an insect chemosterilant and plant growth regulator and as an inhibitor of herpes virus (Becker and Olshevsky, 1973). The therapeutic values of CPT derivatives are highlighted against colon cancer (Giovanella et al. 1989), AIDS (Priel et al. 1991), uterine cervical and ovarian cancer (Takeuchi et al. 1991), and falciparum malaria (Bodley et al. 1998).

The genus *Ophiorrhiza* belongs to the family Rubiaceae, which comprises 150 species. The roots of *Ophiorrhiza* species, *O. mungos* and *O. pumila* have been reported as the sources of CPT and 10-methoxycamptothecin (Tafur et al. 1976; Saito et al. 2001; Sudo et al. 2002; Watase et al. 2004). The *Ophiorrhiza* spp. is also used to provide remedies for ulcers, helminthiasis, snake poison, poisonous wounds, gastropathy, leprosy, and hydrophobia (Kirtikar and Basu, 1975). *O. prostrata* D. Don is an herbaceous perennial medicinal plant, exploited for the production of camptothecin, which is accumulated mainly in the roots. A comparative study of camptothecin content in *Nothapodytes foetida, O. mungos* and *O. rugosa* indicated highest yields of camptothecin and 9-methoxy camptothecin in *N. foetida* (Roja, 2006).

The rate of plant propagation is critical to meet the pharmaceutical demand for camptothecin. A slow propagation rate in *O. prostrata,* because of low seed viability and germination rate as well as a small number of propagules (stem cuttings), has restricted the natural dissemination of the plant. In addition, the destruction caused by harvesting the roots as a source of the drug has threatened the survival of the plant. Thus, the large-scale demand necessitates rapid multiplication of the plant within

Table 1. Direct organogenesis from internode and proximal leaf explants of *O. prostrata* on MS media with various plant growth regulators. Data represents the mean of 20 replicates. Mean values followed by the same letters within columns are not significantly different at the 5% level. Culture duration was 40 days.

Plant Growth regulators (µM)				Percentage of explants forming shoots		Number of shoots per explant	
BA	Kn	NAA	IBA	Leaf	Internode	Leaf	Internode
0	0	0	0	80	90	2.9^{m}	4.0^{l}
2.22				80	85	8.4^{k}	12.3^{j}
4.44				95	100	13.8^{j}	25.9^{g}
6.66				100	100	21.4^{g}	35.1^{e}
8.87				100	100	38.0^{d}	51.5^{d}
13.3				100	100	30.4^{e}	36.5^{e}
17.7				100	100	25.0^{f}	29.3^{f}
22.2				100	100	13.4^{j}	17.6^{h}
	2.32			90	95	4.1^{l}	6.2^{k}
	4.65			100	100	19.1^{g}	27.0^{fg}
	6.97			100	100	16.2^{i}	18.6^{h}
	9.29			100	100	7.5^{k}	14.7^{i}
8.87	0.46			100	100	24.5^{f}	29.5^{f}
8.87	2.32			100	100	21.3^{g}	24.3^{g}
8.87	4.65			100	100	16.1^{i}	21.5^{h}
8.87		0.54		100	100	29.3^{e}	35.6^{e}
8.87		2.69		100	100	24.5^{f}	28.8^{f}
8.87		5.37		100	100	19.2^{g}	24.1^{g}
8.87			0.49	100	100	47.5^{b}	61.0^{b}
8.87			2.46	100	100	76.0^{a}	90.8^{a}
8.87			4.90	100	100	40.7^{c}	56.1^{c}

a short timeframe without a negative impact on the natural resources. *In vitro* morphogenesis without a callus phase is regarded as the most faithful strategy to obtain plants with high speed as well as genetic fidelity. Direct shoot induction as an easy way for the rapid plant propagation has been reported in several medicinal plants *viz*. *Murraya koeningii* (Rout, 2005), and *Euphorbia nivulia* (Martin et al. 2005). Micropropagation of *O. mungo* by using seedling shoot culture has been reported (Jose and Satheeshkumar, 2004). Callus mediated plant regeneration is an easy way to obtain somaclonal variants as has been emphasized in several plants (Dennis and Boban, 2005; Dhar and Joshi, 2005; Faisal and Anis, 2005; Agrawal and Sardar, 2006). So far, no protocol for *in vitro* propagation of *O. prostrata*

has been published. The propagation of *C. acuminata* conventionally as well as through *in vitro* culture is not as easy as that of herbs such as *O. prostrata*. This study establishes rapid propagation protocols using leaf and internode explants in order to meet the demand, which could curtail the impact on the natural population and prevent the plant from becoming endangered.

MATERIALS AND METHODS

Healthy young shoots of *O. prostrata* were collected from potted plants and used as the starting material. Young leaves and internode segments were washed separately under running tap water followed by 5% (v/v) solution of Extran (a neutral liquid detergent, Merck India Ltd., Mumbai) for 5 min followed by surface sterilization using 0.1% (w/v) mercuric chloride. Leaf segments were sterilized for 7-9 min, while internode segments were treated for 10-12 min. Decanting of the mercuric chloride was followed by repeated washes (3 times 5 min each) in sterile water. The sterilized tissues were cut into appropriate sizes (leaf explants of 10 mm^2 and internode of 7-15 mm) using sterile forceps and knife. For the induction of direct shoot formation, the explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with different levels of plant growth regulators (Table 1). Subcultures were carried out on MS medium with various concentrations of plant growth regulators (PGRs) as specified in the text. For indirect organogenesis and *in vitro* rooting MS media supplemented with different PGRs were used as shown respectively in Table 2 and Table 3. Test tubes (25 x 200 mm), 100 ml conical flasks, and jam bottles (53 x 125 mm; 2 mm thick) were used as culture vessels. Test tubes and conical flasks were closed by cotton plugs, while jam bottles were capped with polypropylene autoclavable lids (14 mm height x 48 mm inner diameter with 2 mm thick). The media were adjusted to pH 5.8, and were solidified with 0.8% (w/v) agar. They were sterilized at a pressure of 1.06 kg cm^{-2} at 121ºC for 20 min in an autoclave. All cultures were incubated at 25 ± 2ºC with 16 hrs light (at an irradiance of 25 µmol m^{-2} s^{-1})/8 h dark cycle under white fluorescent tubes.

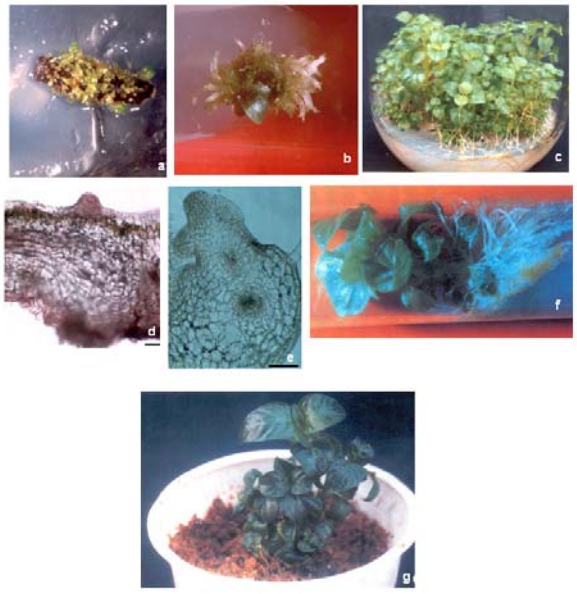

Figure 1. *In vitro* propagation of *O. prostrata* through direct organogenesis.
(a) Shoots from internode explants on MS medium with 8.87 µM BA and 2.46 µM IBA.
(b) Shoots from leaf explants on MS medium with 8.87 µM BA and 2.46 µM IBA.
(c) Shoot multiplication on the above medium during subculture.
(d) Shoot origin from sub-epidermal cells of leaf explants (bar = 0.1 mm).
(e) Shoot origin from sub-epidermal cells of internode explants (bar = 0.1 mm).
(f) Rooted shoot on half-strength MS medium with 10.74 mM NAA and 2.32 mM Kn.
(g) Established plant (after 45 days).

Ontogeny of shoot induction was studied by taking random sections at different growth stages. Sections were stained with a dilute solution of safranin and observed under a Leitz Dialux 20 (Germany) microscope.

Plantlets (derived through *in vitro* rooting) as well as well-grown shoots without roots (*ex vitro* rooting) from the shoot multiplication medium were transferred to small pots containing soil and sand (1:1), covered with moistened polyethylene bags and kept at room temperature (33 ± 2ºC) for acclimation, and subsequently transferred to field conditions.

The experiments were performed in a completely randomized design. Twenty replicates were used for each treatment, and all the treatments were repeated twice for confirmation. The mean values of different treatments were analyzed using Duncan's multiple range test.

RESULTS

Direct organogenesis

Leaf and internode explants cultured on MS basal medium produced directly a mean of 2.9 and 4.0 shoots respectively. Addition of PGRs induced shoot formation and the number of shoots depended on the types and concentrations of PGRs, BA in particular (Table 1) as well as the explant types. MS medium containing BA alone was superior to that containing Kn alone for the induction of shoots (Table 1). On medium with 8.87 µM BA, internode explants developed a mean of 51.5 shoots, while the proximal leaf explants produced a mean of 38.0 shoots within 40 days. Upon medium with 8.87 µM BA, leaf explants initiated shoots within 14 days, while the internode explants induced shoots within 12 days. Lower concentrations of BA induced callus with a reduction in number of shoots. Higher levels of BA increased the amount of callus. The calli later developed shoot buds. Increasing BA concentrations above 8.87 µM exhibited a negative effect on the height of shoots

Table 2. Organogenesis from callus on MS medium with different plant growth regulators. Data represents the mean of 20 replicates. Mean values followed by different letters are significantly different at 5% level. Culture duration was 40 days.

Plant Growth regulators (µM)				Number of shoots per culture
BA	Kn	NAA	IBA	
4.44				5.5[f]
6.66				8.3[e]
8.87				15.7[b]
13.31				8.0[e]
8.87	0.46			11.6[c]
8.87	2.32			7.9[e]
8.87		0.54		10.6[d]
8.87		2.69		9.9[d]
8.87			0.49	14.9[b]
8.87			2.46	20.1[a]
8.87			4.90	12.7[c]

i.e., the shoot height decreased as BA concentrations increased. Kn containing media favoured better shoot elongation than that on BA alone supplemented media, and the shoots on medium having Kn attained > 3 cm height within 40 days. On Kn fortified media, unlike that on BA containing media, the explants as well as the regenerated shoots produced roots as well. Combinations of BA and Kn at different concentrations displayed less numbers of shoots than media with 8.87 µM BA (Table 1).

Synergy between BA and auxins exhibited a positive effect in the induction of shoots. MS medium with 8.87 µM BA and 2.46 µM IBA developed the highest number of shoots; 76.0 and 90.8 shoots from leaf and internode explants respectively (Table 1; Figure 1a and Figure 1b). As on medium with other PGRs, the shoots on leaf explants were developed adaxially, however, with a few shoots on the abaxial side. Of the different regions in the leaf, explants from proximal region were superior in the induction of shoots. Increasing IBA concentration favoured callus induction especially from the cut ends of explants, which later regenerated into shoots. The shoots were longer on media containing BA and auxins than those in media containing BA alone. The regenerated shoots developed roots following further culture. The combination of BA and NAA was inferior for shoot formation (Table 1) but facilitated the formation of a higher amount of callus.

Direct organogenesis is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. As in the present study, direct formation of shoots without an exogenous trigger has been reported in different explants/species, *e.g.* from internodes of *Bacopa monniera* (Tiwari et al. 1998; Shrivastava and Rajani, 1999), and leaf explants of *Drosera binata* (Kawiak et al. 2003). Watase et al. (2004) has reported spontaneous formation of shoots from hairy roots of *Ophiorrhiza pumila*. The development of shoots on basal media may be due to stimulation by endogenous hormones or some signals related to wounding, which play a vital role during the induction of regeneration, or the ratio of ions present in the medium. The difference in the number of shoots formed in leaf and internode explants may be a result of differences in the regeneration potential of different explants, which is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974). Enhancement in the induction of shoots by the synergy of BA and auxins observed in the present study has been documented in *Stevia rebaudiana* (Sivaram and Mukundan, 2003), *Aloe vera* (Liao et al. 2004), *Murraya koeningii* (Rout, 2005) and *Euphorbia nivulia* (Martin et al. 2005). Nevertheless, adventitious shoot regeneration has been accomplished with a mean 10.4 of shoots per shoot explants of *Ophiorrhiza mungo* cultured on MS medium with 2.22 µM BA (Jose and

Satheeshkumar, 2004). Though Kn was inferior for shoot formation in *O. prostrata*, efficacy of Kn or other cytokinins either alone or in combination with BA/auxins in direct shoot formation has been demonstrated in *Asparagus maritimus* (Stajner et al. 2002), *Robinia pseudoacacia* (Shu et al. 2003), and *Bixa orellana* (De Paiva et al. 2003).

High shoot regeneration potential of proximal end explants as to leaf tip explants as in the present study has been emphasized in *Beta vulgaris* (Zhang et al. 2001), *Anthurium andraeanum* cultivars Tinora Red and Senator (Martin et al. 2003), and *Euphorbia nivulia* (Martin et al. 2005). In the view of Welander (1988), the high potential of the proximal end to the distal may be due to the difference in the maturity between proximal and distal end of the leaf, and which is supported by the fact that leaves reach maturity first at distal (tip) and subsequently in a basipetal progression. High frequency shoot induction at the proximal region may also be due to the higher level of IAA and abscisic acid (Rajasekharan et al. 1987).

Callus induction

Leaf and internode explants cultured on MS media with different levels of NAA either alone or in combination with BA or Kn produced calli, roots, and shoots (< 4) (data not shown). MS medium with 5.37 µM NAA alone induced a mean fresh weight of 219 and 188 mg calli per explants respectively from internode and leaf explants. Combinations of NAA with BA or Kn favoured increased numbers of shoots along with callus formation. MS medium with 5.37 µM NAA and 2.22 µM BA induced the highest amount of callus (310 and 295 mg from internode and leaf respectively) with < 6 shoots. The calli developed were pale green to dark green and semi-hard.

Indirect organogenesis

Callus initiated on MS medium with NAA alone or in combination with BA or Kn upon subculture developed adventitious shoots. MS medium with 8.87 M BA and 2.46 µM IBA was the best with a mean of 20.1 shoots within 40 days (Table 2). Increasing the concentration of BA prevented elongation of shoots. Higher concentrations of BA favoured callus proliferation and subsequent culture of the callus favoured better shoot morphogenesis. Callus mediated shoot morphogenesis has been well accomplished in several medicinal plants: *Tylophora indica* (Dennis and

Table 3. Efficacy of half strength MS medium with different plant growth regulators in the induction of roots on *in vitro*-derived shoots. Data represents the mean of 20 replicates. Mean values followed by different letters are significantly different at 5% level. Culture duration was 40 days.

Plant Growth regulators (µM)				Percentage of shoots producing roots	Number of roots per shoot
NAA	IBA	IAA	Kn		
0	0	0	0	95	3.8^{i}
0.54				95	5.9^{h}
2.69				100	19.1^{d}
5.37				100	35.1^{b}
	0.49			95	4.3^{hi}
	2.46			100	12.1^{e}
	4.90			100	10.6^{f}
		0.57		95	5.0^{h}
		2.85		100	13.4e
		5.71		100	11.6f
			4.65	95	8.8^{g}
10.74			2.32	100	48.2^{a}
10.74			4.65	100	26.3^{c}

Boban, 2005; Faisal and Anis, 2005), *Saussurea obvallata* (Dhar and Joshi, 2005) and *Euphorbia nivulia* (Sunandakumari et al. 2005) and *Cassia angustifolia* (Agrawal and Sardar, 2006). High frequency callus mediated shoot regeneration can be utilised for the induction of somaclonal variation for the improvement of this valuable medicinal plant.

Shoot multiplication

The internode and leaf explants derived from established *in vitro* cultures on MS basal medium produced high numbers of shoots with means of 18.3 and 13.7 respectively. A high yield of shoots was achieved by culturing stem and leaf (proximal) segments as well as shoot clumps excised from the primary cultures on medium optimal for direct shoot multiplication (MS medium with 8.87 µM BA and 2.46 µM IBA). The number of shoots developed was difficult to count and was further increased in subsequent cultures (Figure 1c). The shoots cultured on MS medium without PGRs developed long shoots with roots. The roots turned to reddish brown from white through golden yellow. The colour of the roots may be due to the accumulation of secondary metabolites (CPT and its derivatives).

Ontogeny of shoots

The shoots were initiated from sub-epidermal cells especially from the region above the vascular bundles of the explants (Figure 1d and Figure 1e). Both palisade and spongy cells developed shoots. The shoots were shown to contain an extension of the vascular bundles from the source tissue. In the case of leaf explants, the shoots originated from subepidermal cells above the vascular traces. Shoots originating from subepidermal cells have been reported in tobacco stem explants (Creemers-Molenaar et al. 1994). The ability of different tissues to form shoots directly in other species has been demonstrated including leaf epidermis (Lo et al. 1997), vascular cambium (Wenzel and Brown, 1991), cortex (Arai et al. 1997) and cortical, subcortical (Twyford and Mantell, 1996) or epidermis or cortex of root (Knoll et al. 1997).

Rooting

In vitro as well as *ex vitro* root induction was preceded by transfer of healthy shoots with more than 3.0 cm height grown in the shoot induction or shoot multiplication medium to rooting media or soil. Shoots transferred to half-strength MS medium free of PGRs induced more roots than those on full-strength MS medium. Auxins at lower concentrations, supplemented to half-strength MS medium induced a large number of roots (Table 3). Of the different auxins, NAA at 5.37 µM was superior, which induced a mean of 35.1 roots per shoot within 30 days. Auxins also facilitated callus formation in a small number of shoots. The callus developed roots later. Shoots cultured on half-strength MS medium with 4.65 µM Kn alone also induced roots (Table 3). Addition of Kn to auxin containing medium augmented root induction. MS medium with high concentration of NAA and Kn favoured better *in vitro* rooting than with auxins alone. Half-strength MS medium containing 10.74 µM NAA and 2.32 µM Kn produced a mean of 48.2 roots per shoot (Figure 1f). Increased rooting by the addition of cytokinins has been emphasized in *Drosera cunefolia* (Kawiak et al. 2003). However, Jose and Satheeshkumar (2004) observed the best *in vitro* rooting in *Ophiorrizha mungo* on medium with IBA (12.3 µM) plus NAA (1.07 µM). The roots developed on all media became reddish brown through golden yellow from white. The high numbers of roots produced under optimal plant growth regulator regimes opens the possibility of producing camptothecin as the root is the main source for the chemicals.

Transfer of rootless shoots directly into pots containing sand and soil (*ex vitro* rooting) resulted in a survival rate of 50% shoots. The shoots resumed growth after 15 days of transplantation. *Ex vitro* rooting may reduce the micropropagation cost and also the time from laboratory to field. Rooting *extra vitrum* has been reported in *Rotula aquatica* (Martin, 2003a), *Eupatorium triplinerve* (Martin, 2003b) and *Prunus* spp. (Pruski et al. 2005). In the present study, *ex vitro* shoots exhibited only 50% survival. Liu and Li (2001) noticed a high survival rate of plantlets through *ex vitro* propagation. This study showed *ex vitro* rooting gave a lower percentage of survival than *in vitro* rooting though *ex vitro* rooting looks promising considering the reduction in cost by avoiding the *in vitro* rooting and use of auxins and the reduction in labour and time of plantlet establishment from laboratory to land.

Plantlets derived after *in vitro* rooting showed 100% survival in field conditions. The plantlets transferred revived growth after 9 days and grew well (Figure 1g). The field established plants were identical in morphology and growth pattern to that of source plant.

The protocol described in this study enables production of more than 75 plants within two months using single internode explant. This may realize the demand of pharmaceutical industries and minimize the impact of over exploitation of the plants. Besides the propagation of elite cultivars and conservation of this rare medicinal plant, a highly efficient regeneration protocol opens a way for improvement of the plant through genetic transformation strategies.

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