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In vitro Regeneration and Mass Propagation of Medicinally Potential Rare Plant *Cynanchum callialatum* Buch.-Ham. ex Wight

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Abstract

An efficient *in vitro* regeneration protocol for a rare medicinal plant, *Cynanchum callialatum* Buch.-Ham. ex Wight was established. Explants for this study were obtained from aseptically grown healthy green seedlings on MS medium. The shoot apices, nodal and leaf segments from *in vitro* grown seedlings were cultured on MS medium containing different concentrations and combinations of PGRs. The shoot apices and nodal segments underwent direct organogenesis. The highest number of shoot apices (95%) produced multiple shoot buds (5.31 ± 0.14 per explant) when cultured on MS + 2.0 mg/l BAP + 1.0 mg/l NAA. On the other hand, 87% of nodal segments responded to induction of multiple shoot buds (5.26 ± 0.14 per nodal segment) when cultured on MS + 2.0 mg/l BAP + 1.5 mg/l NAA. The highest number of leaf segments (100%) produced whitish friable calli on MS + 1.0 mg/l 2,4-D + 1.0 mg/l BAP. It was noticed that this whitish friable callus did not undergo any kind of differentiation. The elongated shoot buds were individually grown on rooting media. The maximum number of roots (6.82 ± 0.10) per shoot was produced in half-strength MS medium fortified with 2.0 mg/l IBA. After successive phases of acclimatization, 75% of the plantlets were finally survived and established showing normal growth.

Introduction

The genus *Cynanchum* belonged to the family Asclepiadaceae, but presently it has been classified under the Apocynaceae (Oleander family). *Cynanchum callialatum* (commonly known as Pretty-Wing Swallow-Wort) is an annual species growing as twinning vines that wraps around supports. It can measure up to 3 m long. It is a native plant in China,

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India, Myanmar and Pakistan. In Bangladesh, it was first recorded from the sandy bushy areas of Saint Martin Island of Chattogram.

Plants belonging to genus *Cynanchum* have traditionally been used in folk medicine as antifebrile, antitussive, diuretic, expectorant, anticonvulsant, analgesic and tonic agents (Wang and Ng 1999). In Traditional Chinese Medicine (TCM), *Cynanchum* variants are used to cure fever, cough, pneumonia and asthma (Navarra and Lipkowilz 2004). Pharmacological studies have reported various uses for *Cynanchum* plants. The GC-MS analysis shows the presence of 52 compounds in which some have medicinal value. The Liquid chromatography-mass spectrometry (LC-MS) analysis shows the presence of compounds in which most of them have medicinal properties. The phytochemical test on *Cynanchum callialatum* reveals the presence of various constituents like Betulinic acid, Lupeol, Germacrone, Longiverbenone, alkaloids, flavonoids, terpenoids and tanins etc. The presence of Betulinic acid, Lupeol, Germacrone and Longiverbenone ensure the plant may be a potential source for anti-cancer, anti-HIV, anti-inflammatory, and antimicrobial drug discovery. The fresh root paste of this plant is applied on wounds and taken orally in small quantities to cure stomach-ache, while the leaf juice is applied to cure skin diseases (Karthikeyan and Balasubramanian 2014). *Cynanchum callialatum* has been used to treat wounds, headaches, infections and other skin-related problems by tribes in Tamil Nadu, India (Nithyakalyani et al. 2025).

Despite its medicinal significance, this plant remains underexplored in terms of large-scale cultivation and conservation. The over exploitation of wild populations, habitat destruction, and low seed germination rates pose significant challenges to its natural propagation, making *in vitro* regeneration a promising alternative for its conservation and commercial utilization.

In vitro culture techniques are now indispensable for the production of disease-free plants, rapid multiplication of rare plant genotypes, plant genome transformation, and production of plant-derived metabolites of important commercial value (Altpeter et al. 2016). It offers a reliable solution for mass multiplication, conservation, and genetic improvement of medicinal plants. This study aims to develop an efficient and reproducible *in vitro* regeneration system for *Cynanchum callialatum* to ensure a sustainable supply of this plant for future research and therapeutic applications.

Materials and Methods

Mature follicles of *Cynanchum callialatum* were collected and thoroughly washed under running tap water. The follicles were then surface sterilized with 1% Savlon (ACI Ltd., Bangladesh) and liquid soap for 5-10 min with constant shaking. The materials were washed 3-4 times with sterile distilled water for complete removal of detergent and taken under laminar airflow cabinet and transferred to sterilized conical flask. After rinsing with 70% ethanol for less than 60 sec, they were immersed in 0.1% $HgCl_2$ for 2 min. To remove every trace of the sterilant materials were then washed 4-5 times with sterile

distilled water. In the laminar air flow cabinet the explants were kept on a sterile aluminium slab and seeds were separated with a sterilized surgical blade and forceps then cultured on media taken in vessels. The culture vessels with inoculated explants were then taken to the growth room for incubation. The growth room was maintained at a regular cycle of 14 h continuous light and 10 h continuous dark phase with a light intensity of 2000-3000 lux. The room temperature was maintained at 25 ± 2°C. Proliferated multiple shoots were rescued very carefully in aseptic conditions and divided into clusters of 2-3 shoots using a sterile sharp scalpel. Then these were sub-cultured on MS medium containing different concentrations and combinations of auxins and cytokinins for further response at an interval of 15-20 days and culture vessels were maintained in the culture room in the same light and temperature conditions. The calli were produced from leaf explants obtained from *in vitro* grown seedling. This calli were further sub-cultured at a lower density in the PGRs supplemented medium or on the same medium. Different rooting experiments were carried out with full, half and quarter-strength MS medium with or without growth regulators to determine the suitable media composition, and optimum growth requirements. Elongated shoots with 2-3 cm height were rescued aseptically from the culture vessels and then individually transferred to freshly prepared rooting media. In most cases, the adventitious roots were produced from the cut ends of microshoots within 2-3 weeks of culture on suitable medium.

In the rooting medium, the plantlets grew further and produced well developed root system. After sufficient growth, the plantlets were ready for transfer to outside environment. The plantlets grown inside the culture vessels were brought out and established outside the culture room following a successive phase of acclimatization. For acclimatization of *in vitro* developed plantlets in outside environment, the culture vessels were kept outside the culture room with gradual increasing the time up to three days. Finally, the plants were taken out of the culture vessels and washed with running tap water for removal of agar attached to the roots. Then the plantlets were transplanted to small plastic pots containing garden soil and compost in the ratio 1 : 1 and kept in room temperature for 3-5 days. The soil was treated with 0.1% agrosan (fungicide) solution. After gradual acclimatization, plantlets were finally transferred to large earthen pots and kept in outside the natural environment. In all experiments, each treatment consisted of 5 replicates (3 explants each replicate). The mean values of the parameters were compared by analysis of variance using the SPSS (Ver. 26) software.

Results and Discussion

The cent per cent aseptic seed germination rate was observed within five weeks and produced healthy green seedlings in the medium having the combination of 1.0 mg/l BAP + 1.0 mg/l NAA (Fig. 1A, B). Shoot apex, nodal and leaf segments of *in vitro* grown seedlings were further cultured on 0.8% (w/v) agar solidified MS medium.

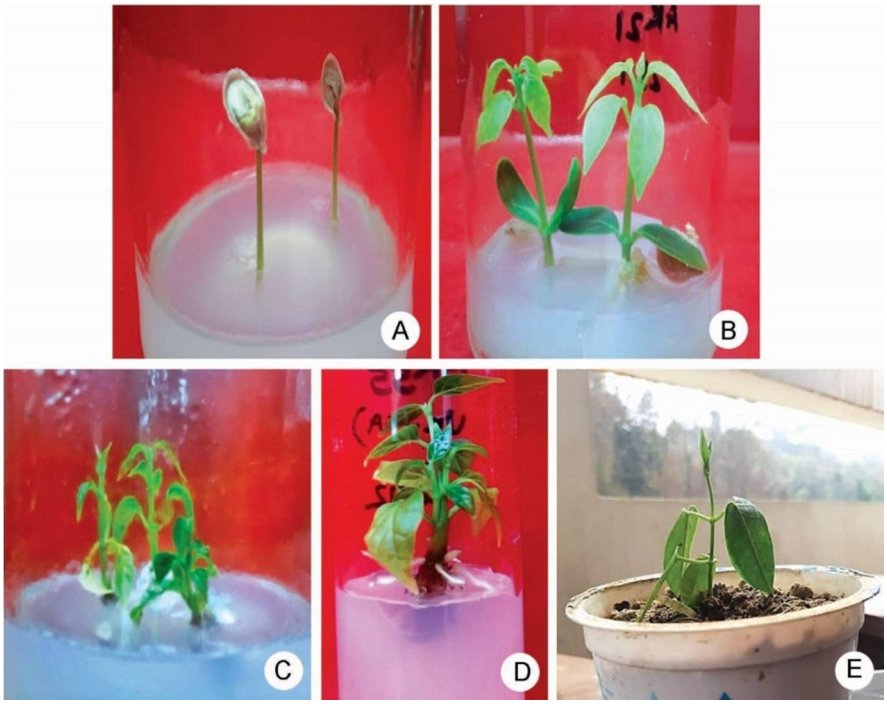

Fig. 1 (A-E). *In vitro* propagation of *Cynanchum callialatum* through direct organogenesis: (A) *In vitro* seed germination, (B) Green seedlings were produced on MS medium containing 1.0 mg/l BAP + 1.0 mg/l NAA, (C) Maximum proliferation of shoot buds on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA, (D) Highest number of roots per micro shoot in half MS medium supplemented with 2.0 mg/l IBA, and (E) Establishment of *in vitro* raised plantlets in plastic pots.

Different concentrations of BAP (0.5-2.0 mg/l) alone and in combination with NAA and Kn in combination with IAA and NAA were used in MS medium for induction and development of shoot buds in shoot apices. The shoot apices underwent direct organogenesis producing shoot buds in some of the media combinations. After 20-25 days of culture the highest average number of shoots per culture was 5.31 ± 0.14 (Fig. 1C, Table 1) in the MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA. It is proved that combination of BAP with NAA is better than the combination of Kn with NAA or IAA for induction of shoot buds. The highest number of nodal segments (87%) produced shoot buds when cultured on MS medium supplemented with 2.0 mg/1 BAP + 1.5 mg/1 NAA and this medium also produced maximum number of shoot buds (5.26 ± 0.14) per nodal segment (Fig. 1D). From the overall result for induction of shoot buds in both explants of *C. callialatum* underwent direct organogenesis but the shoot apices were better than nodal segments. Relatively higher amount of cytokinin in combination with lower amount of auxin promoted direct organogenesis which was better in combination of BAP and NAA than that of any other concentration and combination of PGRs. In addition, BAP was reported to be more stable in tissue culture when compared to other

Table 1. Effects of different concentrations and combinations of PGRs for the induction of shoot buds from shoot apices and nodal segments in *Cynanchum callialatum*.

PGRs concentration (mg/l)					Shoot apices		Nodal segments	
					Percentage* of explants showed proliferation (Mean ± SE)	Average* no. of shoot buds per culture (Mean ± SE)	Percentage* of explants gave response (Mean ± SE)	Average* no. of shoot buds per culture (Mean ± SE)
BAP	BAP + NAA	BAP + IAA	Kn + NAA	Kn + IAA				
0.5	-	-	-	-	78.28 ± 0.03^{a}	2.91 ± 0.16^{a}	64.00 ± 0.08^{jk}	2.38 ± 0.11^{defgh}
1.0	-	-	-	-	87.25 ± 0.02^{a}	4.53 ± 0.19^{a}	68.03 ± 0.05^{klm}	2.59 ± 0.14^{efgh}
1.5	-	-	-	-	77.27 ± 0.03^{a}	2.86 ± 0.23^{a}	74.20 ± 0.08^{ef}	3.27 ± 0.09^{abcdef}
2.0	-	-	-	-	90.02 ± 0.21^{a}	4.69 ± 0.35^{a}	79.05 ± 0.22^{ac}	3.93 ± 0.19^{cde}
-	1.0 + 1.0	-	-	-	80.29 ± 0.01^{a}	0.33 ± 0.21^{a}	81.70 ± 0.07^{ij}	4.29 ± 0.34^{cdefgh}
-	1.0 + 1.5	-	-	-	82.24 ± 0.01^{a}	0.83 ± 0.17^{a}	83.26 ± 0.08^{bc}	3.67 ± 0.03^{abc}
-	2.0 + 1.0	-	-	-	95.04 ± 0.09^{c}	5.31 ± 0.14^{de}	84.07 ± 0.19^{cde}	4.51 ± 0.17^{abe}
-	2.0 + 1.5	-	-	-	86.17 ± 0.29^{f}	4.48 ± 0.11^{bdg}	87.01 ± 0.49^{cd}	5.26 ± 0.14^{cdf}
-	-	1.0 + 0.5	-	-	85.26 ± 0.03^{a}	4.48 ± 0.29^{bcg}	76.01 ± 0.07^{cde}	3.51 ± 0.22^{defgh}
-	-	1.0 + 1.0	-	-	75.31 ± 0.02^{a}	2.73 ± 0.11^{bdf}	75.33 ± 0.07^{fg}	3.34 ± 0.08^{cdgh}
-	-	2.0 + 0.5	-	-	81.29 ± 0.01^{a}	3.17 ± 0.51^{cd}	78.74 ± 0.08^{ab}	3.86 ± 0.29^{acfgh}
-	-	2.0 + 1.0	-	-	80.23 ± 0.14^{c}	2.74 ± 0.08^{b}	72.06 ± 0.08^{ac}	2.81 ± 0.10^{dfgh}
-	-	-	1.0 + 0.5	-	71.28 ± 0.03^{a}	2.33 ± 0.21^{a}	72.61 ± 0.07^{hi}	2.94 ± 0.22^{fgh}
-	-	-	1.0 + 1.0	-	63.31 ± 0.02^{a}	3.67 ± 0.21^{a}	83.18 ± 0.08^{mno}	4.37 ± 0.17^{bcdefgh}
-	-	-	2.0 + 0.5	-	68.27 ± 0.03^{a}	3.50 ± 0.22^{a}	70.23 ± 0.06^{mno}	2.72 ± 0.19^{fgh}
-	-	-	2.0 + 1.0	-	73.24 ± 0.01^{a}	4.67 ± 0.21^{a}	72.11 ± 0.07^{de}	2.00 ± 0.30^{abcde}
-	-	-	-	1.0 + 0.5	45.25 ± 0.02^{a}	1.53 ± 0.27^{a}	62.95 ± 0.06^{kl}	2.83 ± 0.31^{defgh}
-	-	-	-	1.0 + 1.0	56.29 ± 0.04^{a}	1.87 ± 0.51^{a}	66.12 ± 0.08^{lmn}	2.47 ± 0.26^{efgh}
-	-	-	-	2.0 + 0.5	73.27 ± 0.03^{a}	2.68 ± 0.19^{a}	68.92 ± 0.10^{bcd}	3.83 ± 0.40^{abcd}
-	-	-	-	2.0 + 1.0	70.01 ± 0.05^{a}	2.50 ± 0.13^{a}	69.03 ± 0.13^{bcf}	2.64 ± 0.13^{afh}

d = days. *values are the means of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

cytokinins due to its slower rate of metabolism. The importance of plant growth regulators on shoot propagation was highlighted in various studies. The synergistic effect of BAP with NAA has been shown in many other medicinal plants, *Centella asiatica* (Hossain et al. 2000), *Catharanthus roseus* (Islam et al. 2001), *Boerhaavia diffusa* (Roy 2008), *Stemona tuberosa* (Biswas et al. 2011), *Plectranthus amboinicus* (Rahman et al. 2015), and *Aloe vera* (Das and Rout 2018).

On the other hand, leaf segments did not response to produce shoot buds. Therefore, an attempt was made to induce callus tissue from leaf segments. For the induction of callus, when leaf segments were cultured on MS medium fortified with BAP and 2,4-D

individually and in combination with NAA or IAA it produced whitish friable callus in MS medium containing 1.0 mg/l 2,4-D + 1.0 mg/l BAP within 19-25 days of culture. The nature of calli in all the media compositions was whitish and friable. The leaf explants did not produce any callus when cultured them on MS medium containing only Kn. It was found that 2,4-D + BAP combination was better than the other combinations in terms of callus induction. Similar proliferation of callus tissue from leaf segments was also noted in other medicinal plants including *Mirabilis jalapa* (Ling et al. 2009), *Camellia sinensis* L. (Seran et al. 2007), and *Piper carniconnectivum* (Dos-Santos et al. 2015).

It is relevant to mention here that the nodal explants did not induce any callus tissue when cultured on the medium containing PGRs. In order to promote differentiation, the calli that produced from *C. callialatum* were further grown on a broad spectrum of PGRs supplemented MS medium. It was highly noticed that the whitish friable calli did not undergo any kind of differentiation and finally died.

Table 2. Effects of different concentrations and combinations of auxins and cytokinins for the elongation of micro-shoots in *C. callialatum*.

PGRs supplement in MS medium (mg/l)			Average* initial length (cm) of individual shoot bud (Mean ± SE)	Average* increase in length (cm) of shoot bud after 30 d of culture (Mean ± SE)
BAP	BAP + NAA	Kn + IAA		
1.0	-	-	1.62 ± 0.37 ^{ab}	3.87 ± 0.16 ^{adef}
1.5	-	-	1.46 ± 0.13 ^{ac}	3.91 ± 0.25 ^{agh}
2.0	-	-	1.33 ± 0.16 ^{ab}	4.53 ± 0.79 ^{abd}
3.0	-	-	1.53 ± 0.08 ^{agh}	2.91 ± 0.21 ^{aegh}
-	1.0 + 1.0	-	1.25 ± 0.34 ^{cd}	3.76 ± 0.22 ^{abd}
-	1.5 + 1.0	-	1.61 ± 0.25 ^{a}	3.59 ± 0.17 ^{abeg}
-	2.0 + 0.5	-	1.33 ± 0.08 ^{ad}	2.94 ± 0.32 ^{afg}
-	2.0 + 1.0	-	1.82 ± 0.63 ^{bc}	4.93 ± 0.34 ^{acd}
-	2.5 + 1.5	-	1.45 ± 0.17 ^{cd}	3.81 ± 0.19 ^{acgh}
-	3.0 + 0.5	-	1.38 ± 0.10 ^{d}	2.89 ± 0.27 ^{ab}
-	3.0 + 1.0	-	1.69 ± 0.18 ^{ab}	3.47 ± 0.09 ^{afg}
-	-	1.5 + 1.0	1.53 ± 0.33 ^{ac}	2.75 ± 0.32 ^{bch}
-	-	2.0 + 0.5	1.41 ± 0.06 ^{ad}	3.32 ± 0.19 ^{cfh}
-	-	2.0 + 1.0	1.72 ± 0.04 ^{cd}	3.07 ± 0.28 ^{abc}
-	-	2.5 + 1.5	1.38 ± 0.16 ^{d}	2.59 ± 0.32 ^{bcdh}

d = days. *values are the means of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

For further improvement in shoot growth and length, the directly produced shoot buds were sub-cultured and transferred to elongation medium under aseptic condition. The highest increase of shoot buds within 20 d of culture (4.93 ± 0.34 cm) was recorded on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/1 NAA (Table 2) shows the best elongation of shoot buds of *C. callialatum*. The effect of BAP in combination with NAA has been noticed in many other medicinal plant species such as *Plumbago indica* L. (Bhadra et al. 2004), *Vitex negundo* (Hiregoudar et al. 2006), *Plumbago zelynica* (Chaplot et al. 2006) and *Gynura procumbens* (Keng et al. 2009).

Table 3. Effects of different concentrations and combinations of plant growth regulators (PGRs) on the development of roots from elongated shoots of *C. callialatum.*

½MS + IBA	½MS + IAA	½MS + NAA	½MS + IBA + IAA	Percentage* of micro shoot produced roots (Mean ± SE)	Average* number of roots per shoot (Mean ± SE)	Average* length (cm) roots after 30 d of culture (Mean ± SE)
½MS without PGRs				66.05 ± 0.14 ^{cf}	3.42 ± 0.18 ^{abch}	1.89 ± 0.11 ^{acg}
0.5	-	-	-	84.12 ± 0.17 ^{abc}	5.13 ± 0.19 ^{acdg}	3.16 ± 0.20 ^{bdf}
1.0	-	-	-	85.46 ± 0.13 ^{bcg}	5.73 ± 0.14 ^{ab}	3.38 ± 0.43 ^{abcd}
2.0	-	-	-	95.00 ± 0.16 ^{ab}	6.82 ± 0.10 ^{cd}	3.67 ± 0.38 ^{abcgj}
-	0.5	-	-	76.53 ± 0.18 ^{abd}	4.29 ± 0.16 ^{acf}	2.83 ± 0.09 ^{bchi}
-	1.0	-	-	81.15 ± 0.04 ^{afh}	4.93 ± 0.28 ^{abg}	3.03 ± 0.15 ^{abc}
-	2.0	-	-	70.11 ± 0.15 ^{bcd}	3.65 ± 0.13 ^{abc}	1.73 ± 0.10 ^{bc}
-	-	0.5	-	80.23 ± 0.19 ^{ac}	4.57 ± 0.11 ^{ac}	2.97 ± 0.66 ^{ghi}
-	-	1.0	-	75.22 ± 0.33 ^{ad}	4.06 ± 0.32 ^{d}	2.71 ± 0.06 ^{fhj}
-	-	2.0	-	72.45 ± 0.08 ^{ach}	3.84 ± 0.08 ^{abc}	1.68 ± 0.07 ^{ahj}
-	-	-	0.5 + 0.5	88.18 ± 0.11 ^{cef}	6.23 ± 0.14 ^{afg}	3.46 ± 0.24 ^{cej}
-	-	-	1.0 + 1.0	81.39 ± 0.13 ^{bdh}	3.91 ± 0.17 ^{bfh}	2.62 ± 0.04 ^{dhj}
-	-	-	2.0 + 2.0	82.16 ± 0.16 ^{abg}	3.98 ± 0.07 ^{ac}	2.24 ± 0.14 ^{ad}
-	-	-	2.5 + 2.5	76.26 ± 0.03 ^{bce}	4.90 ± 0.10 ^{b}	2.12 ± 0.20 ^{dhi}

d = days. *values are the means of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

In order to produce complete plantlets, elongated shoot buds at a length of 2-3 cm were individually transferred to rooting media. At first, half-strength MS medium without any PGRs was used for rooting experiments. For more efficient rooting, the half-strength MS medium was fortified with different concentrations and combinations of auxins (IBA, IAA and NAA). Development of strong root system was very much dependent on the concentrations of MS basal medium and auxins. Root initiation was noticed within 25-30 days of inoculation. Among three types of auxins, IBA was more effective than NAA and IAA in term of induction of number of root per shoot. The highest rooting percentage (95%) was recorded in half strength MS medium supplemented with 2.0 mg/l IBA. The maximum number of roots (6.82 ± 0.10) per shoot was formed in half-strength MS medium fortified with 2.0 mg/l IBA and the highest

elongation (3.67 ± 0.38 cm) of root was also recorded in this medium (Fig. 1D, Table 3). The elongated shoot buds were also cultured on full and quarter strength MS media with or without any auxins for root induction but the response was not satisfactory. The efficiency of IBA in *in vitro* rooting has been noted in many other medicinal plant species including, *Mentha viridis* (Raja and Arockiasamy 2008), *Heliotropium indicum* (Hassan et al. 2010), *Bacopa monnieri* (Jain et al. 2014), *Satureja punctate* (Teshome et al. 2016).

For acclimatization of the *in vitro* developed complete plantlets in the natural environment, the culture vessels with well-profuse rooted plantlets were taken out of the culture room and kept in ordinary room temperature with gradual increasing the time up to 5 days. Finally, the plantlets were taken out from the culture vessels and washed with tap water for complete removal of agar attached to the roots. Then, the plantlets were planted in small plastic pots containing garden soil and compost in the ratio of 1 : 1 (Fig. 1E) and were kept in room temperature for 3-5 days. After gradual acclimatization, the pots with *in vitro* developed plantlets were finally transferred to outside natural environment. A few of the transferred seedlings died but those survived grown as normally healthy plants. On average, 75% of the plantlets survive finally. There was no detectable variation between *in vitro* raised and normal plants in respect of morphological and growth characteristics indicating the absence of somaclonal variation at morphological level.

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