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Micropropagation of an endangered medicinal herb *Chlorophytum borivilianum* Sant. et Fernand. in bioreactor

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Chlorophytum borivilianum **Sant. et Fernand. is an endangered herb, the tuberous roots of which are source of medicinally important steroidal saponins. In the present study, propagation of *C. borivilianum* using a bench top stirred bioreactor with liquid medium via multiple shoot culture has been reported. One week old shoots along with shoot base part (1.5 cm) obtained from shoots regenerated *in vitro* in liquid medium shake flasks containing 22.2 µM 6-benzylaminopurine, were used as explants. An inoculum density of 120 explants/2.5 L liquid Murashige and Skoog medium supplemented with 22.2 µM 6-benzylaminopurine was found optimal for shoot growth. After three weeks of culture, 4.4-fold increase in biomass (fresh weight) was obtained. Shoots regenerated in bioreactor were rooted *ex vitro* on three-fourth strength liquid MS medium supplemented with 9.8 µM indole-3-butyric acid. Plantlets with 100% rooting of microshoots were hardened and established in the glasshouse with 85% survival rate. Due to rapid and efficient propagation in bioreactor with high survival rate, this protocol may be employed for conservation and large-scale multiplication of *C. borivilianum*.**

Key words: Bioreactor, *Chlorophytum borivilianum*, hyperhydricity, saponins, shoot culture.

INTRODUCTION

Chlorophytum borivilianum commonly known as safed musli (family Liliaceae) is a monocotyledonous plant. Tuberous roots of safed musli contain steroidal saponins (neohecogenin, neotigogenin, stigmasterol, tokorogenin) that are used as tonic and aphrodisiac (Kaushik, 2005; Tandon et al., 1992; Deore and Khadabadi, 2010). Saponins also possess anti-tumour activity (Kumar et al., 2010). They are used in the industrial production of sex hormones, corticosteroids and steroid derivatives. The roots of the plant are widely used in the Indian system of medicine for the treatment of weakness, impotency and sterility; to enhance male potency; as cardiac and brain

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tonic; as a curative agent in various diseases like piles, diabetes and as diuretic and hemostatic. *C*. *borivilianum* has been used along with other plants such as *Asparagus adscendens*, *A. racemosum*, *Curculigo orchioides* and *Withania somnifera* in many herbal and Unani formulations (Thakur et al., 2009; Ramawat et al., 1988). Due to its therapeutic activity and diversified uses, there is demand for safed musli especially *C. borivilianum* in India. Many countries in Gulf, Europe and USA have been major importers of the dry roots of safed musli for preparation of various herbal products in pharmaceutical, phytopharmaceutical and nutraceutical industries. While, the global demand for dry safed musli has been estimated in the order of 35,000 tonnes per annum, the supply stands at 5,000 tonnes per year (www.farmwealthbiotech.com, 2010). Safed musli is a rhizomatous herb propagated vegetatively in nature by shoot buds from perennating root tubers in the soil and sexually by seeds. Seeds have poor germination percentage (11 to 24%), low viability and long dormancy period leading to scarcity of tuberous roots of the plant in nature (Bordia et al., 1995). Conventional techniques for vegetative propagation of planting material are slow and cannot keep pace with the present demand (Bordia et al., 1995). About 95% of the indigenous requirement comes through wild habitats. Due to large-scale and indiscriminate collection of wild material and insufficient attempts to allow its replenishment, *C. borivilianum* is rapidly disappearing. Besides, the scarcity of elite and characterized planting material and non-availability of improved agro technological practices have also limited its commercial cultivation. There is need for conservation and mass multiplication of this plant. The plant has been enlisted as an endangered plant species in Red Data Book of India (Nayar and Sastry, 1988) and National Medicinal Plant Board (NMPB), India has categorized it as one of the prioritized plant species to be promoted for conservation and large-scale cultivation and export of this herb is being actively encouraged by Government of India through NMPB. Therefore to fill the gap between demand and supply of its tuberous roots to meet Industry's requirement and to provide cost-effectiveness, genetically uniform planting material for conventional propagation at a rapid rate in a short span of time, alternate propagation strategies such as micropropagation are urgently needed which will lead towards conservation and mass production of the plant.

Micropropagation serves as a means of clonal propagation of economically important species and as a tool for germplasm conservation. Micropropagation technology is advantageous due to production of high-quality disease-free, true-to-type plants independent of seasonal and other environmental conditions in a comparatively smaller space (Gurel, 2009), but commercial micropropagation is largely limited by the higher cost of plant production (Kozai et al., 1997). Liquid culture was reported to be ideal in micropropagation for automation and reduced manual labour (Roels et al., 2006) and for reducing plantlet production cost (Scheidt et al., 2009). Many plants have been mass-propagated in the liquid medium using bioreactors (Mehrotra et al., 2007). Automation of micropropagation in bioreactors has been advanced as a possible way of reducing cost of micropropagation (Son et al., 1999; Ibaraki and Kurata, 2001; Paek et al., 2001). Various types of simple bioreactors with mechanical or gas-sparged mixing were used in plant and cell cultures to provide stirring, circulation and aeration (Paek et al., 2005; Yesil-Celiktas et al., 2010). Mechanically stirred bioreactors depend on impellers, including a helical ribbon impeller, magnetic stirrers or vibrating perforated plates (Archambault et al., 1994). Aeration, mixing and circulation in bubble-column or airlift bioreactors are provided by air entering the vessel from the side or from the basal opening through a sparger. As the air bubbles rise, they lift the plant biomass and provide the required oxygen (Preil, 1991). Oxygen (O_2) requirements may vary from one species to another and concentration of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow and air bubble size. The other key parameter is mixing, which is necessary to distribute cultured cells or tissues and nutrients equally throughout the liquid phase (Honda et al., 2001). Mixing is normally carried out by sparging, mechanical agitation or a combination of these two, but the magnitude of hydrodynamic forces associated with mixing should be small enough not to cause cell or tissue damage, but sufficient to stimulate desired cell/tissue growth. In the present study, *in vitro* propagation of *C. borivilianum* in a bench top stirred bioreactor of 5 L working capacity, employing liquid medium has been reported for scaling-up cost-effective mass propagation of the plant.

MATERIALS AND METHODS

Bioreactor configuration

In the present study, experiments for large-scale culture of *C. borivilianum* were done by using a bench top stirred bioreactor (Bio Flow-110, M/s New Brunswick Scientific, USA) having working volume of 5 L. The impeller in this air sparged top driven system provided mechanical agitation (75 rpm). The bioreactor consisted of a thick glass jacketed vessel assembly having inner vessel with removable baffle assembly consisting of different probes including dissolved oxygen (DO), pH and temperature probes to control and optimize various culture conditions. The inner vessel assembly was surrounded by an outer thick glass jacket. The external jacket was used as a water temperature controller. The whole glass culture vessel rested on a double walled stainless steel base through which cold water (4°C) was circulated, which maintained the temperature of the culture medium. An exhaust gas condenser was installed to the unit for minimizing the medium evaporation during culture period. All parts of the bioreactor assembly were thoroughly washed and then surface sterilized with ethyl alcohol prior to assembling the unit and lubricated with silicone grease to make the unit air tight. About 2.5 L liquid (without agar), Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented

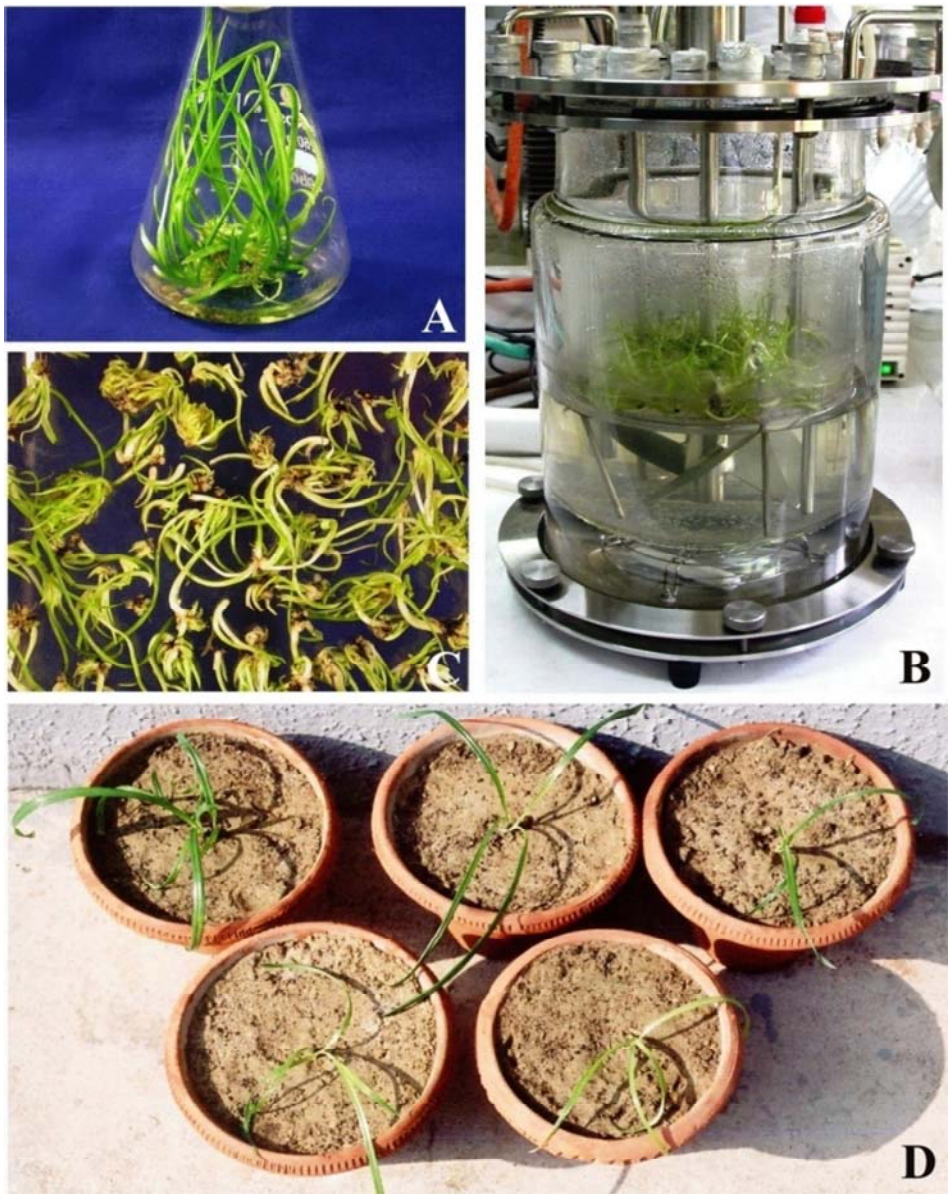

Figure 1. Propagation of *C. borivilianum* in bioreactor: *in vitro* regenerated shoots in liquid medium shake flasks from which explants were obtained (A), shoots cultured in bioreactor (B), shoot biomass harvested from bioreactor after 3 weeks of culture (C) and plants raised from bioreactor culture in earthen pots after 10 weeks of hardening (D).

with 22.2 µM 6-benzylaminopurine (BAP) and 3% (w/v) sucrose were poured in the culture vessel. The pH of the medium was adjusted to 5.8 ± 0.1 using 0.1 N HCl or 0.1 N NaOH prior to autoclaving. Earlier, experiments amongst a wide range of BAP (2.2 to 40.0 µM) tested for their efficacy on *in vitro* shoot multiplication, BAP at 22.2 µM level exhibited optimal response. During the experiments, an autoclavable nylon mesh (pore size 200 µM) tightened just beneath the surface of culture medium on the lower stainless steel semi-circular ring of the baffle assembly prevented the explants from sinking to the bottom or getting submerged in the medium (Figure 1 B). The explants thus remain floating near the medium surface with the support of mesh during the culture period and therefore, they will not become completely immersed in the medium. The incoming air was passed within the medium at 0.5 L/min rate through air sparger after sterilization by a hydrophobic sterile membrane filter (Whatman, USA, 0.22 µ). The complete bioreactor unit was properly assembled and autoclaved at 121°C and 15 lb pressure for 25 min in a vertical cylindrical sterilizer (M/s Yarco, India).

Inoculation of bioreactor vessel

Explants, consisted of one week old shoots along with shoot base part (1.5 cm in length) obtained from shoots regenerated *in vitro* in liquid medium shake flasks (Figure 1A), were aseptically transferred from Erlenmeyer flasks containing MS liquid medium supplemented with 22.2 µM BAP to the sterilized bioreactor culture

Table 1. Shoot regeneration and growth of *Chlorophytum borivilianum* shoot base explants cultured in bioreactor for 3 weeks.

Volume of medium (L)	Inoculum density		Shoot growth response (%)	Final f.wt. of harvested biomass	Percentage increase (biomass)
	Number of explants inoculated	Total f.wt. (g) of the inoculums			
2.50	120	11.90	75	52.36	4.4-fold

f.wt. = fresh weight.

vessel containing same liquid culture medium. Mother plants were obtained from natural habitat (Rajsamand District in the State of Rajasthan). Amongst these mother plants, a high yielding line of *C. borivilianum* selected on the basis of root yield, was maintained in Botanical Garden of the Institute. *In vitro* shoot cultures were established from young shoot apices obtained from the tuberous roots of this selected high yielding line. Approximately, 120 explants (inoculum density) having fresh weight of about 11.90 g were aseptically transferred from Erlenmeyer shake flasks to the sterilized bioreactor through its inoculation port. After inoculation, bioreactor culture vessel was installed on the main control module. Water lines were connected to the water jacket inlet and to the exhaust condenser. All air or gas lines were also reconnected. The pH and DO cables were connected to their respective probes and to the pH and DO control module. The temperature probe was reinstalled into the thermowell. Air sparger was connected to air compressor through silicone tubing fitted with 0.22 µ sterile microfilter (Whatman membrane filter, USA). Culture conditions included 16:8 h light: dark alternating regime of 45 µmol m^{-2} s^{-1} intensity provided by cool white fluorescent tubes, 25 ± 1°C incubation temperature and 50 to 60% relative humidity. Final shoot biomass (fresh weight) and increase in biomass (fresh weight) over initial biomass (fresh weight) were recorded after 3 weeks. The experiment was repeated thrice. Observations are mean of three experiments.

Rooting and acclimatization of *in vitro* regenerated plants

Shoots (3 to 4 cm) regenerated *in vitro* in bioreactor after three weeks of culture of shoot base explants on MS medium supplemented with 22.2 µM BAP, were washed with water and placed at the top of culture tubes filled with three-fourth strength MS nutrient solutions supplemented with 9.8 µM indole-3-butyric acid (IBA) or three-fourth strength MS medium without any growth regulators (control) for *ex vitro* rooting. The shoots regenerated in bioreactor were also kept for *in vitro* rooting on the same culture media as in case of *ex vitro* rooting. The 9.8 µM IBA level was found optimal for rooting of *C. borivilianum* shoots amongst various levels (0.2 to 24.6 µM) of IBA added to three-fourth strength MS liquid medium in the previous experiments. The culture tubes containing shoots regenerated *in vitro* in bioreactor were kept in the culture room for 3 weeks under similar culture room conditions as described above for rooting and then rooted plants were transferred to earthen pots consisting sand: soil: farmyard manure in 1:1:1 (v/v) ratio. Potted plants were covered with transparent polythene bags with small holes for air ventilation to ensure high humidity in initial stages. The polythene bags were removed after 2 to 3 weeks and the surviving plants were maintained in the glass house under 16 h photoperiod at 28 ± 2°C. Number of roots, rooting percentage and root length were recorded after 3 weeks of culture on IBA containing MS nutrient medium as well as on MS medium without growth regulators. The survival percentage was recorded after 12 weeks of transfer of plants in pots.

RESULTS AND DISCUSSION

Up-scaling of shoot cultures of *C. borivilianum* in bioreactor

After 3 weeks of incubation period, the growing shoots were harvested and growth response was recorded (Figure 1C). About 75% response in terms of shoot regeneration and growth was observed and biomass yield of 52.36 g (fresh weight) was recorded after 3 weeks while initial biomass (fresh weight) of all inoculated explants was 11.9 g. Thus, 4.4-fold increase in biomass (fresh weight) over the initial inoculum biomass (fresh weight) was observed in the present investigation after 3 weeks of culture period (Table 1). Large-scale plant tissue culture using bioreactors is promising for industrial plant propagation. It offers various advantages including controlled supply of biochemicals independent of plant availability (cultivation season and pests), consistent quality of the product and it also overcomes the drawbacks of plant cell culture systems. *In vitro* tissue culture using bioreactors enables the production of genetically identical individuals from an elite plant and allows plant multiplication, free of pathogens in less time as compared to conventional methods. The use of liquid culture decrease the plantlet production cost (Scheidt et al., 2009) and they are ideal for automation. Many plants especially medicinal plants have been mass-propagated in the liquid medium using bioreactors (Paek et al., 2005; Mehrotra et al., 2007).

In the present study, the explants size of 1.5 cm resulted in the high shoot growth in bioreactor. While in the case of apple rootstock, the explants size of 0.5 cm or 1 cm resulted in higher shoot production rate as compared to explants size of 1.5 cm (Zhu et al., 2005). The inoculum density in the present investigation was 120 explants in 5 L bioreactor which resulted in high shoot growth in *C. borivilianum*. While optimum inoculum density in a 3 L bioreactor was 200 explants for rhizome multiplication and shoot differentiation in *Cymbidum niveo-maginatum* (Jin et al., 2007). In the present study, high shoot growth was obtained on full strength MS medium supplemented with 22.2 µM BAP. On the other hand, differentiation of *Lilium* bulblets on bulb scales, cultured in bioreactors, was better on half-strength than

on full strength MS medium (Takayama, 1991).

In the present study, in a bench top stirred bioreactor employing liquid medium with helical ribbon impeller and gas sparged mixing, biomass increase of 4.4-fold was obtained. The presumed reasons for faster growth in the liquid medium were thought to be better availability of nutrients and rapid uptake of nutrients. A biomass increase of six-fold was obtained without need for periodic transfer of explants to fresh medium in *Vaccinium corymbosum* (Ross and Castillo, 2009). This represents an effective reduction of cost, where intensive manual handling is the main component. Also, during entire culture period of 3 weeks of *C. borivilianum* in bioreactor, there was no periodic transfer of explants/growing shoots to fresh medium thus removing the need for additional manual labour resulting in cost-reduction. An additional reduction in cost derived from the use of liquid medium thus avoiding the use of agar which otherwise adds up to 65% of the cost of the culture medium (Mitra et al., 1998). However, the use of liquid media in bioreactors can lead to the problem of asphyxia and hyperhydricity in explants as a result of immersion. Hyperhydricity induces morphogenic abnormalities in the developing plantlets (Haq and Dahot, 2007) and subsequently affect their survival. Attempts to control hyperhydric deformities have been largely focussed on better aeration and intermittent or partial plant submergence in the medium using temporary immersion bioreactors (Sajid and Pervaiz, 2008; Farahani and Majd, 2012). In the present study, the explants/growing shoots were supported by nylon mesh in the way that their lower part was continuously immersed in the culture medium, on the other hand, upper portion was exposed to air thus avoiding hyperhydricity of the growing shoots and also preventing sinking of the explants/growing shoots to the bottom of the culture vessel. Similar with our observations, shoot growth and subsequently growth of plantlets in *Anoectochilus formosanus* was more efficient in continuous immersion (with net support) bioreactor as compared to continuous immersion (without net support) and temporary immersion bioreactor culture in liquid media using ebb and flood (Wu et al., 2007). Shoot growth and proliferation were most efficient in balloon type bubble bioreactor (BTBB) having continuous immersion culture with a net to avoid the complete immersion of plant material in garlic as compared to BTBB (immersion culture without net and ebb and flood culture), (Kim, 2002).

In the present study, principles of both mechanically stirred and gas sparged bioreactors were employed. Aeration and circulation were provided by filtered sterile air at the rate of 0.5 L/min throughout the culture duration by two spargers, one above the autoclavable nylon mesh and another just below the mesh and mechanical agitation was provided by a helical ribbon impeller at the rate of 75 rpm for mixing of nutrients and plant growth regulators throughout liquid phase. The aeration at the rate of 0.5 L/min and agitation at 75 rpm were found optimal for the shoot growth in the present investigation. Lack of oxygen in the liquid media containing small explants and asphyxia and hyperhydricity of explants as a results of immersion, are the major limiting factors to their growth (Farahani and Majd, 2012). Supply of compressed air inside the bioreactor chamber for decreasing humidity significantly reduced the hyperhydricity during the bioreactor culture of apple root stock 'M9 EMLA' plantlets (Chakrabarty et al., 2003). Also, supply of filtered sterile air through sparger at 0.5 L/min rate was found to be helpful in reducing hyperhydricity in *C. borivilianum*.

Rooting

In the present investigation, shoots obtained from the bioreactor after 3 weeks of culture, 3 to 4 cm long were rooted *ex vitro*. Shoots kept in the MS medium devoid of growth regulators (control) developed roots at a rate of 60% while shoots in 9.8 µM IBA-supplemented medium showed 100% rooting with 5 to 6 roots per plantlet. The length of roots was 3 to 4 cm. On the other hand, in *in vitro* rooting of microshoots of *C. borivilianum*, 40 and 80% rooting percentage were observed respectively on control medium (MS medium without growth regulators) and IBA supplemented medium which is lower than rooting percentage of *ex vitro* rooted microshoots. Sugarcane shoots regenerated in bioreactor were also rooted *ex vitro* on MS medium supplemented with 2.5 µM IBA and rooting response was noticed in three weeks with a success rate of 83% (Sajid and Pervaiz, 2008). On the other hand, in mass propagation of *V. corymbosum* in bioreactor, 80% rooting was observed *ex vitro* in the control treatment without IBA. While, exogenous addition of IBA resulted in inhibition of root differentiation (Ross and Castillo, 2009). In the present study on *C. borivilianum* micropropagation in bioreactor, roots regenerated *in vitro* were fragile while *ex vitro* derived roots were thick and have a good root system. The survival percentage of plantlets having *ex vitro* rooted shoots was 85% as compared to 70% in *in vitro* rooted shoots. These results show that bioreactor-derived microshoots of *C. borivilianum* are suitable for *ex vitro* rooting thus avoiding the need for *in vitro* rooting which is more time and labour-consuming. Consistent with our investigation, in the studies of Borkowska (2001) on micropropagated strawberry shoots, the *in vitro* formed roots were fragile and easily damaged. Plantlets that were rooted *ex vitro* had a larger root system and more runners than those formed by *in vitro*-rooted strawberry plantlets. The 60% rate of *ex vitro* rooting on growth regulator free medium may add towards the cost-effectiveness of rooting of bioreactor derived *C. borivilianum* microshoots.

Hardening and transplantation

Shoots regenerated in bioreactor with well developed roots after 3 weeks of growth on three-fourth strength MS

medium supplemented with 9.8 µM IBA, were transferred to glasshouse. *Ex vitro* rooted plantlets acclimatized to the glasshouse with survival rate of 85% (Figure 1D), while the survival percentage of plantlets having *in vitro* rooted shoots was 70%. Performance of plants established in glass house was uniform. The plants showed green colour and without mutations. In the studies of McGranahan et al. (2006), due to *ex vitro* rooting of microshoots of walnut rootstock genotypes, survival of rooted microshoots was improved to 80% for *ex vitro* rooted plantlets as compared to 50% for *in vitro* rotted ones of the same genotypes. *Ex vitro* rooted plantlets grew faster in the greenhouse. In low bush blueberry, micro cuttings regenerated in bioreactor performed well in the greenhouse and rooted plants have survival rates of 90 to 99% (Debnath, 2009). While, in the present investigation; *C. borivilianum* plants regenerated in bioreactor and rooted *ex vitro* exhibited 85% survival rate.

Conclusion

In conclusion, this report presents a protocol for *C. borivilianum* micropropagation for the first time in a bioreactor system. About 75% response in terms of shoot regeneration and growth and 4.4-fold increase in biomass (fresh weight) was observed in the present investigation after 3 weeks of culture. The *ex vitro* rooting of bioreactor-derived microshoots of *C. borivilianum* is important in economic sense as it is more cost-effective, less time and labour-consuming as compared to *in vitro* rooting. The rooting at the rate of 60% on growth regulator free medium may be important in cost-effectiveness of micropropagation of *C. borivilianum* in bioreactor. Thus, due to rapid and efficient shoot proliferation, biomass increase, efficient rooting and successful transfer to glasshouse with 85% survival rate, this protocol can be used for large-scale propagation and conservation of this important medicinal plant.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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