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Micropropagation of *Gloriosa superba* L. Through High Frequency Shoot Proliferation

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Abstract

Ninety two per cent of the cultures of apical and axillary buds of young sprouts from naturally grown *Gloriosa superba* plants regenerated four shoots per culture in MS basal medium fortified with 1.5 mg/l BA + 0.5 mg/l NAA. Repeated subcultures in the same medium resulted in rapid shoot multiplication with eight shoots per culture. Addition of 15% (v/v) coconut water (CW) and 2 g/l activated charcoal increased the number of shoots up to 15 per culture. *In vitro* raised shoots rooted on half strength MS with 1.0 mg/l IBA + 0.5 mg/l IAA added as a supplement. Plants in the rooting medium were kept at normal room temperature for seven days before transplanting in pots. Thereafter they were reared for three weeks. The survival rate of regenerants was found to be 85 - 90%.

Introduction

Gloriosa superba L. is a semi-woody herbaceous branched climber, reaching approximately 5 meters in height. One to four stems arise from a single V-shaped fleshy cylindrical tuber. *G. superba* is a native of tropical Africa and is now found growing naturally in many countries of tropical Asia including Bangladesh, India, Sri Lanka, Malaysia and Myanmar. It is also planted outdoors in the southern United States. In cool temperate countries, it is treated as a greenhouse or conservatory plant. In Bangladesh the plant is naturally found to grow in Sal Forests of Dhaka and Gazipur districts. Different parts of the plant have a wide variety of uses especially within traditional medicine practiced in tropical Africa and Asia. The tuber is used traditionally for the treatment of bruises and sprains, colic, chronic ulcers, hemorrhoids, cancer, leprosy and also for inducing labour pains. Because of its similar pharmacological action, the plant is sometimes used as an adulterant of aconite (*Aconitum* sp.). Seeds and root tubers contain a valuable alkaloid, colchicine, as the major constituent. Colchicine is occasionally used in cytological and plant breeding research. Medicinally, the tuber is used as

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abortifacient, and in smaller doses it acts as a tonic, stomachic and anthelmintic. It is also used in the treatment of gout because it contains colchicine. Paste of the tuber is externally applied for parasitic skin diseases (Ghani 1998).

As *G. superba* is considered to be a herbaceous plant, little attention is given towards its propagation and culture. It is commonly grown from seeds and tubers. Since in Bangladesh the seeds and tubers are harvested extensively for their therapeutic uses, the plant stands the chances of becoming a threatened species in Bangladesh. In order to protect such endangered species from possible extinction, the exploitation of medicinal plants must be accompanied by conservation measures (Hamann 1991).

The present study was undertaken with a view to: (a) developing protocol for mass propagation of this important medicinal plant through *in vitro* culture and (b) ensuring production of genetically identical plantlets for further field culture as well as conservation.

Materials and Methods

Healthy and profusely growing vine of *G. superba* was collected from the local Sal (local name of *Shorea robusta*) forest and used as a source of explants. Terminal shoot tips and stem nodes with a single axillary bud were used for this purpose. The explants were washed thoroughly under running tap water, pre-soaked in 0.1% liquid detergent for about 30 min, wiped with cotton and dipped in 70% (v/v) ethanol for 1 min. They were then surface-sterilized with a 0.1% (w/v) mercuric chloride for 5 min, followed by three to five rinses with sterile distilled water inside a laminar air flow cabinet. The surface-sterilized explants were sized to 1 - 1.5 cm length containing a single node with an axillary bud or a shoot tip with an apical bud. The explants were placed vertically on the culture medium. The new shoots induced from the *in vitro* cultures were further used as an explant for adventitious shoot regeneration.

MS basal was used for shoot proliferation and adventitious shoot regeneration and half strength MS was used for *in vitro* rooting. All media were supplemented with 30 g/l sucrose, 7 g/l agar (Difco) and dispensed into 15 × 150 mm culture tubes, 250 ml conical flasks or disposed off jelly-bottles. The pH of the media was adjusted to 5.8 before autoclaving at 121^{o}C for 20 min. The cultures were incubated for a 16 h photoperiod at 24 ± 2^{o}C under a light intensity of 3 mmol m^{-2} s^{-2} emitted from cool white fluorescent tube lamps.

Shoot proliferation from shoot tips and nodal explants was obtained in two separate sets of experiments. In the first experiment 0-2.5 m/l BA and 0-2.5 mg/l Kn were incorporated into MS to select the best cytokinin for the response of shoot induction. In the second set, combinations of BA-Kn and BA-NAA were

assessed for shoot multiplication. For large amounts of shoot proliferation, coconut water and activated charcoal were added to the media. The number of explants showing shoot proliferation was recorded after three weeks of inoculation.

For *in vitro* rooting, individual shoots (3 - 5 cm) were cut from the proliferated shoot cultures and implanted onto half strength MS with different concentrations and combinations of NAA, IBA and IAA.

The rooted plants were taken out from the culture tubes, washed to free agar gel with autoclaved water and transplanted to plastic pots with sterile vermiculite, sand and soil (1 : 2 : 2) for hardening. The plantlets were kept in a polychamber at 80% relative humidity, 32 ± 2°C under a 12 h photoperiod for acclimation. The plants were given fertilizer with 1/8th MS macronutrients biweekly. Established plants were transplanted in earthen pots under natural conditions and the survival rate was recorded.

Results and Discussion

Shoot induction was not found in basal medium even after four weeks of culture. The explants were found to be swollen and they produced two to three shoots within three - four weeks after inoculation in MS containing BA alone but the number of shoots increased up to four when the explants were cultured in MS with 1.5 mg/l BAP + 0.2 mg/l NAA (Table 1, Fig. 1). Both shoot tips and nodal explants responded identically in the same medium. Kn alone and Kn-NAA combinations were not found suitable for shoot induction (data not shown).

Newly initiated shoots were separated and subcultured repeatedly in fresh MS with 1.5 mg/l BA + 0.2 mg/l NAA, where the number of shoots increased up to eight per culture. For further development of the medium, activated charcoal (AC) and coconut water (CW) were added together or individually. Addition of 15% CW and 2 g/l AC was found to increase the number of shoots up to 15 per culture (Fig. 2). Thus, the best medium determined for shoot multiplication was: MS + 1.5 mg/l BA + 0.2 mg/l NAA + 15% CW + 2 g/l AC.

Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently there has been much progress in this technology for conservation of genetic resources and clonal improvement (Barz et al. 1977; Datta and Datta 1985; Kukreja et al. 1989; Jusekutty et al. 1993; Maskay 1996; Wawrosch and Kopp 1999). Rapid shoot regeneration has been achieved with a wide range of species with initial explants being taken from normal aerial shoots of field grown herbaceous medicinal plant species (Jaiswal et al. 1989; Mathur et al. 1993; Maskay 1996; Rai 2002; Hall and Camper 2002). In the present investigations,

shoots were regenerated directly from explants of shoot tips and nodal segments of *G. superba*. Repeated subcultures of explants on fresh shoot proliferation medium helped to achieve continuous production of healthy shoot buds and shoots at least through five to ten subculture cycles. For further

Table 1. Effect of growth regulators in MS on morphogenetic response of *G. superba* shoot tips and nodal segments.

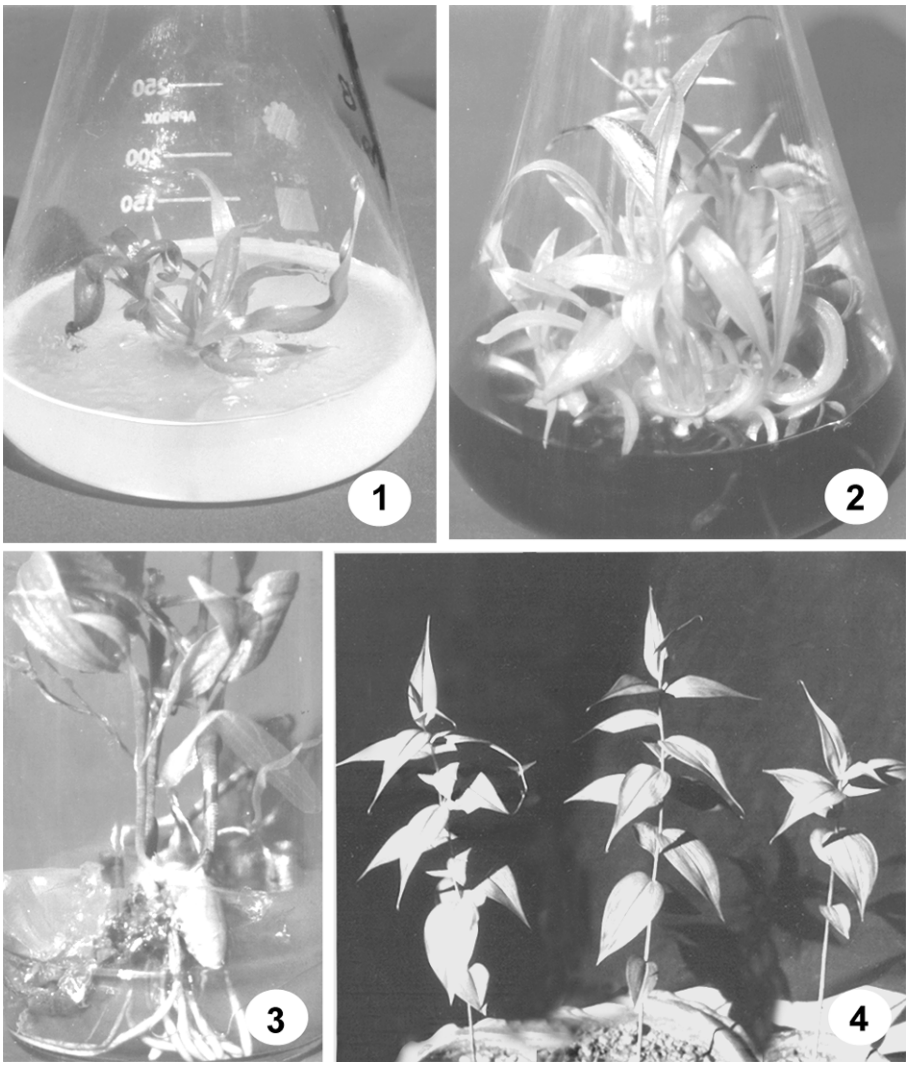
Growth regulators (mg/l)		Shoot tips		Nodal segments	
BAP	NAA	% of explants forming shoots	Mean No. of shoot/explant	% of explants forming shoots	Mean No. of shoot/explant
0.0	0.0	-	-	-	-
0.5	0.0	42 (3.3)	1.6 (0.4)	41 (4.3)	1.8 (0.6)
1.0	0.0	43 (3.6)	1.8 (0.5)	42 (3.8)	1.6 (0.8)
1.5	0.0	46 (4.4)	2.8 (0.5)	48 (3.9)	3.1 (0.7)
2.0	0.0	40 (4.3)	1.4 (0.5)	40 (3.8)	1.6 (0.6)
2.5	0.0	40 (3.5)	1.2 (0.7)	40 (3.8)	1.4 (0.8)
0.5	0.1	59 (5.9)	2.6 (0.9)	60 (4.8)	2.9 (0.7)
0.5	0.2	62 (5.3)	2.5 (0.6)	64 (4.9)	2.4 (0.6)
1.0	0.2	67 (5.1)	3.1 (0.2)	65 (5.3)	3.5 (0.4)
1.0	0.5	70 (6.9)	3.5 (0.4)	73 (6.9)	3.2 (0.5)
1.5	0.2	**92 (6.3)**	**4.1 (0.2)**	**93 (7.0)**	**4.3 (0.3)**
1.5	0.5	66 (4.1)	3.5 (0.5)	68 (3.9)	3.4 (0.4)
1.5	1.0	59 (4.8)	3.3 (0.3)	57 (4.2)	3.1 (0.4)
2.0	0.5	45 (3.9)	3.4 (0.4)	48 (3.8)	3.6 (0.5)
2.0	1.0	31 (3.7)	3.6 (0.4)	30 (2.9)	3.3 (0.6)
2.5	0.5	32 (2.9)	2.4 (0.3)	28 (3.1)	3.0 (0.3)
2.5	1.0	27 (3.5)	2.4 (0.3)	26 (2.9)	2.6 (0.4)

Results are mean ± SE of three experiments with 15 replications.

shoot multiplication and growth, addition of CW and AC in the nutrient media were effective. Positive effect of CW in nutrient medium is also reported by Roy (1998). CW of green nut is very effective in providing an undefined mixture of organic nutrients and growth factors (Gamborg and Phillips 1995). Pierik (1987) showed that the addition of AC often has a promoting effect on growth and organogenesis in plant species. Beneficial effects of activated charcoal were also found in *Muscari armeniacum* (Pierik 1987).

Rooting in regenerated shoots of *G. superba* was achieved at 90 per cent when the excised shoots were cultured individually on root induction medium, consisting of half-strength MS salts with 1.0 mg/l IBA and 0.5 mg/l IAA (Table 2). Roots initiated by the third week of culture and within the fifth week,

showed a tuberous structure at the base of the root (Fig. 3). Use of auxin/s singly or in combination for rooting was also reported by different authors (Sahoo and Chand 1998; Ajith and Seeni 1998; Rai 2002).



Figs. 1 - 4: *In vitro* regeneration of *Gloriosa superba* from shoot tip and nodal explants. 1. Multiple shoot induction in MS + 1.5 mg/l BA + 0.2 mg/l NAA in four weeks. 2. Enhanced shoot proliferation when subcultured in MS + 1.5 mg/l BA + 0.2 mg/l NAA + 15% CM + 2 g/l AC. 3. Rooting of *in vitro* regenerated shoots in half strength MS + 1.0 mg/l NAA + 0.5 mg/l IAA in four weeks. 4. Acclimatized regenerated plants of six months old.

After four - six weeks in the rooting medium the rooted shoots were transferred to pots. None of the plantlets survived when directly transferred from rooting medium to the pot under natural conditions. About 85 - 90 per cent of

the transplanted plants of *G. superba* survived if the plants in the rooting culture tubes were kept in normal room temperature for seven days before transplantation in pots and reared for three weeks. The plantlets were reared under semi-controlled temperature (30 ± 2°C) and light (2000 lux) in a chamber with 80 per cent humidity. During this period of acclimation shoots elongated, leaves expanded and turned deep green looking good and healthier (Fig. 4).

Table 2. Effect of auxin(s) on root induction in regenerated shoots of *G. superba* on half strength MS.

Auxin/s (mg/l)	% of shoots rooted (± SE)	Days required for root induction (± SE)
IBA 0.5	-	-
IBA 1.0	-	-
IBA 1.5	-	-
IBA 2.0	-	-
IBA 2.5	-	-
IBA 0.5 + NAA 0.5	20 (3.2)	26 (1.8)
IBA 1.0 + NAA 0.5	41 (1.2)	20 (1.6)
IBA 1.0 + NAA 1.0	56 (2.1)	24 (1.7)
IBA 1.5 + NAA 1.0	40 (3.9)	24 (2.6)
IBA 2.0 + NAA 1.0	50 (1.2)	24 (1.9)
IBA 2.0 + NAA 2.0	65 (3.1)	25 (1.8)
IBA 2.5 + NAA 2.0	64 (4.2)	24 (2.1)
IBA 0.5 + IAA 0.5	47 (3.9)	24 (1.7)
IBA 1.0 + IAA 0.5	**90 (4.3)**	**23 (1.8)**
IBA 1.0 + IAA 1.0	20 (1.0)	18 (2.8)
IBA 1.5 + IAA 1.0	14 (1.2)	18 (1.8)
IBA 2.0 + IAA 1.0	18 (1.2)	20 (2.1)
IBA 2.0 + IAA 2.0	22 (2.2)	23 (1.9)
IBA 2.5 + IAA 2.0	23 (3.8)	25 (2.4)
NAA 0.5 + IAA 0.5	31 (4.3)	21 (3.7)
NAA 1.0 + IAA 0.5	30 (0.0)	21 (2.6)
NAA 1.0 + IAA 1.0	28 (2.7)	21 (2.1)
NAA 1.5 + IAA 1.0	23 (4.5)	24 (2.0)

Data were recorded after four weeks of culture. Results are mean ± SE of 15 replications.

After three weeks, plants were transferred to an open place and gradually acclimated to outdoor conditions, where 90 per cent of plants survived. The technique described here appears to be readily adaptable for large scale clonal propagation and plantation for sustainable use in the industry. Moreover, by standardizing the protocols for clonal propagation of selected elite plants, it is possible to achieve a tenfold increase in the products per unit area of cultivation.

Clonally propagated plants would also have identical phytochemical profiles (Roja and Heble 1993)

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