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An efficient method of *in vitro* propagation of *Gloriosa superba* L. – an endangered medicinal plant

Tuhin Chatterjee and Biswajit Ghosh[Ψ]

Plant Biotechnology Lab, Post Graduate Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata-700 118, West Bengal, India

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ABSTRACT

Gloriosa superba L. is an important medicinal plant of India. It is an important source of pharmaceutical compound known as colchicine. This plant has become endangered in our country. To protect this species we adopted *in vitro* methodology to develop simple protocols for shoot multiplication as well as *in vitro* tuberization. Maximum shoots (15.67±0.34) were multiplied from tuber explants on MS medium fortified with 5.0 mg/l BAP. The elongated shoots with healthy tubers were subcultured for rooting and best response was on MS medium supplemented with 1.0 mg/l IBA. The *in vitro* raised plantlets were acclimatized in green house and successfully transplanted to natural condition with 75% survival rate. The regenerated plants were cytologically and phenotypically stable.

1. Introduction

Gloriosa superba (Glory lily) an important species of the family Colchicaceae, is a perennial tuberous climbing herb having attractive wavy edged flowers. (Amano et al., 2008). It is known as 'Malabar glory lily' in English, in Hindi as 'Kalihari', in Sanskrit as 'Agnisikha' having its trade name as 'Glory lily'. It is widely scattered in tropical and sub-tropical parts of Africa and Southeast Asia. In India, it is usually found in the Himalayan foot-hills, Tamil Nadu, Andhra Pradesh and West Bengal. Its flower is the national flower of Zimbabwe and also the state flower of Tamil Nadu in India due to its high ornamental value (Jana and Shekhawat, 2011).

Gloriosa superba L is one of the seven Upanishads in the Indian medicine, which cured many ailments, but may prove fatal on misuse (Joshi, 1993). It is an important medicinal plant and is a source of important pharmaceutical compound known as colchicine. Colchicine has been used in medicine for a long time. Colchicine has been effectively used in the treatment of several inflammatory conditions, such as gouty attacks, serositis related to familial Mediterranean fever, Behcet syndrome, and more recently also in acute and recurrent pericarditis. Growing evidence has shown that the drug may be useful to treat an acute attack and may be a way to cope with the prevention of pericarditis in acute and recurrent cases and after cardiac surgery (Ghosh *et al.,* 2002, 2007, Imazio *et al.,* 2009).

Gloriosa superba L. an industrially important medicinal crop of India, with high colchicine content, is still collected from wild. It is mainly vegetatively propagated but the rate is very low (Krause, 1986) as only 2 tubers are produced per year. The medicinal properties of the plant has led to it's over exploitation further inadequate cultivation and unsatisfactory attempts for its replacement has brought marked depletion of its wild resources. So, it has been affirmed as endangered plant by the IUCN Red Data Book (IUCN, 2010; Lal and Mishra, 2011; Sivakumar and Krishnamurthy, 2004, Contu 2013).

Its conventional propagation cannot meet the increasing demand and will ultimately lead to extinction if no attention is given to its conservation and propagation. *In vitro* methods

[Ψ] *Corresponding author; E-mail:* ghosh_b2000@yahoo.co.in

of propagation provide an alternate and effective means for rapid multiplication of species through continuous production to meet the demand for commercial exploitation. The objective of the present study was to develop a simple efficient protocol for large scale plant production of *Gloriosa superba* through tuber culture and to assess genetic stability of regenerants by chromosome analysis.

2. Materials and methods

2.1. Plant material

Gloriosa superba L. were collected from various locations of West Bengal, India and were grown in the experimental garden of RKMVC College, Rahara, Kolkata, India. Healthy tubers were collected from six months old plants used as explants.

2.2. Explants disinfection and implantation

Tubers were cut into round slices with each of them possessing axillary buds. They were washed thoroughly under running tap water for 15 min and then surface sterilized with 2% w/v solution of Bavistin® (systemic fungicide) for 20 min followed by 5% v/v solution of Tween 20 (liquid detergent) for 10 min. Then the explants were thoroughly washed with fresh water to remove the detergent. Finally, the explants were surface sterilized with freshly prepared 0.1% w/v solution of mercuric chloride ($HgCl_2$) for 15 min, rinsed in sterilized distilled water for 3 times to remove traces of $HgCl_2$ under the sterile condition. Then the explants were placed vertically onto sterilized MS media with and without Plant growth regulators.

2.3. Culture media

MS basal medium (Murashige and Skoog, 1962) with 3% sucrose was used for breaking the dormancy of shoot buds as well as for proliferation. The pH of the medium was adjusted to 5.6 ±0.2 using 0.1 N KOH or 0.1 N HCl as and when required and 0.8% (w/v) agar (Merck, India) before autoclaving. Medium was dispensed into each culture tube (25×150 mm), conical flask (250 ml) and jam bottle, plugged with non-absorbent cotton. The tubes were then wrapped in one layer of cheese-cloth and steam sterilized at 15 lb $inch^{-2}$ for 18 min.

2.4. Culture conditions

All the cultures were incubated at 24 ±2 °C temperature and 55 ±5% relative humidity under 16 h photoperiod at 55 ìmol $m^{-2}s^{-1}$ irradiance, supplied by Philips (Trulite 5 star 36 w/82 2700ÚK G84, made in India) fluorescent tubes.

2.5. In vitro shoot multiplication

The excised tuber explants were inoculated vertically on to MS medium supplemented with different concentration of BAP, Kn, 2iP, NAA individually and in combination and replicates thrice of each experiment. The multiple shoots were subcultured at every two weeks for 60 days. A control culture (only basal medium) was maintained to record the frequency of response.

2.6. Shoot elongation

After 40 days induced multiple shoots were excised individually and subcultured on to MS medium supplemented with BAP (1.0 mg/l) and varying concentrations of gibberellic acid (GA_3) (0.2, 0.4, 0.6, 0.8, and 1.0 mg/l) for elongation of shoots. A control culture (basal medium only) was also maintained.

2.7. In vitro root induction

Micro-shoots (1- 3 cm) were excised from the culture and transferred to full- strength MS medium augmented with different concentrations auxins viz., IBA, IAA and NAA (0.2, 0.5, 1.0 and 2.0 mg/l) and 2% sucrose (w/v, Merck, India) for root initiation. Micro-shoots were cultured in one tube each under the same culture conditions as described above. After 4 weeks the percentage of shoots forming roots, the number of roots per shoot and root length were assessed. One culture set was inoculated on basal MS medium without any plant growth regulator and considered as control.

2.8. Acclimatization

For hardening, at first the tissue culture derived healthy rooted plantlets were placed at room temperature for 7-10 days. Then the plantlets were removed from the agar medium, washed thoroughly under running tap water and transferred to earthen pots containing soilrite (Keltech Energies Ltd, Bangalore, India). To preserve moisture, the potted plantlets were covered with transparent polythene bag and the pots were placed on a plastic tray containing water under diffused light (16 h, photoperiod) in the poly-house for 30-35 days, thereafter the acclimatized plants were transferred to field condition under full sunlight.

2.9. Cytological study

Young and healthy root tips of *G. superba* were excised from the source plant as well regenerated plants in order to analyze the chromosome numbers. The root tips were pretreated with saturated solution of PDB for 6 hrs at 14^0C fixed in 1:3 acetic acid:dehydrated ethanol. Aceto-orcein staining technique was adopted. Chromosome plates were observed in Leica DM750 microscope and photographed with Leica DFC 295 camera. Minimum of 5 metaphase plates from each root tip were analyzed to determine the somatic chromosome number at the metaphase stage.

2.10. Statistical analysis

All the experiments were conducted under controlled conditions with three replications. Means and standard errors were carried out for each experiment and data was analyzed using one way Analysis of Variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Tukey's multiple range test at a 5% probability level. Data analysis was performed using SPSS v 16.0 software.

3. Results and Discussion

3.1. Role of plant growth regulators on in vitro *shoot induction and multiplication*

A number of experiments were conducted with a view of finding out optimum culture condition for maximum shoot multiplication from the culture of tuber explants. Multiple shoots developed from the tuber explants when they were cultured on to MS medium supplemented with different combination and concentration of BAP, Kn, 2iP alone and combination with NAA.

It was observed that multiple shoot buds induced from tubers when cultured on MS medium (Murashige and Skoog, 1962) containing cytokinins (BAP, Kn and 2ip) and auxin (NAA) (Table-1). Among the different cytokinins it was found that MS media supplemented with BAP was most effective in shoot induction and proliferation than others. Within 10 days of inoculation, explants swelling were observed. First appearance of buds was observed within 5-7 days. Multiple shoots commenced to emerge from the cut ends in case of BAP and Kn supplemented media. In MS media supplemented with NAA, poor multiplication was observed and a callus like structure was formed. A maximum response of 85% was recorded in MS media supplemented with 5.0 mg/ l BAP alone, where also the number of shoots induced per explants was highest 15.67 ± 0.34 with an average length of 6.56 ±0.03.cm (Fig.1a). It was also reported that BAP was the best cytokinin for *Aegle marmelos* (Nayak *et al.,* 2007). MS medium supplemented with Kn and 2iP showed comparatively less response. MS medium containing 1.0 mg/l Kn showed maximum response as compared to the five concentrations of Kn (0.5, 1.0, 2.0, 3.0 and 5.0 mg/l) assessed and showed maximum 57% sprouting frequency and an average of 5.66 ±0.30 number of shoots per explant segment. MS medium suplimented with 0.5 mg/ l 2iP showed maximum response of 44% as compared with other five concentrations of 2iP (0.5, 1.0, 2.0, 3.0 and 5.0 mg/l). Different combination and concentration among any two types of cytokinins (BAP + Kn, BAP + 2iP, Kn +2iP) or cytokinin with NAA (BAP+NAA, Kn+ NAA, 2iP +NAA), in MS medium failed to exhibit better shoot induction than single cytokinin (Table-1). Some stunted shoots were induced but their multiplication rate was significantly low. Similar results were also observed in *Curcuma* sp. (Yasuda *et al.,* 1987); *Kaempferia galanga* (Shirin *et al.,* 2000) and *Alpinia calcarata* (Amin *et al.,* 2001).

3.2. Shoot elongation

Excised shoots were cultured on to the MS basal medium consisting of 1.0 mg/l BAP in the presence of GA_3 at different concentrations for 2 weeks and were evaluated for shoot length. The result suggest that small amounts of GA_3 (0.2 mg/l) with 1.0 mg/l BAP were effective in stimulating *G. superba* shoot elongation. Shoots attained a maximum height of 10.2 cm during GA_3 treatments (Table-2). Similar nature of response was also found during *in vitro* culture of *Camella sinensis* (Gonbad *et al.,* 2014) and *Plumbago zeylanica* (Chatterjee and Ghosh, 2015).

3.3. Induction of roots from in vitro grown micro shoots

To develop a successful and consistent micropropagation protocol, easy and high frequency rooting from micro shoots is very important. Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil. To induced rooting, elongated shoots (2.0 – 4.0 cm) were cultured on to MS medium supplemented with different types of auxins at different concentration viz., IBA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l), IAA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) and NAA (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) individually. A control set up was also maintained using basal MS medium. Among the three different auxins tested, number of roots and root length varied. Maximum number of excised micro shoot induced roots within 25-30 days of culture. Among different types of auxin used in the present experiment, IBA was found to be the most effective at different concentrations tested for producing roots from the base of micro- shoots. Among different concentrations of IBA, 0.5 mg/l was found to be best concentration auxin for proper rooting of *G. superba* in which 85% shoots rooted within four weeks of culture (Table-3). The similar results were also reported in *Kaempferia galanga* (Shirin *et al.,* 2000), *Tylophora indica* (Haque and Ghosh 2013a) *Luffa acutangula* (Saha and Ghosh, 2014) *Plumbago zeylanica* (Chatterjee and Ghosh, 2015). The present findings are in agreement with those observed in similar rhizomatous plant species such as *Zingiber officinale* (Haque *et al.,* 1999); *Alpinia calcarata* (Amin *et al.,* 2001).

3.4 Acclimatization

The successfully rooted plantlets were transferred to small earthen pots containing soilrite and covered with

Table-1
Influence of different cytokines on shoot bud formation and multiplication from tuber explants of *G. superba*

Cytokinin	Concentration (mgl^{-1})	% of explants showing shoot bud induction	Mean No. of shoots/explants	Mean shoot length (cm)
BAP	0.5	62± 2.0^{c}	7.22±0.33^{de}	3.86±0.08^{f}
	1.0	68± 3.0^{b}	8.67±0.43^{b}	4.97±0.03^{b}
	2.0	72± 1.1^{b}	9.0±0.57^{a}	5.13±0.03^{a}
	3.0	78± 2.0^{a}	14.01±0.50^{c}	5.23±0.08^{d}
	5.0	85± 3.0^{a}	15.67±0.34^{hi}	6.56±0.03^{gh}
Kinetin	0.5	55± 1.0^{bc}	5.34±0.32^{fghi}	3.36±0.06^{i}
	1.0	57± 1.2^{b}	5.66±0.30^{d}	3.30±0.03^{g}
	2.0	45± 0.5^{cd}	4.66±0.35^{c}	2.33±0.05^{d}
	3.0	42± 1.0^{c}	3.34±0.58^{def}	2.03±0.03^{f}
	5.0	41± 1.0^{bc}	3.00±0.32^{ghi}	1.60±0.05^{g}
2iP	0.5	44± 1.3^{c}	3.67±0.34^{efgh}	1.33±0.03^{ih}
	1.0	42± 1.5^{cd}	3.32±0.33^{c}	3.83±0.03^{c}
	2.0	38± 0.1^{f}	2.34±0.56^{d}	2.83±0.03^{e}
	3.0	30± 2.0^{c}	2.00±0.58^{efg}	1.53±0.02^{gh}
	5.0	25± 1.3^{bc}	1.32±0.32^{i}	1.23±0.04^{i}
NAA	0.5	25± 1.0^{c}	1.33±0.44^{ef}	1.03±0.14^{f}
	1.0	20± 1.0^{ef}	1.02±04^{gh}	1.00±0.2^{i}
	2.0	Callus	Callus	Callus
	3.0	Callus	Callus	Callus
	5.0	Callus	Callus	Callus
BAP+Kn	0.5+0.5	57± 1.3^{c}	5.6±0.23^{a}	3.68±0.69^{g}
	1.0+0.5	44± 1.0^{cd}	4.2±0.37^{ij}	4.50±0.58^{cd}
	3.0+0.5	32± 1.1^{e}	3.36±1.02^{c}	3.02±0.51^{bc}
BAP+2iP	0.5+0.5	75± 0.3^{f}	3.30±0.65^{ab}	3.60±0.57^{i}
	1.0+0.5	79± 0.5^{fg}	2.20±0.26^{gh}	3.00±1.03^{e}
	3.0+0.5	75± 1.0^{gh}	1.05±0.95^{fg}	3.40±0.87^{a}
Kn+2iP	0.5+0.5	62± 1.2^{ghi}	2.20±0.91^{ghi}	3.30±0.44^{fg}
	1.0+0.5	70± 1.3^{cd}	2.23±0.65^{ab}	2.80±0.51^{e}
	3.0+0.5	60± 0.1^{cd}	1.20±1.20^{i}	3.70±1.02^{fg}
BAP + NAA	0.5+1.0	50± 1.0^{ghi}	2.0±1.2^{c}	3.12±1.3^{b}
	2.0+1.0	31± 1.0^{f}	1.12± 0.4^{d}	2.0±1.90^{cd}
	3.0+1.0	Callus	Callus	Callus
Kn + NAA	0.5+1.0	29± 1.0^{fg}	2.10±0.32^{cd}	1.79±2.1.2^{bc}
	2.0+1.0	20± 1.1^{gh}	1.17±0.12^{ef}	1.45±2.1^{cd}
	3.0+1.0	Callus	Callus	Callus
2iP + NAA	0.5+1.0	23± 0.2^{cd}	2.0±1.0^{def}	1.20±0.32^{def}
	2.0+1.0	16± 1.0^{gh}	1.0±.2^{efg}	0.07±0.21^{ghi}
	3.0+1.0	Callus	Callus	Callus

(Each value represents the mean ± SD of 10 replicates and each experiment was repeated thrice)

Table 2
Effect of GA_3 on shoot elongation of *G. superba* when cultured on MS medium supplemented with GA_3 and BAP (1.0 mg/l). (Data collected after 14 days of culture.)

BAP (mg/l)	GA_3 (mg/l)	Response for shoot elongation (%)	Mean length of shoot (cm)
1.0	0.2	85± 2.0^{a}	10.2 ± 1.0^{c}
1.0	0.4	82± 1.7^{c}	9.5 ± 0.5^{b}
1.0	0.6	80± 1.2^{b}	9.2 ± 1.5^{a}
1.0	0.8	75± 0.4^{c}	7.0 ± 1.2^{ab}
1.0	1.0	65± 1.1^{b}	5.0 ± 0.2^{ef}

Table -3
In vitro rooting of *G. superba* by using different types of auxins. (Data collected after 30 days of culture).

MSO + Auxins	% of rooting	No. of root / shoot	Root length (cm)
Control	-	-	-
IBA			
0.2	67±0.7^{c}	3.7±1.2^{c}	4.3±0.2^{b}
0.5	85±0.3^{a}	5.8±3.5^{a}	6.3±2.2^{a}
1.0	78±1.1^{b}	4.3±0.7^{c}	4.9±1.1^{c}
2.0	45±0.2^{c}	2.8±1.2^{e}	3.2±0.4^{bc}
IAA			
0.2	53±3.2^{f}	3.8±1.8^{cd}	3.9±2.3^{b}
0.5	65±2.1^{cd}	4.8±3.0^{a}	4.8±2.0^{a}
1.0	48±0.7^{e}	4.0±2.5^{b}	3.1±1.5^{c}
2.0	32±0.4^{c}	2.3±0.5^{e}	2.1±0.5^{bc}
NAA			
0.2	38±0.3^{fg}	3.5±0.5^{f}	3.2±0.2^{f}
0.5	31±0.1^{h}	2.7±1.0^{e}	2.1±0.7^{e}
1.0	23±0.1^{g}	1.8±0.7^{c}	1.2±0.1^{e}
2.0	callus	callus	Callus

transparent poly bags for hardening. All the plantlets were maintained in the culture room (25± 1^{0}C) conditions initially for 6-7 weeks and then transferred to normal laboratory in room temperature conditions and maintained for about 4- 5 weeks. Finally the plantlets were transferred to Poly House and maintained 3- 4 weeks and then were transferred to the experimental field condition (Fig.1c). It was observed that 75% ± 5 of the acclimatized plants survived in field condition. There was no noticeable variation among the acclimatized plants with respect to morphological and growth characteristics. All the micropropagated plants were free from external disease.

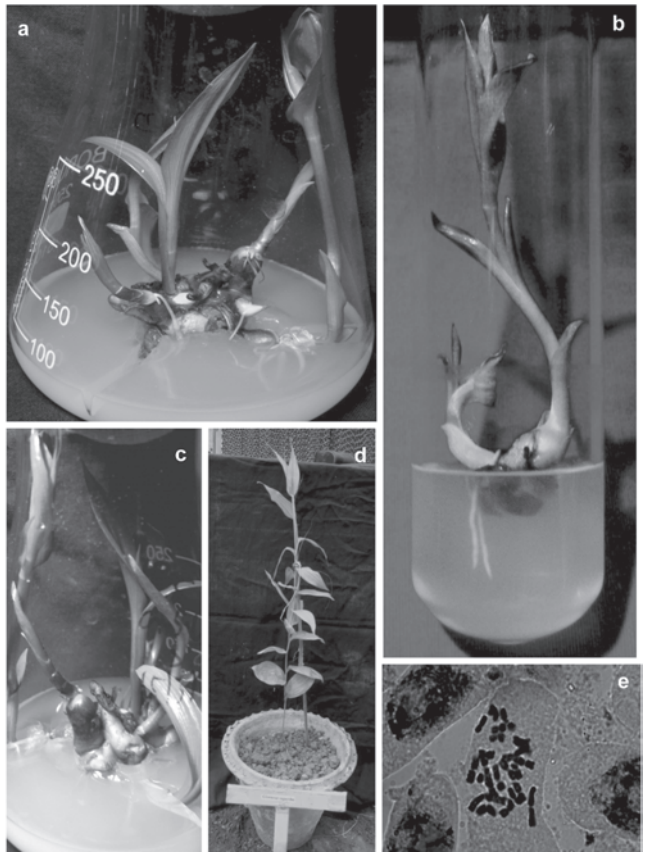

Fig-1. *In vitro* propagated plant of *Gloriosa superba.* (a). Multiplication of shoots from tuber, (b & c) Single complete plantlet, (d) ex-vitro growing tissue culture raised plant, (e) Root tip cell of ex-vitro growing plants showing metaphase stage with (2n= 22) chromosomes.

3.5 Chromosomal study

Chromosomal analysis in our present studies from randomly selected root tips from the source plant showed 2n= 22 chromosomes and cytological preparations from the *in vitro* derived 15 plantlets showed diploid number (2n= 22) of chromosomes (Fig. 1d). Thus the somatic chromosome complements of *in vitro* generated *G. superb* plants remained stable even after passing through three cycles of multiplication. Such mode of propagation can be utilized in other medicinal and economically important plants. A similar cytogenetically stable plant was also observed in *Asparagus cooperi* (Ghosh and Sen, 1992, 1994); *Gloriosa superba* (Ghosh *et al.,* 2007); *Aloe vera* (Haque and Ghosh, 2013b) and *Plumbago zeylanica* (Chatterjee and Ghosh, 2015).

Our studies provide a simple and effective protocol of *in vitro* mass propagation for *in vitro* storage of *G. superba*. In this study we also established genetic stability of regenerants through chromosome analysis reveling diploid chromosome number. The present protocol of *in vitro* propagation of *Gloriosa superba* may be highly useful for

raising quality planting material for commercial and off-season cultivation and also for genetic stock restoration.

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