



Callus proliferation and *in vitro* organogenesis of *Gloriosa superba* : An endangered medicinal plant

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Received: November 12, 2016; Accepted: November 26, 2016

Abstract: *Gloriosa superba* L. an important medicinal plant has become endangered due to over exploitation, as the tubers are rich source of an alkaloid, colchicine. Poor seed germination and tuber dormancy restricts its multiplication and thus the germplasm available is very less. So, there is urgent need to develop a protocol for its conservation and commercial propagation. Only 10-15% of seeds germinated after three weeks following cold treatment. Best shoot multiplication in sprouted tubers (88.67%) was observed in MS supplemented with 2.0 mg/l BAP, 0.5mg/l Kn and 1.0 mg/l GA₃ with 8.7±0.18 average number of shoots per explant. Long and healthy roots were observed in MS medium supplemented with 2.0 mg/l IBA resulting in 84.50% of root growth and average of 7.47±0.29 roots per explant. Organogenesis was obtained from embryogenic callus in MS medium supplemented with BAP, Kn and IBA. Further subculturing of embryogenic callus in MS medium containing 2,4-D, BAP and Kinetin resulted in globular structures while addition of only BAP resulted in heart shaped somatic embryos on the callus. Studies are on way to further use this protocol to increase the content of various alkaloids, with special emphasis on colchicine.

Key words: *Gloriosa superba*; endangered; *in vitro*; callus; somatic embryos; organogenesis

Introduction

Gloriosa superba L. belonging to family Colchicaceae, commonly known as glory lily is an important medicinal plant. It is also known Fire lily or Orange glow due to its showy flowers and is used as an ornamental plant worldwide (Bose and Yadav, 1989). It is a national flower of Zimbabwe and is also a state flower of Tamil Nadu. The plant tubers are toxic due to presence of rich alkaloid, colchicine which plays an important role in cytology by inducing polyploidy. The alkaloid colchicine also plays important role for having antiabortive, antimicrobial and anticancer activity (Reuter *et al.*, 2010; Nikhila *et al.*, 2014; Mahajan, 2015).

The major constraint for its growth is poor seed germination and tuber dormancy and the germplasm available is not much. The propagation in natural habitat is less and the plant has been affirmed as endangered due to its overexploitation for tubers which are the rich source of colchicine. There is threat to its genetic diversity (Rajagopal and Kandhasamy, 2009; Mahajan *et al.*, 2016) due to overharvesting, so there is an urgent need to conserve this plant using biotechnological approaches.

In the present paper, an improved protocol through callus mediated somatic embryogenesis for successful regeneration of plants is presented for *Gloriosa superba* which an endangered medicinal herb.

Material and Methods

Plant material and surface sterilization

The three explants seeds, tubers and intermodal segments were excised from young growing plants of *G. superba* collected from botanical garden, University of Jammu, Jammu and Kashmir, India. For collecting the seeds, the pods were bagged and the collected seeds were kept at 4°C for 24 hrs before sterilization. (Fig. 1A, 1B). The tubers and the intermodal segments were washed thrice with distilled water containing 2-3 drops of Tween 20 in distilled water for 15 minutes. Further sterilization treatments were carried out in Laminar Flow cabinet. The seeds were surface sterilized with 70% ethanol for 60 seconds followed by 0.2% HgCl₂ for 3- 4 minutes and then final washed twice with distilled water. Tubers and intermodal segments were surface sterilized with 0.1% HgCl₂ for 2 minutes and then each explant was washed thrice with distilled water for 5 minutes.

Establishment of cultures

Murashige and Skoog (1962) basal MS medium was prepared and pH 5.8 was maintained after adding 3% Sucrose. 0.7% agar was added to the medium before autoclaving. Surface sterilized seeds, tubers and intermodal segments were then inoculated on basal MS medium for the establishment of cultures and the cultures were kept in culture room at 25 ± 2°C at 16 hrs photoperiod. The three explants were regularly monitored for any further microbial contamination and also for their *in vitro* response. Since the best *in vitro* response was observed in the tubers so these were used for further experiments.

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<http://dx.doi.org/10.21746/aps.2016.12.005>

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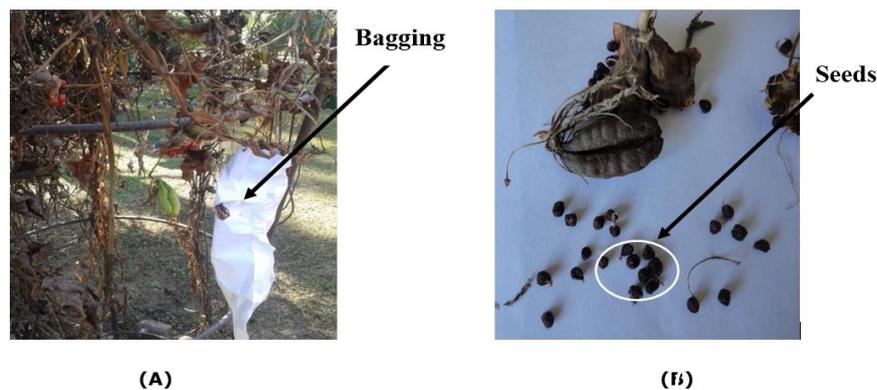


Figure 1: (A) Bagging of the *Gloriosa superba* plants (B) Seeds collected from the dry pods

Shoot induction and multiplication

After the initial establishment of cultures on basal MS medium, the plants were transferred on to modified MS medium containing growth hormones either singly or in different concentrations and combinations (Table 1). The concentration of GA₃ was kept constant at 2.0 mg/l. The plant cultures were maintained by regular subculturing of shoot cultures after every 2-3 weeks. The shoots per explant were counted and the length of the shoots was measured after six weeks.

Table 1: Different concentrations and combinations of growth hormones used in MS medium

Culture media	Conc. mg/l
MS + BAP	0.2,0.5,1.0,1.5,2.0,3.0,4.0
MS + Kinetin	0.2, 0.5, 1.0, 1.5
	1.0 mg/l BAP + 0.5mg/l Kinetin
MS + BAP + Kinetin	0.5mg/l BAP + 1.0mg/l Kinetin
	1.0 mg/l BAP + 1.0mg/l Kinetin

In vitro rooting and Acclimatization

After six weeks the well developed *in vitro* grown shoots were separated and cultured on MS medium supplemented with different concentrations of IBA (0.5 mg/l, 1.0 mg/l, 1.5 mg/l). The plants with well-developed roots were taken out from flasks after four weeks and then washed to remove any agar sticking to it. The rooted plantlets were transferred into small pots containing autoclaved sand and soil in the ratio 1:1. The plants were covered with glass jars to maintain humidity. After two weeks the jars were removed and the plants were allowed to grow further in glass house.

Callus induction and organogenesis

The leaves excised from *in vitro* grown plants of *G. superba* and were then inoculated on MS media supplemented with different concentrations of 2,4-D (1.0, 2.0, 3.0, 4.0 5.0 mg/l). The cultures were maintained at 25°C in dark. After four weeks, the induced callus was further subcultured on the same medium for its proliferation. The proliferated callus after three weeks was transferred to regeneration medium. For shoot induction, the proliferated callus was cut into pieces with sterilized blade and the pieces were sub cultured on MS medium supplemented with different concentrations of cytokinins i.e., BAP (1.0, 2.0, 3.0, 3.5, 4.0 mg/l) and Kn (0.25, 0.50, 0.75, 1.0, 1.5

mg/l) either alone or in combination. For root induction, the proliferated callus was transferred on MS medium supplemented with different concentration and combinations of IAA (1.0, 2.0 mg/l) and BAP (0.5, 1.0 mg/l). The dead and dark brown callus was removed at each step of subculturing.

Somatic embryogenesis

The friable and embryogenic callus was transferred to MS medium containing 3.0 mg/l 2,4-D for further proliferation and somatic embryogenesis. After three weeks, the embryogenic calli pieces were transferred to MS medium, supplemented with different concentrations and combinations of BAP and Kn. 2,4-D at concentration of 1.0 mg/l was kept constant.

Results

Shoot establishment and multiplication

In *Gloriosa superba* the seed germination is very poor, but 10-15% of seed germination was observed after cold treatment after three weeks of culturing (Fig. 2). Explants like nodal segments remained green for two weeks with no further growth. However, after two weeks the explants (nodal segments) turned brown and ultimately die. This could be due to the presence of high alkaloid concentration in *G. superba* plant. However, tubers when cultured on MS basal medium showed sprouting after three weeks (Fig. 3). Even, Hassan and Roy (2005) and Khandel *et al.*, (2011) did not observed any shoot regeneration in *G. superba* on MS basal medium after four weeks of culturing. The established sprouted tubers that showed shoot initiation were subcultured on modified MS medium supplemented with plant growth hormones after four weeks (Fig. 3). Best shoot multiplication was observed in sprouted tubers (88.67%), in MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l Kn and 1.0 mg/l GA₃ with 8.7±0.18 average number of shoots per explant (Table 2; Fig. 4A, 4B).

Table 2: Effect of different concentration of plant growth regulators in the culture medium on *in vitro* plant regeneration

S.No	Concentration (mg/l)	Percentage Shoot growth	Average no. of shoots per explant
1	MS + 0.5 BAP	0	0
2	MS + 1.0 BAP	66.20	2.7± 0.38
3	MS + 1.5 BAP	54.90	2.3± 0.43
4	MS + 0.5 Kn	0	0
5	MS + 1.0 Kn	26.23	1.6±0.76
6	MS + 1.5 Kn	11.54	1.1± 0.62
7	MS + 1.0 BAP + 0.5 Kn	68.60	6.8± 0.21
8	MS + 0.5 BAP + 1.0 Kn	63.41	5.7± 0.25
9	MS + 1.0 BAP + 1.0 Kn	72.49	4.2± 0.51
10	MS + 1.0 BAP + 0.5 Kn+ 2.0 GA ₃	79.63	5.5±0.30
11	MS + 0.5 BAP + 1.0 Kn+ 2.0 GA ₃	76.54	8.2± 0.22
12	MS + 1.0 BAP + 1.0 Kn+ 2.0 GA ₃	78.21	7.8± 0.15
13	MS+1.5 BAP+0.5Kn+2.0 GA ₃	80.21	8.1±0.12
14	MS+2.0 BAP+0.5Kn+1.0 GA ₃	88.67	8.7± 0.18

*Data is from thirty replicates and is represented as mean±SD

**Figure 2:** Seed germination on basal MS medium

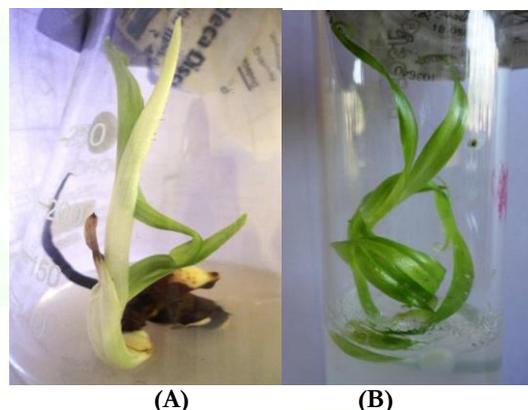
In vitro root regeneration

The addition of auxin in the rooting medium resulted in increase in roots number and root length and as compared to hormone free medium. Direct, healthy root initiation and elongation was observed in *G. superba* after subculturing of elongated single shoots onto MS basal medium supplemented with different concentrations of IBA (Table 3- Fig. 5A, 5B). Best rooting was observed in MS medium supplemented with 2.0 mg/l IBA resulting in 84.50% of root growth. The average number of roots per explant was 7.47 ± 0.29 . The roots were long and healthy. Success of *in vitro* propagation depends upon the survival rate of the plantlets from *in vitro* to field conditions. The rooted plants were removed from flasks, washed carefully to remove agar and transferred to a potting mixture in plastic pots containing sand: soil

Table 3: Effect of IBA concentration on *in vitro* rooting

S. No.	MS + Growth regulators (mg/l)	% age rooting	Average no. of roots per explant (mean ±SD)	Types of roots
1.	MS +0.5 IBA	25.42	1.11±0.26	Small thread like roots no root hairs.
2.	MS +1.0 IBA	45.70	3.93±0.52	Thin, fragile roots with less root hair.
3.	MS +1.5 IBA	78.51	6.14±0.24	Thin, fragile roots with root hairs.
4.	MS +2.0 IBA	84.50	7.47±0.29	Thin, fragile roots with few root hairs

(1:1). Right concentration of the potting mixture also ensures high survival rate (Kaur *et al.*, 2011). The plants were covered with jars to maintain the humidity because when shoots or plantlets desiccate or wilt rapidly when transplanted from culture room to greenhouse conditions due to the change in environment conditions (Mahajan, 2016). Ninety percent survival rate was obtained upon hardening.

**Figure 3:** Initiation of shoot from the tuber**Figure 4:** Multiplication of shoots from (A) tuber and (B) nodal segment in MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l Kn and 2.0 mg/l GA₃

Callus induction and organogenesis

Callus was induced from the leaves taken from *in vitro* grown shoots on different concentrations of 2,4-D after six weeks of inoculation. Creamy white, friable callus, large in size and with good growth rate was observed in MS medium supplemented with 3.0mg/l 2,4-D (Table 4- Fig. 6) but as the concentration of 2,4-D was increased to 5.0 mg/l, the leaf explants turned brown. Though callus initiation was also observed in MS medium containing 2.0mg/l and 4.0 mg/l 2,4-D, but there was no callus proliferation even after six weeks of culturing.

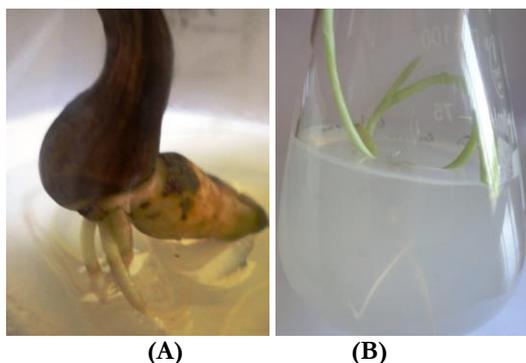


Figure 5: Initiation of roots from (A) tubers in basal MS medium (B) *in vitro* shoots in MS containing 2.0 mg/l IBA

Pieces of friable callus was transferred to MS medium containing different concentration and combination of BAP and Kinetin along with 2,4-D (1.0 mg/l). After six weeks of culturing maximum shoot regeneration (92.60%), with an average number of (4.2±0.22) shoots per explant was recorded in MS medium containing

BAP (3.0 mg/l), 2,4-D (1.0mg/l) and Kn (0.5 mg/l) (Table 5- Fig. 7). As the concentration of BAP and Kinetin was increased to 4.0 mg/l and 1.0 mg/l respectively the number of shoots decreased. Ngomuo *et al.*, (2014) reported that the different plant species, their source and type of explant used for *in vitro* culture has a great effect on callus induction when different concentrations of plant growth regulators are used.

However, *in vitro* generated callus when subcultured on MS medium supplemented with different concentrations of IBA resulted in root formation (Table 6). The best roots with root hair were obtained on MS medium supplemented with 2.5 mg/l IBA resulting in 91.80% root regeneration after 4 weeks of subculturing, with an average of 3.4±0.21 roots per explant (calli piece) (Fig. 8) while MS medium containing 3.0 mg/l of IBA resulted in 78.83% root induction. However, no root initiation was observed in MS medium with IBA at the concentration of 0.5 mg/l.

Table 4: Genotypic response of *Gloriosa superba* on callus induction after 6 weeks on MS media supplemented with different concentrations of 2,4-D.

S. No	MS+2,4-D (mg/l)	Type of Response in <i>G. superba</i>	Rate of callus induction
1	MS+1.0 2,4-D	No callus	---
2	MS+2.0 2,4-D	Creamy white, friable, small in size	++
3	MS+3.0 2,4-D	Creamy white, friable, large in size	+++
4	MS+4.0 2,4-D	Creamy white, friable, large in size	++
5	MS+5.0 2,4-D	Small callus with browning of the callus	+

--- Absent, + Less callus, ++ moderate growth, +++ Good growth rate

Table 5: Effect of plant growth hormones on shoot regeneration from the callus after 6 weeks

S. No.	MS medium with BAP+2,4-D + Kn (mg/l)	% Shoot initiation	Average number of shoots per explant (mean±SD)	Morphology of shoots
1	1.0 + 1.0 + 0.50	59.80	2.8±0.19	Light green shoots
2	2.0 + 1.0 + 0.50	63.60	3.1±0.75	Light green shoots
3	3.0 + 1.0 + 0.50	92.60	4.2±0.22	Dark green shoot
4	3.5 + 1.0 + 1.00	71.80	3.7±0.72	Dark green shoots
5	3.0 + 1.0 + 1.50	66.20	2.2±0.56	Dark green shoots
6	4.0 + 1.0 + 1.50	22.40	1.2±0.43	Browning of shoots

Table 6: Effect of plant growth hormones on root regeneration from callus after 4 weeks

S. No.	Concentration (mg/l)	% Root initiation	Average number of roots per explant (mean±SD)	Root morphology
1	MS +0.5 IBA	-----	-----	-----
2	MS +1.0 IBA	32.80	1.2±0.32	Very few fragile roots
3	MS +2.0 IBA	67.20	2.8±0.16	Fragile roots
4	MS+ 2.5 IBA	91.80	3.4±0.21	Well developed roots with numerous root hair
5	MS+3.0 IBA	78.83	2.3±0.45	Well developed roots with few root hair

The result is the mean ± Standard error of 3 replicates



Figure 6: Callus proliferation in MS medium containing 3.0 mg/l 2,4-D



Figure 7: Shoot initiation from callus in *G. superba* in MS medium containing 3.0 mg/l BAP, 1.0 mg/l 2,4-D and 0.5 mg/l Kn

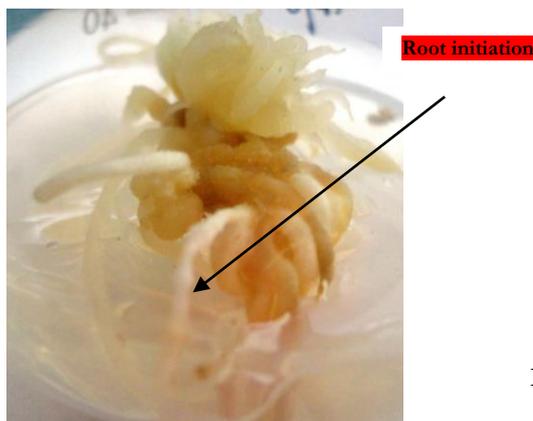


Figure 8: Root initiation from the callus in MS medium supplemented with 2.5 mg/l IBA

Somatic embryogenesis

After regular subculturing of embryogenic callus for three weeks on MS medium supplemented with 2,4-D (1.0 mg/l) along with BAP and Kn at a concentration of 0.5 mg/l each resulted in formation of globular structures on the callus (Fig. 9). MS medium supplemented with BAP (0.5 mg/l) and 2,4-D (1.0 mg/l) resulted in heart shaped somatic embryos (Fig. 10). As the concentration of BAP was increased to 1.5 mg/l, it resulted in formation of hard callus. Similar results were observed by Puhanand and Rath (2012) in *Desmodium gangeticum* where the percentage of somatic embryos was low in MS medium supplemented with Kn along with 2,4-D (1.0 mg/l), so Kn was found to be less competent as compared to BAP in initiating somatic embryo in callus cultures. Even, Mujib *et al.*, (2013) reported Kn to be less efficient in somatic embryo formation in *G. superba*. Also, MS medium without 2,4-D and containing only BAP and Kn resulted in very low percentage of somatic embryos.



Figure 9: Somatic embryogenesis in MS medium supplemented with BAP, Kn and 2,4-D

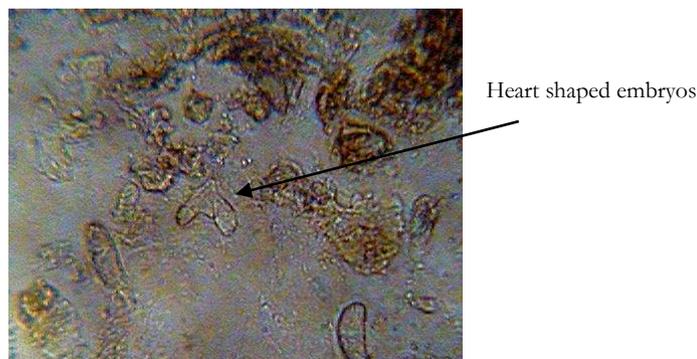


Figure 10: Heart shaped somatic embryos in *G. superba*

Conclusions

Different biotechnological approaches help in conservation and multiplication of some highly important medicinal plants that are overexploited for their bioactive components. A protocol for mass multiplication and somatic embryogenesis in *G. superba* was developed in present study for its rapid propagation. The significant result of our investigation is that addition of high concentration of BAP and low concentration of Kn in MS medium to proliferated callus, is suitable for plantlet conversion. Further addition of BAP along with 2,4-D in MS medium enhances the formation of cotyledonary embryos from heart and globular shaped embryos. Efforts are on way to increase the content of various alkaloids, with special emphasis on colchicine which is one of the most widely studied alkaloid from this plant.

Acknowledgements

The authors are thankful to UGC, New Delhi for their financial support. The authors are also thankful to School of Biotechnology, University of Jammu, Jammu for providing the basic facilities to carry out this research work.

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Cite this article as:

Ritu Mahajan, Nisha Kapoor, Pallavi Billowria. Callus proliferation and *in vitro* organogenesis of *Gloriosa superba*: an endangered medicinal plant. *Annals of Plant Sciences* 5.12 (2016): 1466-1471.

DOI: <http://dx.doi.org/10.21746/aps.2016.12.003>

Source of support: UGC, New Delhi, India.

Conflict of interest: Nil