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In vitro Micropropagation using Corm Bud Explants: An Endangered Medicinal Plant of *Gloriosa superba* L.

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ABSTRACT

An efficient protocol was developed *in vitro* micropropagation of *Gloriosa superba* by using corm bud explant. MS basal medium supplemented with different concentration and combination of 2,4-D (2,4-dichlorophenoxy acetic acid), IAA (Indole-3-acetic acid), BAP (6-benzyl aminopurine), GA_3 (Gibberellic acid), Zen (Zeatin), NAA (α-naphthaleneacetic acid), AC (Activated Charcoals) and CW (Coconut Water) were used. The 98.30±0.84% of yellowish Callus initiation was observed in MS media with (2, 4-D 1.0 mg L^{-1}, IAA 0.5 mg L^{-1}) after 4 week culture. This corm bud callus transferred to the shoots initiation medium. The maximum number (94.00±2.92) of multiple shoots was obtained on half strength of MS medium with (Kn (Kinetin) 1.0+BAP1.5+20% CW). The shoot lets transferred to the roots initiation medium. The 96.20±2.59% of multiple roots was obtained at the concentration of MS medium with BAP(8.0)+GA_3(1.0)+Zen(0.5)+NAA(1.0)+2 g L^{-1} AC. In other case without addition of activated charcoal in the MS medium, only 92.20±1.92% of root initiation was occurred, 16% of root induction depends on the addition of activated charcoal present in the MS medium. The rooted plantlets were transferred into small plastic nursery tray which was containing vermi compost, sand and red soil in the ratio of 1:2:2 and kept in a mist house. After acclimatization in the mist house for 2-months, the regenerated plantlets were hardened in the greenhouse and successfully transferred into soil which shows 90% survival rate. This new protocol was standardized for easy mass propagation of such an endangered medicinal plant *G. superba* by using corm bud explants.

Key words: *Gloriosa superba*, micropropagation, callus regeneration, corm bud, endanger medicinal plant

INTRODUCTION

Gloriosa superba Linn. (Liliaceae) is a climber, tuberous and monocot plant. It is native to the tropical and southern part of Africa. *G. superba* is national flower of Zimbabwe, Tamil Eelam and state flower of Tamil Nadu. The altitudinal range of this species is above 1500-2530 m from the surface of the sea level. In India, it is a rare and endangered medicinal plant on southern part of Andhra Pradesh, Karnataka, Kerala and Tamil Nadu (Chopra *et al*., 1956; Chandel *et al*., 1996). Its vernacular name is Kanvali poo, Kaandal, Kalappai kizhangu, Karthigai poo in Tamil. Traditionally since 2000 BC, it is used as a medicinal plant by the tribes (Ade and Rai, 2009). All the parts of *G. superba* are used as medicinal purpose in Siddha, Ayurveda and Yunani system of medicine. The tuber is effective against paralysis rheumatism, snake bite, insect bites, intermittent

fevers, wounds, anti-fertility, gonorrhea, leprosy, piles, debility, dyspepsia, flatulence, haemorrhoids, helminthiasis and inflammations (Pulliah, 2002; Warrier *et al.*, 1995; Ambasta, 1986), anthelmintic activity (Pawar *et al.*, 2010). It is also considered to be useful in promoting labor pain and expulsion of placenta (Evans *et al.*, 1981). Seeds are used for relieving rheumatic pain and as a muscle relaxant (Nadkarni, 2002).

The seeds and tubers contains high quantity of colchicine ($C_{22}H_{25}NO_6$: molecular weight 399.4: melting point (140-152°C) and colchicosides ($C_{27}H_{33}O_{11}N$: molecular weight 547.5). Colchicine content in tuber (0.3%), stem (0.33-0.41%), flower (1.18%) and ovary (0.08%) (Clewer *et al.*, 1915; Finnie and Van Staden, 1994) reported. The seeds contain 0.6% colchicines and 0.8% colchicosides (Sarin *et al.*, 1974). The seeds are the best source of colchicines as their content is 2-5 times higher than in tubers and other compounds such us lumicolchicine, 3-demethyl-N-deformyl-N-deacetylcolchicine, 3-demethylcolchicine, N-formyldeacetylcolchcine have been isolated from the plant (Sugandhi, 2000; Suri *et al.*, 2001). The isolation of 3-demethylcolchicine from *G. superba* sludge and coupling with α-acetobromoglucose to yield colchicoside and thiocolchicoside (Nautiyal, 2011). Antimicrobial and mutagenic properties (Hemaiswarya *et al.*, 2009), antibacterial activity (Banu and Nagarajan, 2011), FTIR spectroscopic study for antifungal activity (Ravi *et al.*, 2011a) and micro and macro nutrient analysis (Ravi *et al.*, 2011b) in *G. superba* were studied. Colchicine has an inhibitory effect on the growth of certain tumors in plants and animals. It inhibits spindle formation by arresting the polymerization of tubulin proteins and thereby checks karyokinesis (Andreu *et al.*, 1998). The effect of colchicine extracted from six different *Gloriosa* species was studied in onion root tip treated with 30 mg L^{-1} colchicine for 2 h (Bharathi *et al.*, 2006). Morphological and biochemical traits were employed for characterizing 18 germplasm accessions of *G. superba* representing different geographical regions of Tamil Nadu and Andhra Pradesh. The Accession GS 15 (Andhra wild) excelled in the accumulation of starch, soluble protein and phenol in tuber (Chitra and Rajamani, 2010). Colchicine is occasionally used in cytological and plant breeding research and it is used for the cancer treatment (Sayeed Hassan and Roy, 2005; Chopra *et al.*, 1956). The aromatic amino acids like Phenylalanine, tyrosine and tryptophan which are derived from the shikimate pathway. They are required as building blocks for protein synthesis and production of secondary metabolites like colchicine (Sivakumar *et al.*, 2004: Ghosh *et al.*, 2002). *G. superba* proved to be 96.15% of the endangered medicinal plant (Siddique *et al.*, 2005). This species is on the verge of extinction and included in the Red data book (Sivakumar *et al.*, 2003a,b, 2006). Due to the medicinal value, these plants are collected from the wild and used as raw material for large-scale medicinal industry, leading to over exploitation and it becomes an endangered plant species. One of the main problems for commercial cultivation is that the seed viability is poor (Mamatha *et al.*, 1993). It takes four or five vegetative cycles to complete a reproductive phase (Samarajeewa *et al.*, 1993). These plants are commercially propagated through asexual reproduction by using tubers (Sivakumar *et al.*, 2003b). Thus mass clonal multiplication through tissue culture is urgently needed not only to conserve this taxon but also to meet the demand for its need in medicinal field. Some of the reports are available on *in vitro* regeneration of this plant callus induction and regeneration of using different explant in MS and B5 media (Rishi, 2011). The root tuber inducted from leaf, nodal explants (Madhavan and Joseph, 2010) and shoot tip explants (Sivakumar and Krishnamurthym, 2000). It is a matter of great concern to conserve this plant otherwise we will be loosing it by 2020 (Ade and Rai, 2009).

The present study was conducted to standardized a simple and efficient protocol for *in vitro* micro-propagation through the corm bud explant of *G. superba* which would be highly useful for the conservation of this endangered plant species.

MATERIAL AND METHODS

Collection of plants: *Gloriosa superba* plants, rhizomes and seeds were collected from the Nathapalayam, Tamil Nadu, India.

Surface sterilization: Seed were washed in running tap water for 2 min and then with tween 1% (v/v) for 5 min. Then the seeds were sterilized in 70% alcohol for 1 min and 0.01% $HgCl_2$ for 2 min remove the traces for $HgCl_2$ (mercuric chloride).

Seed germination: Seeds were surface sterilized aseptically germinated in the MS (Murashige and Skoog, 1962) medium with 30% sucrose and solidified with 0.8% agar.

Sub-culturing: From 4 to 6 week old seedling, 0.5-1 cm corm bud were isolated and cultured on MS medium. The corm bud explants were produced the callus, MS medium supplemented with 2,4-D (1.0-10.0 mg L^{-1}) along with IAA (0.5-5.0 mg L^{-1}) was used. Shoots multiplication MS medium with BAP (1.0-5.0) and Kn (0.5-10 mg L^{-1})+BAP (1-5 mg L^{-1})+CW 20%. The roots multiplication of the MS medium with BAP (8.0-16 mg L^{-1})+GA_3(1.0-5.0 mg L^{-1})+Zen (0.5-2.5 mg L^{-1})+NAA (1 mg L^{-1}) and AC 2 g. Hormones were added to medium, adjusted to pH 5.8±1 with 0.1 N NaOH or 0.1 N HCl before autoclaved for 20 min at 121°C and 15 lb.

Culture conditions: All cultures were maintained at 22±1°C under 16 h photoperiod at a photosynthetic flux of 12.6 μmol m^{-2} sec^{-1} provided by cool daylight fluorescent lamps.

Hardening: The hardening 7 to 8 week old rooted plantlets (Fig. 1i) were removed from the culture flacks. After washing away the agar with water they were transferred in to plastic nursery tray containing sterile vermi compost, sand and red soil in the ratio of 1:2:2 and were kept in a mist house. After acclimatization in the mist house for 2-months (Fig. 1j, k), The regenerated plantlets were hardened in the greenhouse and successfully transferred to field (Fig. 1l).

RESULTS AND DISCUSSION

The corm bud explants inoculated in different concentration of 2,4-D (1.0-10.0 mg L^{-1}) and IAA (0.5-5.0 mg L^{-1}) in MS media (Table 1). The 39.00±3.94 to 98.30±0.84% of yellowish callus initiated in 25-30 days cultures (Fig. 1a-c). Increasing the hormone concentration of 2,4-D and IAA which

Table 1: Effect of different concentration of 2,4-D and IAA callus induction in corm bud explants of *G. superba*

MS medium with 2,4-D+IAA (mg L^{-1})	Corm-bud callus initiation (%)	Callus morphology
1.0+0.5	98.30±0.84	Yellowish callus
1.5+1.0	97.50±1.58	Yellowish callus
2.0+1.5	88.40±6.07	Yellowish callus
2.5+2.0	78.80±9.36	Yellowish callus
3.0+2.5	75.60±2.70	Yellowish callus
3.5+3.0	71.80±6.02	Yellowish callus
4.0+3.5	61.40±6.02	Yellowish callus
4.5+4.0	57.80±5.26	Yellowish callus
5.0+4.5	49.80±4.44	Yellowish callus
10.0+5.0	39.00±3.94	Yellowish callus

The result are the Mean±SE of 5 replicates, SE: Standard error

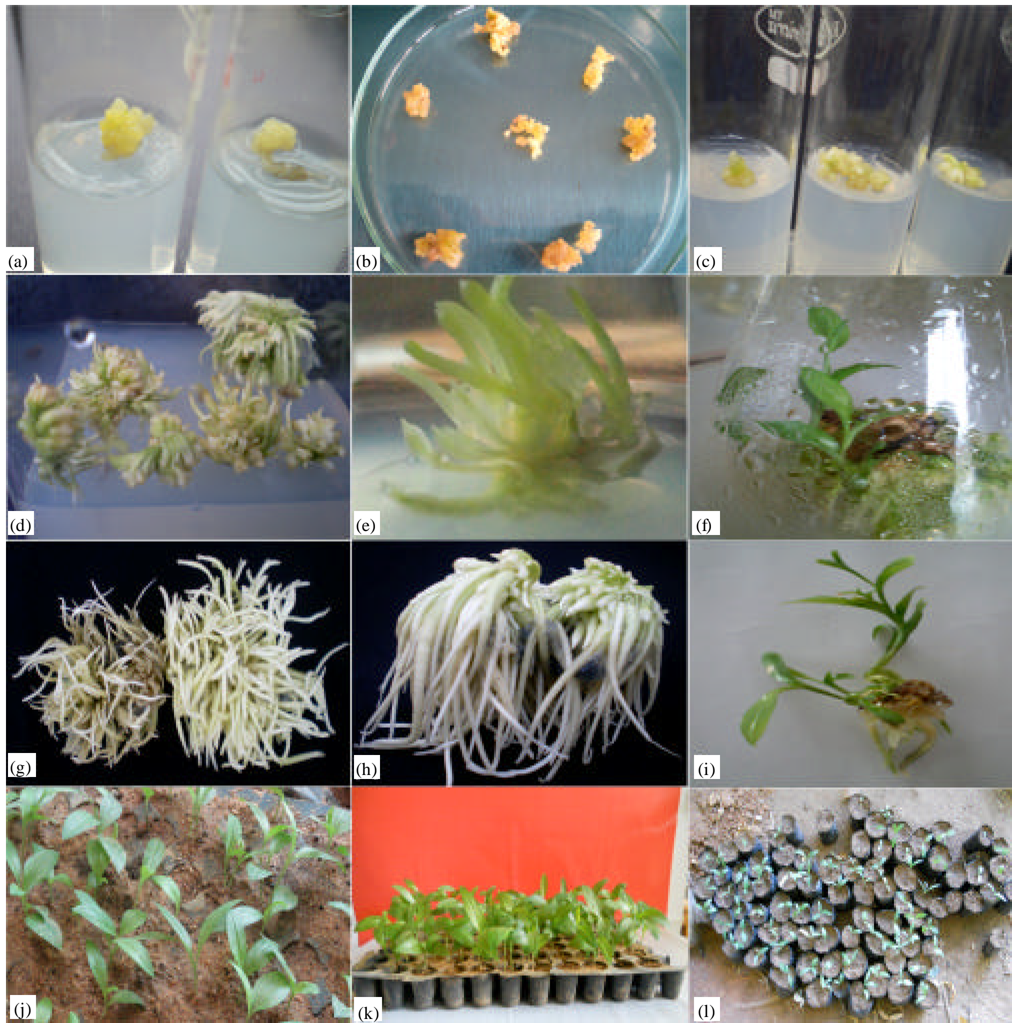

Fig. 1(a-l): *Gloriosa superba* (a-c) Proliferated yellowish callus initiation by corm bud explants, (d-f) Regeneration of multiple shoots initiation from the corm bud callus, (g-h) Root initiation form regenerated shoot lets, (i) Well developed rooted shoot lets, (j-k) Rooted shoots were transfer in to small plastic nursery tray acclimatization in the mist house for 2 months and (l) Regenerated plantlets were transplanted with polythene bags to hardening for *in vivo* condition

reduced callus initiation in corm bud explant. The highest profuse white, friable callus production was observed at 4.52 μM 2,4-D and 2.32 μM Kin in six different explants (Sivakumar *et al*., 2003a). *Podophyllum hexandrum* callus initiation occurs from root segments of established *in vitro* grown seedlings on B5 medium (half strength) supplemented with 2,4-D (0.5-1.5 mg L^{-1}) and BA (0.2-1.0 mg L^{-1}) (Sultan *et al*., 2006). *Aloe barbadensis* shoot tip explant used for *in vitro* regeneration on MS medium supplemented with 4 mg L^{-1} BA and 1 mg L^{-1} IAA (Supe, 2007).

Shoot initiation from the corm bud callus: The 30 days old corm bud callus transferred to the half strength MS medium contains different concentration and combination of cytokinins. The 93.20±2.39% of greenish shoot formation was obtained half strength of MS medium with BAP 2.0 mg L^{-1} (Fig. 1d, e), lower concentration of BAP can be used for obtaining the desirable shoot regeneration. Shoot initiation was reduced, when increasing the concentration of BAP on the medium (Table 2). *Boesenbergia pulcherrima* shoot tip explants achieved better response in BAP than in Kn. Addition of Kn (0.5 mg L^{-1}) to BAP (1.0 mg L^{-1}) containing medium promoted the rate of multiplication and obtained average 5.8 shoots in 5 weeks (Anish *et al.*, 2008). *Alpinia officinarum* optimum shoot multiplication was observed on MS medium containing 3% (w/v) sucrose and 3.0 mg L^{-1} Kinetin (Kn) and 1.0 mg L^{-1} Naphthalene Acetic Acid (NAA) each rhizome bud gave rise to an average of 11 shoots per explant (Selvakkumar *et al.*, 2007). In another case shoot multiplication, the maximum number (94.00±2.92%) of dark greenish multiple shoots was obtained on half strength of MS medium with (Kn 1.0+BAP 1.5+20% CW) (Fig. 1f). The greatest number of shoots was obtained in the presence of 9.84 μM 2iP combined with 4.64 μM Kin after 21-day culturing (Sivakumar *et al.*, 2003a). The best medium determined for shoot multiplication was obtain concentration of MS+1.5 mg L^{-1} BA+0.2 mg L^{-1} NAA+15% CW+2 g L^{-1} AC (Sayeed Hassan and Roy, 2005). Coconut Water is very effective in providing an undefined mixture of organic nutrients and growth factors (Gamborg and Phillips, 1995). The effectiveness of BAP, Kn and CW varied for *in vitro* multiple shoot regeneration from rooted callus. *Curcuma haritha* using rhizome tip explants, the best shoot multiplication of 11 shoots were achieved on MS medium containing (BA 4.4 μM) and IAA (2.9 μM) (Bejoy *et al.*, 2006). Aloe vera rhizomatous stem explants to initiate the multiple shoot bud in MS with 0.25 mg L^{-1} NAA and 1.5 mg L^{-1} BAP (Gantait *et al.*, 2010).

Root initiation from the shoot let explant: Regenerated shoot lets were transferred into root induction medium supplemented with BAP (8.0-16.0 mg L^{-1})+GA_3 (1.0-5.0 mg L^{-1})+Zen (0.5-2.5 mg L^{-1})+NAA (1.0 mg L^{-1}). After 23-25 days culture of this shoot lets expalnts to develop the multiple roots initiation (Fig. 1g, h). In another way to rapidly initiate we add activated

Table 2: Shoots multiplication with combination of growth regulators using corm bud callus of *G. superba*

½MS medium	Shoot initiation of rooted callus (%)	Developmental stage of shoot morphology
BAP (mg L^{-1})		
1.0	28.60±2.70	Greenish shoot formation
1.5	38.00±2.83	Greenish shoot formation
2.0	93.20±2.39	Greenish shoot formation
2.5	90.60±2.70	Greenish shoot formation
3.0	85.60±4.62	Greenish shoot formation
4.0	51.40±5.13	Greenish shoot formation
5.0	34.60±4.77	Greenish shoot formation
Kn+BAP+CW (%)		
0.5+1.0+20	64.40±5.32	Light greenish multiple shoot formation
1.0+1.5+20	94.00±2.92	Dark greenish multiple shoot formation
2.0+2.0+20	74.80±4.32	Light greenish multiple shoot formation
3.0+2.5+20	70.60±4.04	Dark greenish multiple shoot formation
4.0+3.0+20	61.40±4.93	Light greenish multiple shoot formation
5.0+4.0+20	52.00±4.95	Light greenish multiple shoot formation
10.0+5.0+20	45.40±4.77	Dark greenish multiple shoot formation

The result are the Mean±SE of 5 replicates, SE: Standard error

charcoals (AC-2 g L^{-1}) within the MS media, 16% induction of root initiation for best result was observed (Table 3). The shoot let explants highly regenerated up to 96.20±2.59% of roots formation obtained (Fig. 2a-d) at the concentration of MS+BAP (8.0)+GA_3 (1.0)+Zen (0.5)+NAA (1.0)+2 g AC and (Fig. 2e-h) with out addition of activated charcoals in the MS medium at produced 80.30±1.86

Table 3: Effect of activated charcoals induced the root initiation of shoot let explants of *G. superba*

MS medium with BAP+GA_3+Zen+NAA (mg L^{-1})	Mean number of roots initiation of corm bud callus±SE Add to 2 g L^{-1} of AC	Mean number of roots initiation of corm bud callus±SE without AC
8.0 +1.0+0.5+1.0	96.20±2.59	80.30±1.86
10.0+2.0+1.0+1.0	95.60±3.21	92.20±1.92
12.0+3.0+1.5+1.0	93.40±3.36	89.50±4.18
14.0+4.0+2.0+1.0	92.60±2.30	88.40±3.65
16.0+5.0+2.5+1.0	90.20±1.92	84.20±3.19

The result are the Mean±SE of 5 replicates, SE: Standard error

Fig. 2(a-h): *Gloriosa superba*, (a-d) 90.20±1.92 to 96.20±2.59%, Multiple roots formation at the concentration of MS+BAP (8.0)+GA_3 (1.0)+Zen (0.5)+NAA (1.0)+2 g AC and (e-h) with out activated charcoals on the MS medium to produced 80.30±1.86 to 92.20±1.92% of roots initiation

to 92.20±1.92% rhizogenesis initiation (Table 3). 4-9.9% of roots induction depends on the addition of activated charcoal present in the MS medium. The addition of AC to effectively promotes the organogenesis of *Muscari armeniacum* (Pierik, 1987). Root organ cultures initiated from root-tip explant on MS medium supplemented with NAA and BA or Kn for 35 days culture of *G. superba* (Jha *et al.*, 2005).

CONCLUSION

The present investigation pertained the simple and an efficient protocol was developed the micropropagation of an endangered medicinal plant *G. superba* in under *in vitro* condition by using corm bud explants. This protocol provides a successful and rapid technique that can be used for ex-sit conservation. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of the endangered flora of the southern India.

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