

A Reliable Protocol for Micropropagation of *Gloriosa superba* L. (Colchicaceae)

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Abstract. An efficient, rapid and improved *in vitro* plant regeneration protocol has been established for large scale multiplication of *Gloriosa superba*, an endangered ornamental and medicinal plant with limited reproductive capacity. Shoot tip explants from mature plants were sterilized using different concentrations (0.5- 1.0 w/v) of sodium hypochlorite (NaOCl) and then cultured on BAP or Kn (0.5-2.0 mg/l) alone or in combination with NAA (0.5 mg/l) at 25±2°C under a 16/8 hour light/dark photoperiod cycle. The most effective sterilization was achieved using 1.0% NaOCl treatment for 8 minutes. The highest regeneration frequency (76.6%) and average number of shoots (1.2) were obtained on MS medium fortified with BAP (2.0 mg l⁻¹) + NAA (0.5 mg l⁻¹). A high frequency of rooting (66.6%) with early root initiation (20.2 days) and maximum growth response was obtained when *in vitro* shoots were transferred onto half strength MS medium supplemented with NAA (1.0 mg l⁻¹) + IBA (0.5 mg l⁻¹) at 3% (w/v) sucrose. Simultaneously, a successful attempt was made to acclimatize the tissue culture-raised plants of *G. superba* using different organic manures as hardening media. Among all the combinations tried, sand: soil: vermicompost (1:2:1) produced the highest percentage survival (93.3%) upon transplantation of plants to the field conditions, followed by sand: soil: farm yard manure (1:2:1) which produced 86.7% survival. The hardened plantlets were successfully acclimatized in the greenhouse and transferred to open field conditions. Parameters including plant height, number of leaves per plant, leaf area, root length and tuber length were also monitored periodically. The protocol can be successfully applied for the mass multiplication of this valuable threatened taxon as well as to facilitate experiments involving its genetic modification.

Keywords: Acclimatization, Endangered, *Gloriosa superba*, Ornamental, Sterilization

INTRODUCTION

Gloriosa superba L., also known as Kalihari, Flame lily and Glory lily, belongs to the family Colchicaceae. It is a perennial, tuberous, climbing herb, widely distributed across tropical and sub-tropical parts of Africa and Southeast Asia (Sivakumar and Krishnamurthy, 2002). In Haryana, India, it is occasionally found in the Kurukshetra, Pinjoor and Morni regions (Jain *et al.*, 2000). This plant is used for its ornamental value and its flowers are used as cut flowers. Its attractive, wavy-edged, orange-red flower (Figure 1) is the national flower of Zimbabwe and also the state flower of Tamil Nadu in India (Dey, 1998). A postal stamp has been issued by the Postal Department of India for this flower. Colchicine (C₂₂H₂₆O₆H), the main valuable alkaloid present in this plant, is used for treating gout, controlling cancer and inducing polyploidy in plants. The plant is also used to cure arthritis, Mediterranean fever, rheumatism, inflammation, ulcers, bleeding piles, skin diseases, leprosy, impotency and snake-bites (Jana and Shekhawat, 2011).

Poor seed germination, susceptibility to pests and unscrupulous collection for its horticultural value and commercial use has pushed this taxon to a position of serious threat (Sivakumar and Krishnamurthy, 2000). Therefore,

alternative methods such as *in vitro* propagation would be crucial for disease-free, rapid, uniform mass multiplication and conservation of this highly valuable medicinal plant. Establishment of a successful protocol for *in vitro* multiplication of a plant species is species-specific and depends upon several factors, including culture media, alternate substratum (Deb and Imchen, 2010), hydroponic systems (Lopes da Silva 2006) and concentration of agar and sucrose in the medium (Paudel and Pant, 2013).

Explants collected from the open field are naturally contaminated with exogenous and endogenous microbial contaminants from the environment (Howell, 2002; Ahmad *et al.*, 2012). Contamination of plant tissue cultures with different microorganisms (bacteria, fungi, viruses and yeast) can cause variable growth, tissue necrosis, reduced shoot proliferation or reduced rooting (Omamor

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Figure 1. Close-up view of *Gloriosa superba* plant showing buds and flowers.

et al., 2007). These microbes compete adversely with plant tissue cultures for nutrients and ultimately result in increased culture mortality (Tiwari *et al.*, 2012). Maintenance of aseptic conditions prior to culture initiation is a prerequisite for successful tissue culture procedures.

Acclimatization is an essential and critical step in micropropagation which may result in a high mortality rate if not completed successfully (Torres *et al.*, 2006; Deb and Imchen, 2010). Plantlets are produced *in vitro* under a controlled environment, characterized by high humidity, low light intensity, constant temperature, supplementary sugar supply and growth regulators. When transferred to *ex vitro* conditions these plantlets are usually very susceptible to desiccation because of transfer shock (Mokhtarzadeh *et al.*, 2013), due to the exposure of plantlets that have poor root-stem connection, poor epicuticular and cuticular development, malfunctioning stomata, poor hydraulic conductivity of roots and poor photosynthetic ability to new *ex vitro* conditions such as low humidity, high level irradiation and water deficit (Hazarika, 2006). Some medium components, such as sucrose, also affect the morphology of plantlets. Many species of horticultural plants are easily micropropagated *in vitro* but exhibit poor acclimatization and subsequent survival *ex vitro* (Debergh and Zimmerman, 1991). So, it is necessary to transfer the *in vitro* raised plants to the field through weaning stages in order to increase survival. The selection of substrates to be used during acclimatization is also a key issue determining the growth and survival of micropropagated plants in *ex vitro* conditions (Kaur *et al.*, 2011; Yadav *et al.*, 2015).

Although earlier attempts have been made to propagate *G. superba* *in vitro* using different explants (Yadav *et al.*, 2012; 2013a; 2013b; Sivakumar and Krishanmurthy,

2000; 2002. (Hassan and Roy, 2005 considerable effort is still required to make it more practicable. It is extremely necessary to contrive an alternative rapid and efficient micropropagation protocol for the sustainable management of *G. superba* using shoot tip explants. The present study was therefore undertaken in an attempt to standardize a regeneration protocol for *G. superba* using shoot apex explants. This protocol is simple, reproducible and suitable for mass production as well as conservation of this highly valuable medicinal and ornamental plant.

MATERIALS AND METHODS

Explant collection and disinfection. Shoot apex explants (1.0 cm) of *Gloriosa superba* were collected from mature plants procured from Ch. Devi Lal Herbal Garden, Chuaharpur, Yamuna Nagar, Haryana, India. These were washed under running water with Tween-20 (2 drops per 100 mL water) for 1-2 hours to remove the adhering soil particles and then sterilized with 0.5- 1.0% (w/v) sodium hypochlorite solution (NaOCl) for 5- 10 minutes followed by a dip in ethanol (70%). Shoot tips were rinsed with sterile distilled water 4 - 5 times to remove traces of NaOCl. Survival was calculated using the following equation:

$$\% \text{ Survival (shoot tip/explants)} = (\text{Number of survived cultured} / \text{Total number of explants cultured}) \times 100$$

Culture media and conditions. After sterilization, the shoot tip explants were inoculated onto full strength solidified MS (Murashige and Skoog, 1962) medium augmented with various growth regulators, 3.0% (w/v) sucrose and 0.8% (w/v) agar (Hi-Media labs, India). The pH of the medium was adjusted to 5.8 using 1 N NaOH or 0.1 N HCl prior to autoclaving at 15 psi for 15-20 minutes. The cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16/8 hour light/dark photoperiod cycle provided by cool white fluorescent tubes with a photosynthetic photon flux density (PPFD) of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 60 - 70% humidity. All aseptic manipulations were carried out under a laminar airflow chamber.

Shoot induction and multiplication. The surface sterilized explants were then trimmed carefully with a sterilized surgical blade to remove the sterilizing agent-affected brown parts and inoculated onto MS media supplemented with different concentrations (0.5–2.0 mg/l) of benzylaminopurine (BAP) or kinetin (Kn) individually or in combination with 0.5 mg/l of 1-naphthaleneacetic acid (NAA) for culture initiation. The explants which produced shoots were sub-cultured onto fresh media every 6 weeks.

A treatment containing MS medium without any of these growth regulators served as the control.

***In vitro* rooting and acclimatization.** To find out the optimum concentration of sucrose for rooting, 3–4 cm of the *in vitro* regenerated shoots were excised aseptically and transferred to half strength MS medium containing 1.0 mg/l indole-3-butyric acid (IBA) + 0.5 mg/l NAA, 0.8% (w/v) agar and sucrose concentrations ranging from 2 to 4% under the same culture conditions described for the previous experiment to evaluate the effect of sucrose concentration on rooting success.

Plantlets of *G. superba* with well developed shoots and roots were taken out from the culture vessels, washed gently with tap water and transferred to one of three different planting substrates, viz. sterile soil: sand (3:1), sand: soil: farm yard manure (FYM) (1:2:1) or sand: soil: vermicompost (1:2:1), for acclimatization. Potted plantlets were covered with transparent polyethylene bags to maintain humidity and watered every 2 days with half strength MS salt solution without vitamins or sucrose for 15 days. Thereafter, the plastic bags were gradually opened and the plantlets were kept in the culture room for another 15 days. Plantlets were monitored and watered periodically. The hardened plantlets were then transferred to the glass house for maintenance under ambient daylight conditions at an average temperature of $25 \pm 2^\circ\text{C}$, relative humidity of 70% and constant ventilation for another 4 weeks before they were transferred to field conditions. During this period, plants become photosynthetically active and sturdy with well developed and efficient root system. Parameters including survival, plant height (cm), number of leaves per plant, leaf area (cm^2) and tuber length (cm) were observed for 120 days after inoculation. Leaf area was measured using a leaf area meter (211; Systronics Ltd., Ahmedabad, India).

Statistical analysis. All the experiments were conducted with a minimum of 10 replicates per treatment and each treatment was repeated three times. The results are expressed as mean $\pm$ SE of the three experiments. The data were analyzed using one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test (DMRT) at $P \leq 0.05$. All statistical analyses were performed using SPSS software (version 11.5, SPSS Inc., USA).

RESULTS AND DISCUSSION

The present investigation was carried out in three steps, which included comparison of the effect of different sterilization treatments, of cytokinins on proportion bud break and shoot multiplication followed by comparison of the effect of different concentrations of sucrose on optimized rooting media. Thereafter, the effect of different substrates were compared and optimized for the hardening and acclimatization steps of propagation.

Effect of different concentrations of NaOCl and exposure time on explant survival. Sterilization is the process of making explants contamination-free before establishment of cultures (Badoni and Chauhan, 2010). Sodium hypochlorite, ethanol, calcium hypochlorite, mercuric chloride, silver nitrate, bromine water and hydrogen peroxide have all been commonly used for surface sterilization of plant and seed material of different plant species (Talei *et al.*, 2012; Daud *et al.*, 2012; Olowe *et al.*, 2014).

Many sterilants are also toxic to the plant tissues, and hence it is essential to optimize and standardize concentration, exposure time and the sequence of sterilants used before starting a culture to minimize explant injury and to achieve optimal survival rate (CPRI, 1992). Sodium hypochlorite has been demonstrated to be a better sterilant than mercuric chloride which is one of the most dangerous chemicals used for sterilization, being highly toxic and requiring safe handling during the sterilization procedure, while the resulting hazardous waste requires special collection and disposal (Barampuram *et al.*, 2014).

Concentration of NaOCl and exposure time significantly affected survival of the shoot tip explants of *Gloriosa superba* (Table 1). The greatest proportion survival was observed when shoot tips were exposed to 1.0% NaOCl for 8 minutes followed by 0.75% NaOCl for 10 minutes (86.66% survival). In this study, increasing the sodium hypochlorite concentration had the greatest effect against microbiological contamination, although survival was reduced. When dissolved in water, the hypochlorite salts lead to the formation of HOCl, which is responsible for the bactericidal activity. Even a micromolar concentration of sodium hypochlorite is enough to significantly reduce bacterial populations (Nakagawara *et al.*, 1998). Many workers have used different concentrations of NaOCl for different exposure times for sterilization of a range of plant species (Webster *et al.*, 2003; Badoni and Chauhan, 2010; Sen *et al.*, 2013; Olowe *et al.*, 2014).

Effect of different growth regulators on bud break and shoot multiplication. MS basal medium

Table 1. Effect of different concentrations of sodium hypochlorite (NaOCl) with varying durations of exposure on contamination and percentage survival on shoot tip explants of *Gloriosa superba* recorded after 6 weeks of culture on MS medium containing 2.0 mg/l BAP + 0.5 mg/l NAA.

Concentration of Sodium hypochlorite (NaOCl w/v)	Exposure time (min)	Contamination (%)	Survival (%)
0.5%	5	83.33 ⁱ	6.66 ⁱ
	8	66.66 ^h	20.00 ^h
	10	43.33 ^f	40.00 ^g
0.75%	5	46.66 ^g	46.66 ^f
	8	20.00 ^d	76.66 ^d
	10	10.00 ^c	86.66 ^b
1.0%	5	23.33 ^c	66.66 ^e
	8	6.66 ^b	90.00 ^a
	10	3.33 ^a	80.00 ^c

Values represent mean $\pm$ standard error, n = 30.

Means were compared using the least significant difference (LSD) test ($P \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $P \leq 0.05$.

without growth regulators did not induce bud break or shoot multiplication, and nor was bud break observed on medium supplemented with Kinetin (Kn) (0.5 mg/l). Higher concentrations of BAP, or BAP in combination with NAA, were more effective for shoot formation when compared to Kn alone or in combination with NAA (Table 2). Maximum shoot bud induction and shoot sprouting was observed on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA followed by MS medium with 2.0 mg/l BAP (Figure 2a - c). Further, decreasing the BAP concentration not only increased shoot initiation time but also decreased the level of shoot initiation. Addition of Kn resulted in a decrease in bud break. This result was analogous to the multiple shoot production previously observed from shoot apex segments by Kaur *et al.* (1992) in *Anogeissus sericea*, by Perezeparron *et al.* (1994) in *Fraxinus angustifolia*, and by Mao *et al.* (2000) in *Litsea cubeba*. Kn at higher concentrations alone or in combination with NAA could produce only a single shoot per explant. The proportion bud break was maximized (to 30%) in the medium with 2.0 mg/l Kn + 0.5 mg/l NAA. The superiority of BAP over other cytokinins in plant

tissue culture of explants has been well demonstrated in many other important medicinal plants including *Prosopis cineraria* (Kumar and Singh, 2009), *Spilanthes acmella* (Yadav and Singh, 2010) and *Glycyrrhiza glabra* (Yadav and Singh, 2012).

A lower concentration of auxin with a higher concentration of cytokinins was found to be most effective for induction and production of shoots. Lal *et al.* (2010), Verma *et al.* (2011) and Yadav and Singh (2012) have all also noted the synergistic effect of BAP in combination with an auxin for efficient shoot regeneration. NAA regulates not only vegetative growth but also organ growth whereas BAP facilitates cell division and sprouting (Pan, 2001). The influence of the genotype on *in vitro* shoot multiplication is demonstrated, as well as the importance of the balance between concentrations of different plant growth regulators (PGRs) in explant medium.

Effect of sucrose concentration on rooting. Production of plantlets with profuse rooting under *in vitro* conditions is

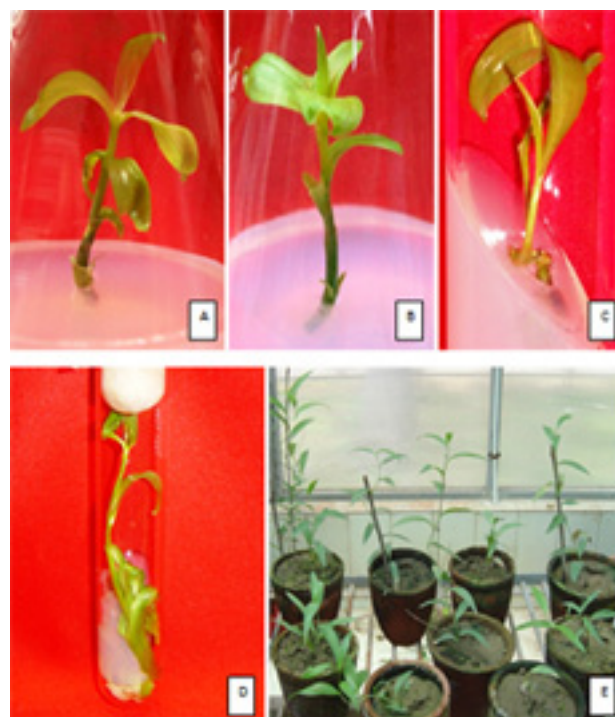


Figure 2. *In vitro* propagation of *Gloriosa superba*. (A) Shoots from apical shoot buds grown *in vitro* on MS medium containing 2.0 mg/l BAP + 0.5 NAA after six weeks of culture. (B, C) Multiple shoot formation on MS medium + BAP (2.0 mg/l). (D) *In vitro* derived plantlets on 3% sucrose. (E) Plantlets growing in a glass house.

Table 2. Effect of various concentrations of cytokinins provided alone and in combination with NAA on shoot induction in shoot tip explant of *Gloriosa superba* after 6 weeks of culture.

Plant growth regulators concentration (mg/l)		Bud break (%)	Number of days required for bud break	Number of shoots per explant	Shoot length (cm)
BAP (0.5)	-	13.33 ^h	28.28 ± 2.05 ^c	1.00 ± 0.00 ^b	3.2 ± 0.20 ^{ef}
BAP (1.0)	-	36.66 ^c	18.0 ± 1.84 ^d	1.00 ± 0.00 ^b	4.2 ± 0.21 ^d
BAP (2.0)	-	53.33 ^b	13.9 ± 1.38 ^c	1.12 ± 0.34 ^{ab}	6.6 ± 0.21 ^b
Kn (0.5)	-	-	-	-	-
Kn (1.0)	-	16.66 ^g	38.4 ± 1.67 ^g	1.00 ± 0.00 ^b	2.7 ± 0.25 ^g
Kn (2.0)	-	26.66 ^c	35.7 ± 1.28 ^{fg}	1.00 ± 0.00 ^b	3.2 ± 0.19 ^{ef}
BAP (1.0)	NAA (0.5)	53.33 ^b	12.3 ± 1.45 ^b	1.18 ± 0.40 ^{ab}	6.2 ± 0.20 ^c
BAP (2.0)	NAA (0.5)	76.66 ^a	9.17 ± 1.46 ^a	1.21 ± 0.42 ^a	7.0 ± 0.17 ^a
Kn (1.0)	NAA (0.5)	20.00 ^f	34.50 ± 1.51 ^{fg}	1.00 ± 0.00 ^b	2.9 ± 0.16 ^f
Kn (2.0)	NAA (0.5)	30.00 ^d	32.11 ± 0.92 ^f	1.00 ± 0.00 ^b	3.4 ± 0.23 ^c
-: No response.					
Values represent mean ± standard error, n = 30.					
Means were compared using the least significant difference (LSD) test (P ≤ 0.05). Data within each column followed by dissimilar letters differ significantly at P ≤ 0.05.					

often problematic and losses at this stage lead to vast economic consequences (Fatima and Anis, 2012). In the present study, excised shoots failed to develop roots on either full or half strength MS medium without provision of PGRs. MS medium lacking any PGR supplement has proven to be completely unable to induce rooting in many plant species (Lal *et al.*, 2010; Verma *et al.*, 2011; Yadav and Singh, 2011; 2011a). Our earlier study (Yadav *et al.*, 2012) evaluated the rooting success of healthy and uniform sized *in vitro*-regenerated shoots and showed that half-strength MS medium with 1.0 mg L⁻¹ indole-3-butyric acid (IBA) + 0.5 mg L⁻¹ NAA produced the greatest root induction (Figure 2d). The effectiveness of IBA with NAA in rooting of *in vitro* regenerated shoots has been well documented in plant including *Gymnema sylvestra* (Komalavalli and Rao, 2000) and *Anethum graveolens* (Jana and Shekhawat, 2011a).

Exogenous sucrose is commonly used as a carbon source to induce optimal growth and morphogenesis in plant tissue culture systems. It also represents the major osmotic component of the medium and is necessary for various metabolic activities. The previously observed difference between plants in the specific requirement for sugar in the media might be due to genotypic effects (Singh and

Shymal, 2001). Among the different sugar concentrations tried in this study, a 3% sugar concentration was found to be the most suitable, inducing 66.6% of explants to root with early root initiation (20.2 days); 2% and 4% sucrose concentration induced 40% and 53% of explants to root, respectively. Sucrose concentration was positively correlated with rooting success and extent (Table 3). However, a conspicuous variation was noted among the shoot morphology on these different concentrations of sucrose. The plants growing on MS medium supplemented with 3% sucrose showed better rooting as well as morphologically normal shoots and greater mean plant length when compared to those grown at higher and lower concentrations. It may be due to the relatively higher or lower osmotic pressure caused by the respective sucrose concentrations resulting in positive or negative water stress (Cui *et al.*, 2010). The beneficial effect of different strengths of culturing medium, sucrose concentration and agar concentration during rooting phase has also been discussed by Hazarika *et al.* (2000), Singh and Shymal (2001), and Barpete *et al.* (2014).

Effect of various substrates on hardening and acclimatization. The most crucial step in the

Table 3. Effect of different levels of sucrose on *in vitro* rooting on half strength MS medium containing 2.0 mg/l IBA + 0.5 mg/l NAA after 9 weeks.

Sucrose concentration	Days required for root induction	Rooting %	Extent of rooting	Plant morphology
2%	26.91 ± 1.16 ^c	40.00 ^c	++	Weak, smaller and thin
3%	20.20 ± 1.19 ^a	66.66 ^a	+++	normal with optimum height
4%	23.06 ± 1.43 ^b	53.33 ^b	++	normal with lesser height
The number of '+' signs donates the extent of rooting. +: fair rooting; ++: good rooting; +++: extensive rooting. Values represent mean ± standard error, n = 30. Means were compared using the least significant difference (LSD) test ($P \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $P \leq 0.05$.				

micropropagation process is the hardening and acclimatization as it is the process which makes the plantlets capable of tolerating and surviving in external environmental conditions (Ortega-Loeza *et al.*, 2011). Amongst different hardening mixtures used, the micro-plants hardened in plastic pots filled with sand: soil: vermicompost (1:2:1), covered with transparent polythene bags showed the greatest survival rate followed by sand: soil: FYM (1:2:1) (Table 4). The application of different organic manures not only influenced survival but also the growth and total yield in *G. superba*. The beneficial effect of vermicompost on growth and acclimatization has also been observed for *Bacopa monnieri* (Sharma, 2005), *Chlorophytum borivillianum* (Kumar *et al.*, 2010) and *Tylophora indica* (Rani and Rana, 2010). The more successful growth could be attributed to the release of different growth hormones (auxin and cytokinins) and chemical exudates during the biological activity promoted by organic manures and vermicompost (Kale and Krishnamoorti, 1981). Various workers have employed

different potting mixtures during the transplantation stage of acclimatization to increase the survival rate of *in vitro* raised plants, such as soaked cotton for *Saccharum officinarum* (Gill *et al.*, 2004), solrite for *Carica papaya* (Agnihotri *et al.*, 2004), cocopeat for *Garcinia indicia* (Chabukswar and Deodhar, 2005), charcoal, bricks and decayed wood chips for orchids plantlets (Deb and Imchen, 2010) and perlite for *Bambusa tulda* (Mishra *et al.*, 2011). In contrast, some propagators have reduced explant mortality rate by using biofertilizers like *Azotobacter*, *Pseudomonas*, or AM fungi along with the basic potting mixture during transplantation (Vasane and Kothari, 2006; Kaur *et al.*, 2011; Yadav *et al.*, 2013). It is important that as well as effective the hardening substrate should also be inexpensive and easily available.

After successful hardening, plantlets were transferred to pots containing normal soil and maintained in a greenhouse under ambient day length conditions (Figure 2e). The importance of a successful acclimatization process and field transfer of *in vitro*

Table 4. Effect of different hardening substrates on *ex vitro* acclimatization of *in vitro* raised plantlets of *Gloriosa superba*.

Planting substrates	Plantlet survival (%)	Plant height (cm)	Number of leaves/ plant	Leaf area (cm ²)	Tuber length (cm)	Tuber girth (cm)
sterile soil: sand (3:1)	73.33 ^c	118.0 ± 3.16 ^c	30.6 ± 1.94 ^c	29.7 ± 0.69 ^c	3.0 ± 0.16 ^c	1.26 ± 0.10 ^c
sand: soil: F.Y.M. (1:2:1)	86.66 ^b	128.00 ± 3.08 ^b	40.20 ± 1.48 ^b	31.20 ± 0.83 ^b	3.26 ± 0.08 ^a	1.31 ± 0.02 ^b
sand: soil: vermicompost (1:2:1).	93.33 ^a	141.00 ± 2.64 ^a	45.00 ± 2.23 ^a	32.80 ± 0.83 ^a	3.42 ± 0.16 ^a	1.40 ± 0.01 ^a

Values represent mean ± standard error, n = 30.

Means were compared using the least significant difference (LSD) test ($P \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $P \leq 0.05$.

regenerated plantlets have also been highlighted by Lattoo *et al.* (2006), Vadodaria *et al.* (2007) and Verma *et al.* (2011). There was no detectable variation between the regenerated plants with respect to morphological or growth characteristics.

CONCLUSION

The present protocol describes an improved and successful *in vitro* system of *Gloriosa superba* propagation from shoot tip explants through direct organogenesis. The method removes the need for an intervening callus phase, and avoids the use of mercuric chloride, thus assuring the species' effective establishment, multiplication, rhizogenesis and acclimatization throughout the year, irrespective of seasonal constraints. The technique could support the conservation of this valuable plant species to protect it from indiscriminate exploitation, which is increasing to keep pace with commercial needs. It could work as a useful tool to increase the biomass and yield of pharmaceutically important alkaloid colchicine, and other useful phytochemicals, accumulated in *G. superba*.

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REFERENCES

- Ahmad, F., Daglish, G.J., Ridley, A.W. and Walter, G.H. 2012. Responses of *Tribolium castaneum* to olfactory cues from cotton seeds, the fungi associated with cotton seeds, and cereals. *Entomologia Experimentalis et Applicata* 145: 272–281.
- Badoni, A. and Chauhan, J.S. 2010. *In vitro* sterilization protocol for micropropagation of *Solanum tuberosum* cv. 'Kufri Himalini'. *Academia Arena*, 2: 24–27.
- Barampuram, S., Allen, G. and Krasnyanski, S. 2014. Effect of various sterilization procedures on the *in vitro* germination of cotton seeds. *Plant Cell Tissue and Organ Culture* 118: 179–185.
- Barpete, S., Khawar, K.M. and Ozcan, S. 2014. Differential competence for *in vitro* adventitious rooting of grass pea (*Lathyrus sativus* L.). *Plant Cell Tissue and Organ Culture* 119(1): 39–50.
- CPRI (Central Potato Research Institute). 1992. Tissue culture technique for potato health, conservation, micropropagation and improvement. *CPRI, Shimla*, p. 23.
- Cui, X.H., Murthy, H.N., Wu, C.H. and Paek, K.Y. 2010. Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. *Plant Cell Tissue and Organ Culture* 103:7–14.

- Daud, N.H., Jayaraman, S. and Mohamed, R. 2012. An improved surface sterilization technique for introducing leaf, nodal and seed explants of *Aquilaria malaccensis* from field sources into tissue culture. *Asia Pacific Journal of Molecular Biology and Biotechnology* 20:55–58.
- Deb, C.R. and Imchen, T. 2010. An efficient *in vitro* hardening technique of tissue culture raised plants. *Biotechnology* 9: 79-83.
- Debergh, P.C. and Zimmerman, R.H. 1991. Micropropagation, technology and application. London: Kluwer Academic. p. 71-93.
- Dey, S.C. 1998. Glory Lily: its propagation by microtubers. *Industrial Horticulture* 42: 24-25.
- Fatima, N. and Anis, M. 2012. Role of growth regulators on *in vitro* regeneration and histological analysis in Indian ginseng (*Withania somnifera* L.) Dunal. *Physiology and Molecular Biology of Plants* 18: 59–67.
- Hazarika, B.N. 2006. Morpho-physiological disorders in *in vitro* culture of plants. *Scientia Horticulturae* 108: 105–120.
- Hazarika, B.N., Parthasarathy, V.A., Nagaraju, V. and Bhowmik, G. 2000. Sucrose induced biochemical changes in *in vitro* microshoots of Citrus species. *Indian Journal of Horticulture* 57: 27–31.
- Howell, C.R. 2002. Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. *Phytopathology* 92: 177–180.
- Jain, S.P., Verma, D.N., Singh, S.C., Singh, J.S. and Kumar, S. 2000. *Flora of Haryana*, CIMAP, Lucknow.
- Jana, S. and Shekhawat, G.S. 2011. Critical review on medicinally potent plant species: *Gloriosa superba*. *Fitoterapia* 82: 293-301.
- Jana, S. and Shekhawat, G.S. 2011a. Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and *in vitro* flowering of *Anethum graveolens*. *Acta Physiol. Plant.* 33: 305-311.
- Kale, R.D. and Krishnamoorthi, R.V. 1981. Environment of soil fertility by earthworm's activity: In: Progress in Soil Biology and Ecology in India. G.K. Veeresh (ed.). USA. Technol. Serv. pp. 64-66.
- Kaur, G., Singh, R.P., Rathor, T.S. and Shekhawat, N.S. 1992. *In vitro* propagation of *Anogeissus sericea*. *Indian Journal of Experimental Biology* 30: 788-791.
- Kaur, H., Anand, M. and Goyal, D. 2011. Optimization of potting mixture for hardening of *in vitro* raised plants of *Tylophora indica* to ensure high survival percentage. *International Journal of Medical and Aromatic Plants* 1: 83- 88.
- Komalavalli, N. and Rao, M.V. 2000. *In vitro* micropropagation of *Gymnema sylvestre* – A multipurpose medicinal plant. *Plant Cell Tissue and Organ Culture* 61: 97-105.
- Kumar, A., Aggarwal, D., Gupta, P. and Reddy, M.S. 2010. Factors affecting *in vitro* propagation and field establishment of *Chlorophytum borivilianum*. *Biologia Plantarum* 54: 601-606.
- Lal, D., Singh, N. and Yadav, K. 2010. *In vitro* studies on *Celastrus paniculatus*. *Journal of Tropical and Medicinal Plants* 11: 169-174.
- Lattoo, S.K., Bamotra, S., Saprudhar, R., Khan, S. and Dhar, A.K. 2006. Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker- an endangered medicinal herb. *Plant Cell Reproduction* 25: 499–506.
- Lopes da Silva, A.L., Henz, F.E.T., Horbach, M.A. and Walter, J.M. 2006. Aclimatização de mudas de *Cattleya tigrina* A. Rich. Ex Beer (Orchidaceae) em sistema hidroponico. *Cuaderno de Pesquisa Sér Bio.* 18: 129–139.
- Mao, A.A., Wetten, A., Fay, M.F. and Caligari, P.D.S. 2000. *In vitro* propagation of *Litsea cubeba* Pers., a multipurpose tree. *Plant Cell Reproduction* 19: 263-267.
- Mishra, Y., Patel, P. and Ansari, S.A. 2011. Acclimatization and macroproliferation of micropropagated plants of *Bambusa tulda* Roxb. *Asian Journal of Experimental and Biological Science* 2: 498-501.
- Mokhtarzadeh, S., Hajyzadeh, M., Ahmad, H.A. and Khawar, K.M. 2013. The problems in acclimatisation of *in vitro* multiplied plants of *Lavandula angustifolia* Miller under field conditions. *Acta Horticulturae* 988:71–76.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.
- Nakagawara, S., Goto, T., Nara, M., Ozawa, Y., Hotta, K. and Arata, Y. 1998. Spectroscopic characterization and the pH dependence of bactericidal activity of the aqueous chlorine solution. *Analytical Sciences* 14: 691–698.

- Olowe, O., Adesoye, A., Ojobo, O., Amusa, O. and Liamngee, S. 2014. Effects of sterilization and phytohormones on shoot tip culture of *Telfairia occidentalis*. *Journal of Natural Sciences Research* 4: 53-58.
- Omamor, I.B., Asemota, A.O., Eke, C.R. and Ezia, E.I. 2007. Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). *African Journal of Agricultural Research* 2: 534-537.
- Ortega-Loeza, M.M., Salgado-Garciglia, R., Gómez-Alonso, C. and Ávila-Díaz, I. 2011. Acclimatization of the endangered Mexican epiphytic orchid, *Laelia speciosa* (H.B.K.) Schltr. *European Journal of Environmental Sciences* 1: 48-54.
- Pan, R.C. 2001. *Phytophysiology* 4th edn. Higher Education Press, Beijing, China, pp. 176-186.
- Paudel, M.R. and Pant, B. 2013. A reliable protocol for micropropagation of *Esmeralda clarkei* Rchb. f. (Orchidaceae). *Asia Pacific Journal of Molecular Biology and Biotechnology* 21: 114-120.
- Perez-Parron, M.A., Gonzalez-Benito, M.E. and Perez, C. 1994. Micropropagation of *Fraxinus angustifolia* from mature and juvenile plant material. *Plant Cell Tissue and Organ Culture* 37: 297-302.
- Rani, S. and Rana, J.S. 2010. In vitro Propagation of *Tylophora indica* influence of explanting season, growth regulator synergy, culture passage and planting substrate. *Journal of American Science* 6: 385-392.
- Sen, M.K., Jamal, M.A.H.M. and Nasrin, S. 2013. Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured *in vitro*. *Environmental and Experimental Biology* 11: 119-123.
- Sharma, N. 2005. Micropropagation of *Bacopa monneiri* L. Penn. –an important medicinal plant. - MSc Thesis, Thapar University, Patiala.
- Singh, S.K. and Shymal, M.M. 2001. Effect of media and physical factors on in vitro rooting in roses. *Horticultural Journal* 14:91-97.
- Sivakumar, G. and Krishnamurthy, K.V. 2000. Micropropagation of *Gloriosa superba* L. -an endangered species of Asia and Africa. *Current Science* 78: 31-32.
- Sivakumar, G. and Krishnamurthy, K.V. 2002. *Gloriosa superba* L. – a very useful medicinal plant. In VK Singh, JN Govil, S Hashmi, G Singh, eds, Recent Progress in Medicinal Plants, Vol. 7, Ethnomedicine and Pharmacognosy, Part II. *Series Sci. Tech. Pub., Texas, USA*, pp. 465-82.
- Talei, D., Valdiani, A., Abdullah, M.P. and Hassan, S.A. 2012. A rapid and effective method for dormancy breakage and germination of King of Bitters (*Andrographis paniculata* Nees.) seeds. *Maydica* 57: 98-105.
- Tiwari, S., Arya, A. Kumar, S. 2012. Standardizing sterilization protocol and establishment of callus culture of sugarcane for enhanced plant regeneration *in vitro*. *Research Journal of Botany* 7: 1-7.
- Torres, J., Laskowski, L. and Sanabria, M.E. 2006. Efecto del ambiente de desarrollo sobre la anatomía de la epidermis foliar de *Cattleya jenmanii* Rolfe. *Bioagro* 18: 93-99.
- Vadodaria, H.K., Samantaray, S. and Maiti, S. 2007. Micropropagation of *Glycyrrhiza glabra* Linn.: An important medicinal plant. *Cell and Tissue Research* 7: 921-926.
- Vasane, S.R. and Kothari, R.M. 2006. Optimization of secondary hardening process of Banana plantlets (*Musa paradisiacal* L. var. grand Nain). *Indian Journal of Biotechnology* 5: 394-399.
- Verma, S., Yadav, K. and Singh, N. 2011. Optimization of the Protocols for Surface Sterilization, Regeneration and Acclimatization of *Stevia rebaudiana* Bertoni. *American- European Journal of Environmental and Agricultural Science* 11: 221-227.
- Webster, S., Mitchell, S.A. and Ahmad, M.H. (2003) A novel surface sterilization method for reducing fungal and bacterial contamination of field grown medicinal explants intended for *in vitro* culture. In: Proceedings of 17th SRC conference entitled 'Science and Technology for Economic Development: Technology Driven Agriculture and Agro-Processing' SRC, Jamaica.
- Yadav, K. and Singh, N. 2011. Effect of seed harvesting season and sterilization treatments on germination and in vitro propagation of *Albizia lebbeck* (L.) Benth. *Analele Universității din Oradea. Fascicula Biologie* 18: 151-156.

- Yadav, K. and Singh, N. 2011a. In vitro propagation and biochemical analysis of field established Wood apple (*Aegle marmelos* L.). *Analele Universității din Oradea. Fascicula Biologie* 18: 23-28.
- Yadav, K. and Singh, N. 2012. Factors influencing *in vitro* plant regeneration of Liquorice (*Glycyrrhiza glabra* L.). *Iranian Journal of Biotechnology* 10: 161-167.
- Yadav, K., Aggarwal, A. and Singh, N. 2012. Actions for *ex situ* conservation of *Gloriosa superba* L. - an endangered ornamental cum medicinal plant. *Journal of Crop Science and Biotechnology* 15: 297- 303.
- Yadav, K., Aggarwal, A. and Singh, N. 2013. Arbuscular mycorrhizal fungi (AMF) induced acclimatization, growth enhancement and colchicine content of micropropagated *Gloriosa superba* L. plantlets. *Industrial Crops and Products* 45: 88-93.
- Yadav, K., Aggarwal, A. and Singh, N. 2013a. Evaluation of genetic fidelity among micropropagated plants of *Gloriosa superba* L. using DNA based markers- a potential medicinal plant. *Fitoterapia* 89: 265-270.
- Yadav, K., Aggarwal, A. and Singh, N. 2015. Influence of arbuscular mycorrhizal fungi (AMF) on acclimatization and growth enhancement of micropropagated medicinal plants, In: *Advances in Plant Physiology*, Volume 15 (2014-15) Ed. A. Hemantaranjan, *Scientific Publishers*, Jodhpur, pp. 495-512.