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 (C\(_{22}\)H\(_{26}\)O\(_{6}\)H), 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 (Omanor
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 Krishnamurty, 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, Chuharpur, 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:

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

**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±2°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 μmol m⁻² 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 0.5mg/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 ± 2°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 (cm²) 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 ± 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 ≤ 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 HOCI, which is responsible for the bactericidal activity. Even a micromolar concentration of sodium hypochlorite is enough to significantly reduce bacterial populations (Nakagawa *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.

<table>
<thead>
<tr>
<th>Concentration of Sodium hypochlorite (NaOCl w/v)</th>
<th>Exposure time (min)</th>
<th>Contamination (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>5</td>
<td>83.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>66.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>43.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.75%</td>
<td>5</td>
<td>46.66&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.66&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>76.66&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0%</td>
<td>5</td>
<td>23.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.66&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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.

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
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.

<table>
<thead>
<tr>
<th>Plant growth regulators concentration (mg/l)</th>
<th>Bud break (%)</th>
<th>Number of days required for bud break</th>
<th>Number of shoots per explant</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP (0.5)</td>
<td>13.33b</td>
<td>28.28 ± 2.05e</td>
<td>1.00 ± 0.00b</td>
<td>3.2 ± 0.20ef</td>
</tr>
<tr>
<td>BAP (1.0)</td>
<td>36.66c</td>
<td>18.0 ± 1.84d</td>
<td>1.00 ± 0.00b</td>
<td>4.2 ± 0.21d</td>
</tr>
<tr>
<td>BAP (2.0)</td>
<td>53.33b</td>
<td>13.9 ± 1.38c</td>
<td>1.12 ± 0.34a</td>
<td>6.6 ± 0.21b</td>
</tr>
<tr>
<td>Kn (0.5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kn (1.0)</td>
<td>16.66d</td>
<td>38.4 ± 1.67e</td>
<td>1.00 ± 0.00b</td>
<td>2.7 ± 0.25e</td>
</tr>
<tr>
<td>Kn (2.0)</td>
<td>26.66d</td>
<td>35.7 ± 1.28b</td>
<td>1.00 ± 0.00b</td>
<td>3.2 ± 0.19df</td>
</tr>
<tr>
<td>BAP (1.0) NAA (0.5)</td>
<td>53.33b</td>
<td>12.3 ± 1.45b</td>
<td>1.18 ± 0.40a</td>
<td>6.2 ± 0.20f</td>
</tr>
<tr>
<td>BAP (2.0) NAA (0.5)</td>
<td>76.66e</td>
<td>9.17 ± 1.46c</td>
<td>1.21 ± 0.42c</td>
<td>7.0 ± 0.17c</td>
</tr>
<tr>
<td>Kn (1.0) NAA (0.5)</td>
<td>20.00f</td>
<td>34.50 ± 1.51f</td>
<td>1.00 ± 0.00b</td>
<td>2.9 ± 0.16f</td>
</tr>
<tr>
<td>Kn (2.0) NAA (0.5)</td>
<td>30.00d</td>
<td>32.11 ± 0.92f</td>
<td>1.00 ± 0.00b</td>
<td>3.4 ± 0.23c</td>
</tr>
</tbody>
</table>

:: 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 orlower 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 Barpate 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.

<table>
<thead>
<tr>
<th>Sucrose concentration</th>
<th>Days required for root induction</th>
<th>Rooting %</th>
<th>Extent of rooting</th>
<th>Plant morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>26.91 ± 1.16 c</td>
<td>40.00 c</td>
<td>++</td>
<td>Weak, smaller and thin</td>
</tr>
<tr>
<td>3%</td>
<td>20.20 ± 1.19 a</td>
<td>66.66 a</td>
<td>+++</td>
<td>normal with optimum height</td>
</tr>
<tr>
<td>4%</td>
<td>23.06 ± 1.43 b</td>
<td>53.33 b</td>
<td>++</td>
<td>normal with lesser height</td>
</tr>
</tbody>
</table>

The number of ‘+’ signs donate 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 ≤ 0.05). Data within each column followed by dissimilar letters differ significantly at P ≤ 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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