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In vitro Tuberization and Colchicine Content Analysis of *Gloriosa superba* L.

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ABSTRACT

Gloriosa superba L. is an herbaceous climber distributed in tropical parts of the world. Pharmaceutically important alkaloid-colchicine, present in its tubers and seeds and due to overexploitation it becomes vulnerable in the forests. In the present investigation, *in vitro* tuber production was carried out for its propagation and conservation. The plant possesses a very strong apical dominance. Consequently, any damage to the plant apical meristem is fatal for it which was also exhibited during *in vitro* culture. Only apical meristems were able to produce a single and un-branched shoots and nodal explants were remained dormant even in the presence of exogenous cytokinin. The *in vitro* propagation was accomplished by the microtuber formation technique, in two steps. Maximum number of microtubers 9.8±0.8 per culture in eight weeks, were produced *in vitro* on Murashige and Skoog medium with sucrose (60 g L^{-1}) and in the presence of 35.5 µM 6-benzyladenine (BA) with citric acid and polyvinyl pyrrolidone-40. Subsequently the induced microtubers were sub-cultured on to the medium with lower cytokinin level, 8.88 µM BA. The individual microtubers with shoots were subjected to a single step rooting and *in vitro* acclimatization in coco-pit containing vessels, exhibited 90% survival. *In vitro* grown tubers contained less percentage of colchicine than the natural field-grown plant tubers. However, microtubers showed increased colchicine content, as they grow older.

Key words: Apical dominance release, bud culture, cytokinin, glory lily

INTRODUCTION

Gloriosa superba L. (Glory Lily or Kalihari) belongs to the family Liliaceae. It is a perennial and herbaceous small climber, which produces underground tubers. It is native of Africa and is in the tropical and sub-tropical regions of Asia (Royal Botanical Gardens, 2011) known for its glorious red and yellow flowers. It is a national flower of Zimbabwe and also is state flower of Tamil Nadu state of India. Seeds and underground tubers of the plant contain commercially important alkaloid-colchicine. After the discovery of the colchicine in *G. superba*, its commercial importance has increased due to higher colchicine contents as compared to *Colchicum* plant (Finnie and van Staden, 1991). Furthermore due to the high demand of colchicine in national and international markets, *G. superba* is included into the list of 32 nationally prioritized medicinal plants of India (NMPB, 2014). Colchicine is used to treat inflammatory diseases (Cronstein and Terkeltaub, 2006), lipophilic drug action on tumors (Baker *et al*., 1996) and used to treat pulmonary fibrosis (Addrizzo-Harris *et al*., 2002). Because of the economical and medicinal values, *G. superba* has been over harvested from forests; consequently, its population is declining in India, Sri Lanka, Bangladesh and Southern Africa (Royal Botanical Gardens, 2011). The plant is now a vulnerable species, in most of the Indian states (FRLHT., 2003). Its seeds are with poor germination capacity; furthermore, its conventional practice of cultivation by underground tubers is also very slow, because only two tubers produced in one year cycle (Ghosh *et al*., 2007). Therefore, *in vitro* tuber production of this plant is a good alternative for conservation and production of disease free planting material. Several attempts were made for micropropagation (Hassan and Roy, 2005; Sivakumar and Krishnamurthy, 2000; Custers and Bergervoet, 1994), somatic embryogenesis (Jadhav and Hegde, 2001), embryoid formation (Sivakumar *et al*., 2003a) and Organogenesis (Sivakumar and Krishnamurthy, 2004) of *G. superba.* However, few reports are available on microtuberization of *G. superba* (Yadav *et al*., 2012; Ghosh *et al*., 2007; Shimasaki *et al*., 2009; Sivakumar *et al*.,

2003b). The present study is undertaken to investigate an efficient protocol for *in vitro* tuberization as well as colchicine analysis in the regenerated microtubers of this threatened plant.

MATERIALS AND METHODS

***In vitro* culture:** *Gloriosa superba* explants were collected from the nursery of Biotechnology Department of Pt. Ravishankar Shukla University, Raipur (India). Three different explant types were used to initiate *in vitro* cultures of the plant. Apical shoot tips and nodal segments of the aerial shoots growing on rainy season were used to initiate shoot cultures; meristematic region of the underground tuber was also used as third explant type. The explants were washed thoroughly in running tap water and then were treated with fungicide solution (0.1% Carbendazim and 0.25% Mancozeb) for 20 min. Further, the explants were surface disinfested in laminar airflow cabinet using 70% ethanol for few seconds and then with aqueous mercuric chloride (0.2%) for 10 min, followed by rinsing 4 times in sterile distilled water. Finally, the explants were cut to final size of approximately 1 cm long and were transferred aseptically onto explant establishment medium. Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with 3% sucrose was used as culture medium supplemented with the different concentrations of 6-benzyladenine (BA) or kinetin. The media was solidified with 0.7% agar and the pH was adjusted to 5.7, was sterilized in autoclave for 20 min at 1.05 kg cm^{-2} pressure at 121°C. After inoculation of explants, cultures were incubated at 25±2°C under 16 h light and 8 h dark regime with 150 µmol m^{-2} sec^{-1} light intensity provided by cool white fluorescent tubes. For explant establishment experiments, 25×150 mm glass tubes were used containing 15 mL medium in each. After four weeks of culture, data was recorded for each explant, i.e., bud break, shoot number, shoot length, node number, microtuber number and length. After the growth of four weeks on the explant establishment medium, the shoots elongated from the original explants, were divided in to shoot tips and nodes and then were sub cultured on to fresh medium for further shoot multiplication. Effect of BA and kinetin at different concentrations were tested in MS medium, for this stage also.

For microtuberization experiments, modified MS medium was used, containing 100 mg L^{-1} citric acid and 1 g L^{-1} polyvinyl pyrrolidone-40 (PVP-40). Microtuber production experiments were carried out in 300 mL polypropylene jars, containing 50 mL medium in each. To investigate the effect of sucrose level on *in vitro* microtuber production; 30, 60, 90 or 120 g L^{-1} of sucrose was incorporated in to the modified MS medium. Effect of BA level was also examined for *in vitro* tuber formation. Number of micro-tubers and their lengths were recorded after eight weeks of culture. Regenerated microtubers with shoot and roots were transferred on to sterilized coco peat containing vessels for *in vitro* acclimatization; were incubated in culture room for eight weeks and were irrigated with MS inorganic solution only. After eight weeks of incubation, the *in vitro* hardened plantlets were remove from the vessels and were planted in plastic pots containing coco-pit, irrigated with fungicide (0.1% Carbendazim and 0.25% Mancozeb) solution and were kept in green house for further growth.

Statistical analysis: In all the experiments, each treatment was consisted of 10 replicates and repeated three times. The data were analyzed by analysis of variance (ANOVA) and the means were separated by the Duncan's Multiple Range (DMR) test. The p-values and F-values are derived from the one way ANOVA. Mean values followed by similar letters do not differ significantly at 5% level, using the statistical package version 6.4, COSTAT.

Colchicine analysis: Colchicine content was analyzed in the underground tubers, *in vitro* developed microtubers and from acclimatized plantlets microtubers using HPLC according to the protocol developed by Alali *et al*. (2004). Identification of colchicine was carried out by comparing the retention time of the sample with authentic colchicine. The chromatograms were recorded at 245 nm.

RESULTS

Bud culture: At culture establishment stage, all the three explants types were behaved differently. The shoot tip explants exhibited 70% bud break response on hormone free basal MS medium (Table 1, Fig. 1a). However, 90% bud break and shoot elongation were observed, in presence of 2.22 µM BA in MS medium from the apical shoot tip explants. Also it was noticed that although the apical shoot tips were showed good bud break percentage but each explant was always produced only a single shoot moreover, which was never branched. On the other hand the 2nd explant type, the nodal segments were failed to produce shoots *in vitro* in the presence of cytokinin (BA or kinetin) in the medium (Fig. 1b). The third explant type, underground tuber meristems showed 70% bud break as well as shoot elongation in the presence of 8.88 µM BA in the MS medium (Table 2) producing single shoot from each explant as in the case of the apical tip explants. However, the major difference was the formation of microtubers from the underground tuber explant apart from the production of shoot (Table 2, Fig. 1c). The *in vitro* microtubers were not produced when aerial shoot tip and nodal were used as explants.

***In vitro* tuber formation:** The established cultures from underground tuber meristem explants were produced *in vitro* tubers along with the single shoot probably due to the availability of the storage food in these underground tuber explants. Therefore, these cultures were subject to further microtuber production using higher concentrations of sugar and manipulation of BA contents to enhance the microtuber production rate. Hence, the cultures from underground tuber origin were further sub cultured on MS medium to optimize the *in vitro* tuber production. However, the cultures were turning black gradually and finally died. It was check by the incorporation of antioxidant and absorbent in MS medium, citric acid and PVP-40, respectively.

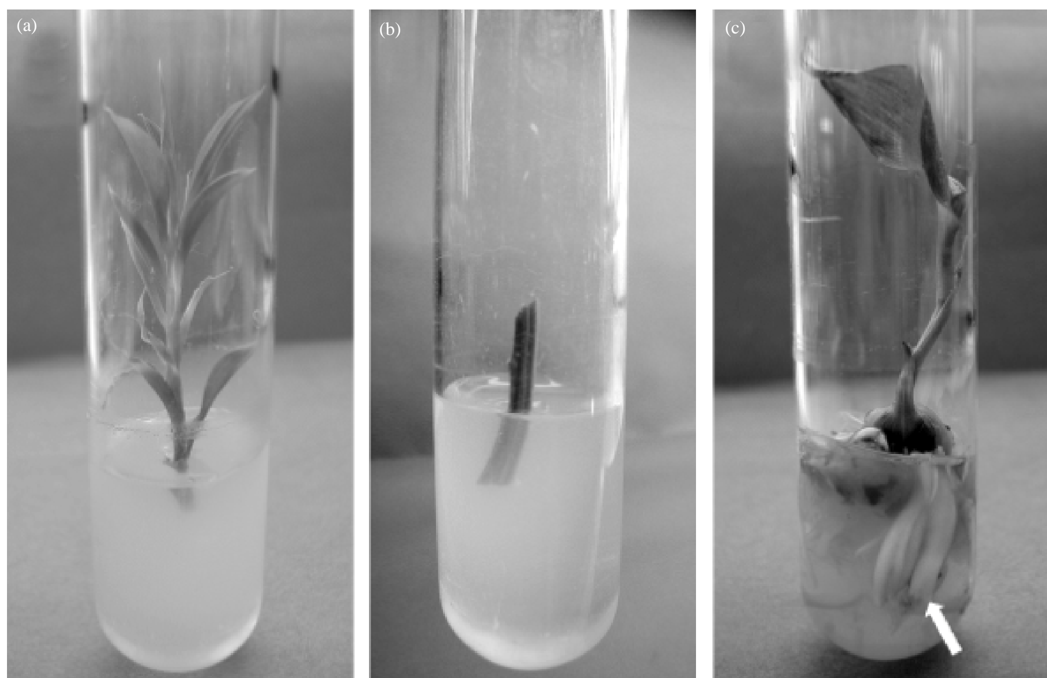

Fig. 1(a-c): Shoot initiation from three different explant types of *G. superba*, on MS medium, (a) Shoot tip explant, (b) Nodal explant and (c) Meristematic zone of underground tuber explant (arrow indicates the formation of microtubers)

Table 1: Effect of 6-benzyladenine on shoot initiation of *Gloriosa superba* on MS medium using shoot tip as explants

BA (μM)	Bud break (%)	No. of shoot per explants	Shoot length (cm)	No. of node per explant
		(Mean±SE)		
0	70	1±0	3.7±0.3^{bc}	1.9±0.2^{b}
2.22	90	1±0	6.3±0.4^{a}	3.3±0.2^{a}
8.88	90	1±0	3.9±0.2^{b}	2.2±0.2^{b}
17.76	80	1±0	2.9±0.2^{d}	1.4±1.0^{c}
26.64	60	1±0	3.0±0.1^{cd}	2.1±0.1^{b}
F	-	-	23.5768	20.0087
P	-	-	<0.0001	<0.0001

Each experiment consisted of 10 replicates (explants) and each experiment was repeated three times. The p-value and F-value are derived from the one way ANOVA. Mean value followed by similar letters do not differ significantly at 5% level, by DMR test

Table 2: Effect of 6-benzyladenine (BA) on shoot initiation and microtuberization of *Gloriosa superba* using underground tuber as explants on MS medium

BA (μM)	Bud break (%)	Shoot No. per explant	Shoot length (cm)	Node No. per explant	Microtuber No. per explant	Microtuber length (cm)
		(Mean±SE)				
00	70	1±0	2.1±0.1^{b}	2.4±0.2^{a}	3.0±0.2^{c}	1.1±0.05^{b}
8.88	70	1±0	3.0±0.3^{a}	2.7±0.3^{a}	6.1±0.3^{a}	1.6±0.07^{a}
17.76	50	1±0	2.6±0.2^{ab}	2.2±0.1^{a}	4.0±0.3^{b}	0.9±0.05^{c}
F	-	-	3.8468	1.6134	34.0652	4.5368
P	-	-	0.0251	0.2000	<0.0001	<0.0001

Each experiment consisted of 10 replicates (explants) and each experiment was repeated three times. The p-value and F-value are derived from the one way ANOVA. Mean value followed by similar letters do not differ significantly at 5% level, by DMR test

Sucrose at 60 g L^{-1} was observed as the optimum concentration inducing highest number of microtubers in PGR free modified MS medium (Fig. 2). The BA concentration was optimized in modified MS medium with 60 g L^{-1} sucrose. Maximum microtubers were obtained in the presence of 35.5 μM BA containing 60 g L^{-1} sucrose (Table 3, Fig. 3a). Continuous exposure of cultures to high BA level (35.5 μM) has resulted in the induction of numerous secondary microtubers and stunted shoots. Therefore, after eight weeks, the induced microtubers were sub-cultured on the same modified MS medium but with lower BA conc. (8.88 μM) for another eight weeks. In this medium, the microtubers were grown in size and produced shoots (Fig. 3b). The *in vitro* tubers were produced continually, in this two-stepped sub-culture cycle. The individual microtubers with shoots from the microtuber production cycle were harvested for *in vitro* acclimatization. The microtubers with shoots were transferred aseptically in to vessels filled with coco-pit and were irrigated. The microtubers in coco-pit produced functional roots, shoots and photosynthetically active leaves. Moreover, in the absence

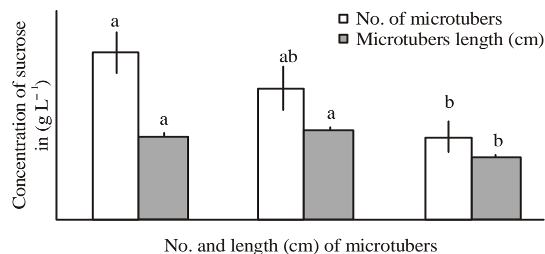

Fig. 2: Effect of sucrose on microtuber formation of *G. superba* on MS basal medium with citric acid and PVP-40, observations were recorded after eight weeks. Each experiment consisted of 10 replicates and each experiment was repeated three times. Mean values followed by similar letters do not differ significantly at 5% level by DMR test

Fig. 3(a-b): *In vitro* tuberization of *G. superba* (a) Microtuber formation on MS medium supplemented with citric acid, PVP-40, 60 g L^{-1} sucrose and 35.5 μM BA and (b) Shoot formation from microtubers on MS medium supplemented with citric acid, PVP-40, 60 g L^{-1} sucrose and 8.88 μM BA

Table 3: Effect of 6-benzyladenine (BA) on microtuber formation of *Gloriosa superba*, on MS medium supplemented with citric acid, PVP-40 and 60 g L^{-1} sucrose

BA (μM)	No. of microtuber per culture	Length of microtuber (cm)
00	2.4±0.3^{c}	1.2±0.07^{b}
2.22	3.0±0.2^{c}	0.9±0.03^{c}
4.44	4.5±0.3^{b}	1.4±0.09^{ab}
8.88	4.8±0.4^{b}	1.6±0.14^{a}
17.76	5.3±0.4^{b}	1.4±0.11^{ab}
35.52	9.8±0.8^{a}	1.4±0.13^{ab}
F	35.3501	6.0121
P	<0.0001	<0.0001

Each experiment consisted of 10 replicates (explants) and each experiment was repeated three times. The p-value and F-value are derived from the one way ANOVA. Mean value followed by similar letters do not differ significantly at 5% level, by DMR test

of organic supplements there was no contamination. The *in vitro* acclimatized plantlets with functional leaves and roots were successfully transferred to green house with 90% survival.

Colchicine analysis: The HPLC analysis was carried out to compare colchicine content in the field grown plant tubers and *in vitro* raised tubers of *G. superba*. The field-grown natural plant tubers contained 0.36% colchicine whereas one-month-old and three-months-old microtubers of *G. superba* in cultures showed 0.08 and 0.11% colchicine, respectively. Tubers from six-months-old tissue culture raised and acclimatized plantlets in the field contained 0.22% colchicine.

DISCUSSION

A very strong apical dominance is present naturally, in *G. superba*. Any damage to the apical shoot tip of this plant resulted in the death of complete plant due to its inability to produce branches before flowering. It may be the possible reason of its shrinking populations in forests, exposed to cattle grazing. It was also observed in our nursery plants, whenever the apical shoot tips were removed for explants has resulted in death of hundreds of the decapitated plants (personal observation). For herbivorous tolerance, activation of dormant axillary buds after the apical shoot damage is an important adaptation. It was observed that the field gentian (*Gentianella campestris*) was able to recover from damage even if the damage was high up to 75% (Huhta *et al*., 2000). The strong apical dominance nature in *G. superba* was also recorded during its *in vitro* culture when only the apical buds were able to produce shoots; the nodal meristems were remained dormant even when the apical shoot tip was absent and exogenous cytokinin was present in the medium. While, the *in vitro* release of apical dominance and axillary buds growth using cytokinin in the culture medium, was reported in another monocot species *Alstroemeria* (Pumisutapon *et al*., 2011).

During microtuber production, *in vitro* culture blackening was checked via incorporation of citric acid and PVP-40 in the culture medium. Similar strategy was also adopted for *Cleistanthus collinus* cultures (Quraishi and Mishra, 1998) to

solve the problem of blackening and death of cultures. For microtuber induction, 35.5 μM BA in the presence of 6% sucrose in the modified MS medium was recorded as the most suitable combination. Previously, Sivakumar *et al*. (2003b) have used MS medium with B_5 vitamins, 6% sucrose supplemented with cytokinin and Ancymidol, which induced multiple shoot formation and a single corm formation *in vitro* at the base of the each shoot of *G. superba*. Similarly, Ghosh *et al.* (2007) have used MS medium with normal sucrose level (3%) without any PGR and were observed three *in vitro* tubers per culture in twelve weeks, in the same plant. Yadav *et al.* (2012) also reported about three tubers per explant of *G. superba* in nine weeks using MS with normal sucrose level (3%) and combination of BA and α-naphthalene acetic acid (NAA). Shimasaki *et al.* (2009) reported maximum nine tubers formation of *G. superba,* using 3 μM thidiazuron with 80 g L^{-1} sucrose or 40 g L^{-1} trehalose. On the other hand, in the present study about 10 microtubers of *G. superba* per culture in eight weeks were produced, combined with single step *in vitro* rooting (without auxin) and acclimatization followed by the good survival rate.

The tissue culture raised tubers showed the lesser content of colchicine than the field-grown tubers probably due the fact that the field grown tubers were bigger in size and more than two years old. Nevertheless, the *in vitro* raised tubers showed increasing pattern of colchicine content, as they were growing bigger and older. Yadav *et al*. (2012) have reported higher colchicine content present in the tubers from micropropagated plantlets of *G. superba* than the natural ones. However, they have not mentioned the age of the tubers. On the other hand, in the other reports on microtuber production of this plant (Ghosh *et al*., 2007; Shimasaki *et al*., 2009; Sivakumar *et al*., 2003b), the colchicine content present in the regenerated microtubers was not examined.

CONCLUSION

Gloriosa superba L. is an herbaceous climber and important medicinal plant, distributed in tropical parts of the world. The plant becomes vulnerable in India due to its mass harvesting from forests because of medicinal and economic importance. The plant possess a very strong apical dominance that has also reflected in the present *in vitro* study. Only apical meristem explants were able to produce a single and un-branched shoots and nodal explants were remained dormant on MS medium supplemented with different concentrations of BA. Therefore, the *in vitro* propagation was accomplished by the microtuber formation technique. Maximum number of microtubers were produced on 35.5 μM BA in the presence of 6% sucrose in the modified MS medium. Moreover, rooting and *in vitro* acclimatization were achieved in a single step, combined with good survival when transferred to field. However, lesser colchicine content was recorded from *in vitro* raised plants in compare to naturally growing plants. But the colchicine content was increasing with the growing size and age of the *in vitro* raised tubers. Therefore, the present *in vitro* study may be useful for the efficient propagation and conservation of threatened medicinal plant *G. superba*.

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REFERENCES

Addrizzo-Harris, D.J., T.J. Harkin, K.M. Tchou-Wong, G. McGuinness, R. Goldring, D. Cheng and D.W.N. Rom, 2002. Mechanisms of colchicine effect in the treatment of asbestosis and idiopathic pulmonary fibrosis. Lung, 180: 61-72.

Alali, F., K. Tawaha and R.M. Qasaymeh, 2004. Determination of colchicine in *Colchicum steveni* and *C. hierosolymitanum* (colchicaceae): Comparison between two analytical methods. Phytochem. Anal., 15: 27-29.

Baker, M.A., B.D. Gray, B.M. Ohlsson-Wilhelm, D.C. Carpenter and K.A. Muirhead, 1996. Zyn-Linked colchicines: Controlled-release lipophilic prodrugs with enhanced antitumor efficacy. J. Controlled Release, 40: 89-100.

Cronstein, B.N. and R. Terkeltaub, 2006. The inflammatory process of gout and its treatment. Arthritis Res. Ther., Vol. 8.

Custers, J.B.M. and J.H.W. Bergervoet, 1994. Micropropagation of *Gloriosa*: Towards a practical protocol. Scientia Horticulturae, 57: 323-334.

FRLHT., 2003. Medicinal plants of conservation concern. Foundation for Revitalisation of Local Health Traditions (FRLHT), India.

Finnie, J.F. and J. van Staden, 1991. Isolation of Colchicine from *Sandersonia aurantiaca* and *Gloriosa superba*. Variation in the alkaloid levels of plants grown *in vivo*. J. Plant Physiol., 138: 691-695.

Ghosh, S., B. Ghosh and S. Jha, 2007. *In vitro* tuberisation of *Gloriosa superba* L. on basal medium. Scientia Horticulturae, 114: 220-223.

Hassan, A.K.M.S. and S.K. Roy, 2005. Micropropagation of *Gloriosa superba* L. through high frequency shoot proliferation. Plant Tissue Cult., 15: 67-74.

Huhta, A.P., T. Lennartsson, J. Tuomi, P. Rautio and K. Laine, 2000. Tolerance of *Gentianella campestris* in relation to damage intensity: An interplay between apical dominance and herbivory. Evol. Ecol., 14: 373-392.

Jadhav, S.Y. and B.A. Hegde, 2001. Somatic embryogenesis and plant regeneration in *Gloriosa* L. Indian J. Exp. Biol., 39: 943-946.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

NMPB, 2014. Annual demand of 32 prioritised medicinal plants and its uses. National Medicinal Plants Board (NMPB), Government of India, New Delhi, India.

Pumisutapon, P., R.G.F. Visser and G.J. de Klerk, 2011. Hormonal control of the outgrowth of axillary buds in *Alstroemeria* cultured *in vitro*. Biol. Plant., 55: 664-668.

Quraishi, A. and S.K. Mishra, 1998. Micropropagation of nodal explants from adult trees of *Cleistanthus collinus*. Plant Cell Rep., 17: 430-433.

Royal Botanical Gardens, 2011. *Gloriosa superb* (Flame lily). http://www.kew.org/science-conservation/plants-fungi/gloriosa-superba-flame-lily.

Shimasaki, K., K. Sakuma and Y. Nishimura, 2009. Tuber formation of *Gloriosa superb* using stem sections of branches under cultivation. Acta Horticulturae, 812: 245-250.

Sivakumar, G. and K.V. Krishnamurthy, 2000. Micropropagation of *Gloriosa superb* L.: An endangered species of Asia and Africa. Curr. Sci., 78: 30-32.

Sivakumar, G., K.V. Krishnamurthy and T.D. Rajendran, 2003a. *In vitro* corm production in *Gloriosa superba* L., an Ayurvedic medicinal plant. J. Horticult. Sci. Biotechnol., 78: 450-453.

Sivakumar, G., K.V. Krishnamurthy and T.D. Rajendran, 2003b. Embryoidogenesis and plant regeneration from leaf tissue of *Gloriosa superba*. Planta Med., 69: 479-481.

Sivakumar, G. and K.V. Krishnamurthy, 2004. *In vitro* organogenetic responses of *Gloriosa superba*. Russian J. Plant Physiol., 51: 713-721.

Yadav, K., A. Aggarwal and N. Singh, 2012. Actions for *ex situ* conservation of *Gloriosa superba* L.-an endangered ornamental cum medicinal plant. J. Crop Sci. Biotechnol., 15: 297-303.