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ASSESSMENT OF SOME FACTORS FOR SHOOT PROLIFERATION POTENTIAL IN TUBER EXPLANTS OF *Gloriosa superba* L. - A HIGH VALUE INDUSTRIAL MEDICINAL HERB

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ABSTRACT

***Gloriosa superba* L., an important medicinal plant, is included in the list of endangered plants so needs a special attention for its conservation through micropropagation. An efficient *in vitro* regeneration protocol was established using tubers as explants. Briefly, surface sterilized explants were cultured on MS medium containing different concentrations and combinations of 6-benzylaminopurine (BAP), kinetin and 1-naphthaleneacetic acid. Highest shoot regeneration frequency (88%) was recorded in MS medium containing 2.0 mg L^{-1} BAP. Half strength MS media supplemented with indole-3-acetic and indole-3-butyric acid (IBA), either alone or in combination, were used for rooting. Highest root regeneration (71%) was recorded in 2.0 mg L^{-1} IBA. Genetic fidelity of *in vitro*-raised plants was evaluated using RAPD and ISSR molecular markers, as there is always a danger of somaclonal variations in tissue culture technology. Out of the 17 (10 random amplified polymorphic DNA (RAPD) and 7 inter simple sequence repeats (ISSR) primers screened, 2 RAPD and 2 ISSR primers produced clear, distinct and reproducible amplicons. The results revealed genetic homogeneity and true-to-type nature of *in vitro* raised plants using RAPD and ISSR analysis. The developed protocol may prove helpful in regenerating valuable resource material for easy growth.**

Keywords: Explant, genetic fidelity, *Gloriosa superba*, micro-propagation, molecular markers

INTRODUCTION

Medicinal plants are considered to be the factories of bioactive compounds and are used as remedy against many diseases (Mishra and Sharma, 2020). *Gloriosa superba* L., commonly known as Kalihari, is an herbaceous glabrous branching annual climber belonging to the family Liliaceae (Soumya, 2018). The plant grows in sandy-loam soil in mixed deciduous forest in sunny locations and is usually found in the Himalayan foothills, Central India, Tamil Nadu, Andhra Pradesh and Bengal (Kumar and Murthy, 2002). It has several applications in medicines, pharmaceuticals and cosmetics and is widely used in Siddha, Ayurveda and Unani systems. It possesses antiinflammatory, antimicrobial, antibacterial, antidepressant and anticancerous properties due to the presence of highly active alkaloid colchicine and other bioactive compounds (Ade and Rai, 2009; Hemaiswarya *et al*., 2009; Nautiyal 2011). The presence of highly active alkaloid colchicines has created exploitation pressure on natural forests due to increasing demand. Owing to its overutilization, such

as harvesting from natural forests, habitat destruction and forest fires the plant is declared as endangered plant by IUCN (Pattanaik *et al*., 2009). Hence, there is need to conserve this plant. However, through the use of tissue culture techniques such as *in-vitro* mass multiplication and *in-vitro* regeneration, production of its bioactive compounds can be increased in less period of time and in an effective way. The bio molecular function can also be imitated through technology as there are no geographical barriers for the production of the plant materials through micro-propagation. Keeping the above things in mind, the present investigation was conducted with an aim to establish an *in vitro* regeneration protocol and to assess the genetic homogeneity of *in vitro* raised plants from indigenous *Gloriosa superba*.

MATERIALS AND METHODS

Surface sterilization and media preparation

The present study was conducted in the Department of Biotechnology, College of Horticulture and Forestry, Dr. Y.S. Parmar University of Horticulture and Forestry, Neri-Hamirpur (H.P.) from 2017 to 2019. The explants tubers (non-dormant) of *G. superba* were collected from the Herbal Garden, Neri, Hamirpur, H.P., India. The explants were surface sterilized under aseptic environment in a laminar air flow with 1% carbendazim 50% WP (Bavistin) for 1 min, followed by rinsing with autoclaved distilled water and were subsequently treated with 0.01-0.5% mercuric chloride solution for 30-60 sec. Subsequently, the explants were cultured in MS medium fortified with different concentrations of growth regulators either alone or in combination. The pH of medium was adjusted to 5.8 with 1N HCl. The cultures were maintained aseptically at 25±2ºC under a 16 h light and 8 h dark photoperiod.

In vitro shoot proliferation

The explants inoculated on MS medium were supplemented with varied concentrations (0.5-0.75 mg L^{-1}) of cytokinins namely 6-benzylaminopurine (BAP) and kinetin [Kn). BAP was used alone in concentration 0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1}, whereas, Kn was also used in combination with BAP (BAP + Kn) in the concentration of 0.5 + 0.25, 1.0 + 0.5 and 2.0 + 0.75 mg L^{-1}.

In vitro rooting and hardening

Regenerated shoots were transferred to MS medium containing different concentration of auxins [indole acetic acid (IAA) and indole-3-butyric acid (IBA)] for root induction. The concentrations of IAA and IBA each evaluated alone were 0.5, 1.0 and 2.0 mg L^{-1}, whereas IAA and IBA were used in different combinations (IAA + IBA) of 1.0 + 0.5, and 1.0 + 1.0 mg L^{-1}. Healthy shoots with well-developed roots were then hardened. Plantlets with well-developed shoots and roots were transferred to the potting mixture containing sand: soil: vermicompost (1:2:1). The potted plantlets were covered with transparent polythene bags so as to maintain proper humidity. The plants irrigated at regular intervals. IBA @ 0.5, 1.0, and 2.0 mg L^{-1}. was also used alone, and IAA in combination with IBA (IAA + IBA) @ 1.0 + 0.5 and 1.0 + 1.0 mg L^{-1}. Healthy shoots with well-developed roots were then hardened.

DNA extraction and PCR amplification

Total genomic DNA was isolated by using CTAB method (Doyle and Doyle, 1987) with slight modifications from young leaves of mother plants and *in vitro*-raised plants of *Gloriosa superba*. For RAPD analysis, 10 primers were screened and PCR reactions performed in 25 µL reaction mixture having 0.5 units of Taq DNA polymerase, 200µM of each dNTPs, 1 X assay buffer 0.2 µM primers and 50 ng template DNA. The PCR reactions were carried out in a thermal cycler. The PCR amplification conditions for RAPD consisted of initial denaturation at 94ºC for 7 min, followed by

39 cycles of denaturation at 94ºC for 45 sec, annealing at 37ºC for 45 sec and initial extension at 72ºC for 2 min, followed by final extension at 72ºC for 7 min. The reaction products were mixed with 3 µL of 6X loading buffer and then assessed on 1.2% agarose gel. After the separation gels were documented using gel documentation system.

For ISSR analysis, 7 primers were screened and PCR reactions were same as followed in RAPD analysis, except 1.0 units of Taq DNA polymerase. In case of ISSR, the PCR amplification reaction consisted of initial denaturation at 94ºC for 7 min, followed by 39 cycles of denaturation at 94ºC for 45 sec, annealing for 30 sec at specific temperature for each primer and initial extension at 72ºC for 2 min, followed by extension at 72ºC for 7 min. The data were analyzed using one-way and two-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

To solve the contamination problems encountered in tuber culture of *Gloriosa superba*, variable concentrations of $HgCl_2$ (0.01-1.0%) were evaluated. The maximum survival rate (73.33%) was observed in 0.5% of concentrations of $HgCl_2$ for 30 sec.

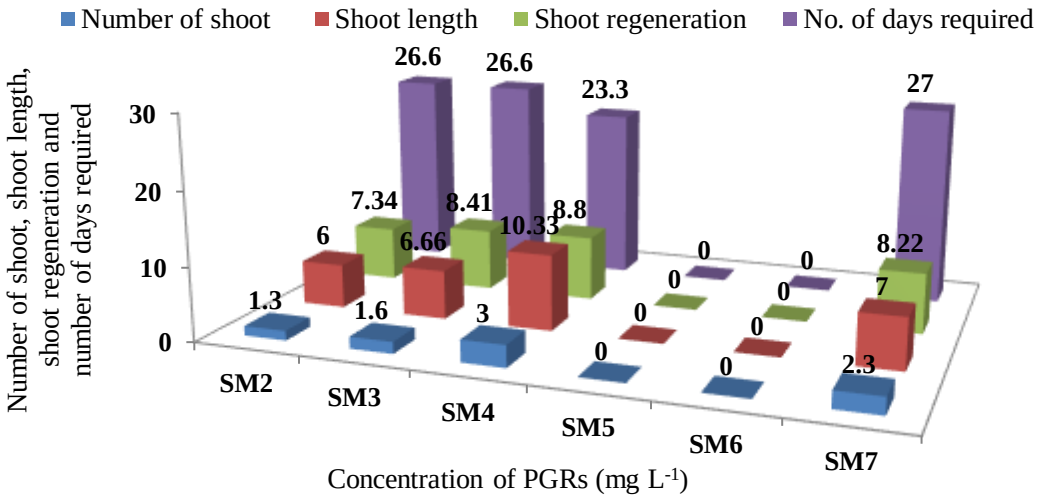

**Fig. 1: Effect of various concentrations of growth regulators on number of shoots explants^{-1}, shoot regeneration, number of days required for shoot regeneration and shoot length from tuber as explants in *Gloriosa superba;* ***SM = Shooting medium; SM2 = BAP (0.5 mg L^{-1}), SM3 = BAP (1.0 mg L^{-1}), SM4 = BAP (2.0 mg L^{-1}), SM5 = BAP + Kn (0.5 mg L^{-1} + 0.25 mg L^{-1}), SM6 = BAP + Kn (1.0 mg L^{-1} + 0.5 mg L^{-1}), SM7 = BAP + Kn (2.0 mg L^{-1} + 0.75 mg L^{-1})

Effect of different plant growth regulators on in vitro shoot regeneration

For establishment of tuber explants, the surface sterilized explants were cultured on MS medium supplemented with varying concentrations of BAP and Kn for shoot regeneration. MS medium without growth regulators did not induce bud break or shoot multiplication. Of the various treatments, highest shoot frequency (88%) was observed in MS medium containing 2.0 mg L^{-1} BAP with highest number of shoots explants (3.00 ± 0.58) and shoot length (10.33 ± 0.92 cm) after 26 days (Fig. 1).

Effect of growth regulators on root regeneration

In vitro grown shoots were taken and used for studying *in vitro* induction of rooting. The study revealed that the excised shoots failed to develop roots on medium without provision of PGRs. Among various treatments half strength MS basal and 2.0 mg L^{-1} IBA was recorded to be the best

medium for *in vitro* root induction and showed the highest root regeneration (71%), number of roots shoot^{-1} (8.00 ± 0.58) and root length (5.00 ± 0.58 cm) after 18 days. The success of IBA in promoting efficient root induction has been reported for *Ceropegia candelabrum* (Beena *et al.* 2003), *Mucunapruriens* (Faisal *et al.* 2006). *Kaempferia galanga* (Shirin*et al*., 2000), *Tylophora indica* (Haque and Ghosh 2013), *Luffa acutangula* (Saha and Ghosh, 2015). IBA was found to be the most effective at different concentrations among different types of auxins in *Gloriosa superba* (Chatterjee and Ghosh, 2015) and among different concentrations IBA 0.5 mg L^{-1} was found to be best concentration of auxin for proper rooting (Fig. 2).

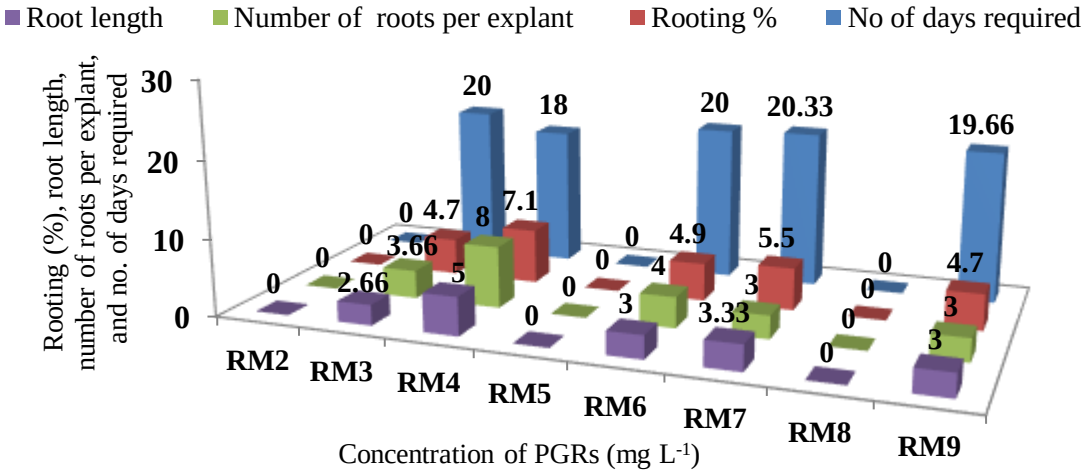

Fig. 2: Effect of different concentrations of IAA and IBA on root length, number of roots explants^{-1}, rooting (%) and number of days required for *in vitro* rooting in *G. superba;* *RM = Rooting medium; RM2 = IBA (0.5 mg L^{-1}), RM3 = IBA (1.0 mg L^{-1}), RM4 = IBA (2.0 mg L^{-1}), RM5 = IAA (0.5 mg L^{-1}), RM6 = IAA (1.0 mg L^{-1}), RM7 = IAA (2.0 mg L^{-1}), RM8 = IAA + IBA (1.0 mg L^{-1} + 0.5 mg L^{-1}) and RM9 = IAA + IBA (1.0 mg L^{-1} + 1.0 mg L^{-1})

Effect of substrates on hardening

For hardening and establishment of *G. superba* plantlets under natural conditions, the well-rooted plantlets were transferred to the plastic pots containing sand: soil: vermicompost (1:2:1) and covered with polythene bags (Fig. 3). Micro-plants hardened in these pots showed higher survival rate of 73.3%. Thakur *et al.* (2016) in *Pittosporum eriocarpum* reported 73% survival rate. The beneficial effect of vermicompost on growth and acclimatization has also been reported for *Chlorophytum borivilanum* (Kumar *et al.* 2010) and *Tylophora indica* (Rani and Rana, 2010). In many other taxa, high mortality rate has been reported for *in vitro*-raised plants, transferred to natural field conditions, as they possess weak root system at this stage (Mathur *et al.* 2008; Chandra *et al.* 2010).

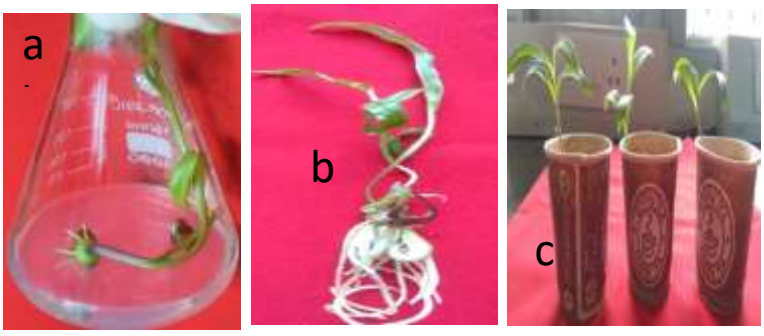

Fig. 3: *In vitro* regeneration of *Gloriosa superba:* a) shoot initiation in MS medium with 2.0 mg BAP L^{-1} + 0.75 mg Kn L^{-1}; b) root regeneration; c) hardened plant after 10 days in substrate sand: soil: vermicompost (1:2:1)

DNA amplification using RAPD and ISSR primer

Of 10 primers screened, only 2 primers *viz*., GCC114 (sequence TGACCGAGAC) and GCC111 (sequence AGTAGACGGG, produced amplification. Finally, 2 anchored RAPD primers were used

for the analysis of genetic diversity amongst 4 samples of *in vitro*-raised plants and mother plant. Primer GCC114 produced total of 4 scorable bands and out of these bands 3 were monomorphic and 1 was polymorphic. The RAPD banding pattern of GCC111 produced 1 scorable band with size 490 bp. The band was monomorphic. The matrix obtained was analyzed with NTSYS-pc software. The similarity coefficient ranged from 0.833 to 1.000 and the dendrogram was constructed using UPGMA between mother plant and *in vitro* plants. Three *in vitro* plants were 100% similar to the mother plant and one plant showed 83% similarity. RAPD-mediated DNA fingerprinting has extensively been used for detection of polymorphism among the micro-propagated medicinal plants (Ramesh *et al.,* 2011). In case of ISSR, out of 7 primers screened 2 primer sequences *viz*., GCC810 and GCC846 produced amplification and were used for the analysis of genetic diversity amongst 4 samples of *in vitro*-raised plants and mother plant (Fig. 4). Dendrogram was plotted using UPGMA between mother plant and *in vitro*-plants. Three *in vitro* plants showed 100% similarity and one plant showed 80% similarity with mother plant. ISSR has been used for detecting the somaclonal variations in many economical micro-propagated plants like apple rootstock (Pathak *et al.,* 2012).

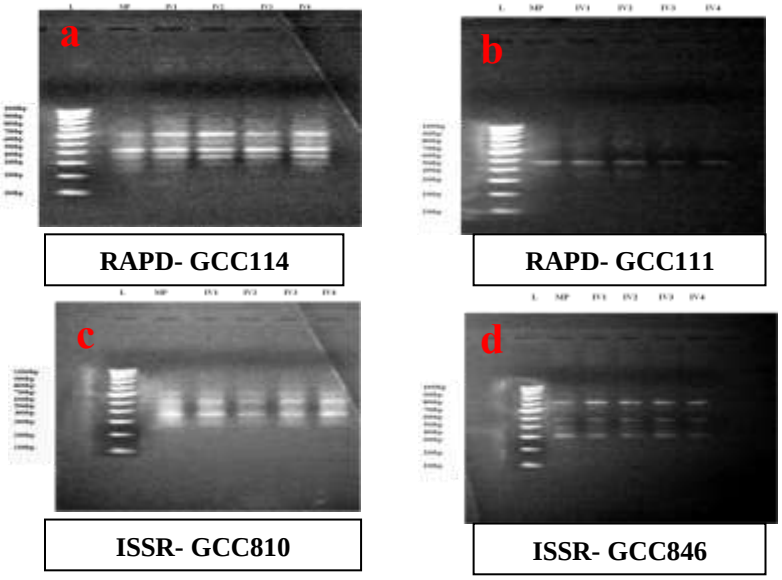

Fig. 4: RAPD and ISSR profiles of mother and *in vitro*-raised plants of *Gloriosa superba*

Conclusions: The present study reports important base-line information regarding *in vitro* regeneration, greenhouse acclimatization and field transfer of *G. superba*. The results reveal the genetic homogeneity and true-to-type nature of *in vitro*-raised plants using RAPD and ISSR analysis. Such protocols will be helpful in providing valuable resource material and help in delisting of species from red data book.

Conflict of interest: The authors declare that there is no conflict of interest.

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