Fitoterapia 89 (2013) 265–270

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Evaluation of genetic fidelity among micropropagated plants of *Gloriosa superba* L. using DNA-based markers — a potential medicinal plant

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ARTICLE INFO

Article history:
Received 19 March 2013
Accepted in revised form 11 June 2013
Available online 28 June 2013

Keywords:
Gloriosa superba
Micropropagation
Genetic fidelity
Molecular markers
RAPD
ISSR

ABSTRACT

Malabar glory lily (*Gloriosa superba* L.) is a medicinally potent plant species used for the production of alkaloid colchicine. With ever increasing demand, there is a pressing need to conserve it through biotechnological approaches. A large number of complete plantlets were obtained by direct regeneration from the non-dormant tuber explants on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l 6-benzylaminopurine (BAP) + 0.5 mg/l α-naphthalene acetic acid (NAA). Large number of plants can be produced in vitro under aseptic conditions, but there is always a danger of producing somaclonal variants by tissue culture technology. Thus, the genetic stability of micropropagated clones was evaluated using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis. During the study a total of 80 (50 RAPD and 30 ISSR) primers were screened, out of which 10 RAPD and 7 ISSR primers produced a total of 98 (49 RAPD and 49 ISSR) clear, distinct and reproducible amplicons. The amplification products of the regenerated plants showed similar banding patterns to that of the mother plant thus demonstrating the homogeneity of the micropropagated plants. This is the first report that evaluates the use of genetic markers to establish genetic fidelity of micropropagated *G. superba* using RAPD and ISSR, which can be successfully applied for the mass multiplication, germplasm conservation and further genetic transformation assays for colchicine production to meet the ever increasing demand of this medicinally potent plant for industrial and pharmaceutical uses.

1. Introduction

Malabar glory lily (*Gloriosa superba* L.), the sole species in the family Colchicaceae is a perennial tuberous climbing herb widely scattered in tropical and sub-tropical parts of Africa and Southeast Asia. In India, it is usually found in the Himalayan foot-hills, Tamil Nadu, Andhra Pradesh and Bengal [27]. Its attractive wavy edged orange–red flower is the national flower of Zimbabwe and also the state flower of Tamil Nadu in India.

The tubers and seeds of this plant contain more colchicine content than the genera *Colchicum* [3]. Colchicine alkaloid (Fig. 1) is highly demanded due to its uses for treating arthritis, Mediterranean fever, gout, rheumatism, inflammation, ulcers, bleeding piles, skin diseases, leprosy, impotency and snakebites [7]. Colchicine also has antimitotic activity, preventing growth of cancer cells by interacting with microtubules, which could lead to the design of better cancer therapeutics [29]. Due to the action of colchicine on spindle-fiber formation during cell-division, it is also capable of inducing polyploidy and used in cytological and plant breeding research [10].

Abbreviations: dNTPs, deoxyribonucleotide triphosphates; PCR, polymerase chain reaction; BAP, 6-benzylaminopurine; NAA, α-naphthalene acetic acid; ISSR, inter simple sequence repeats; RAPD, random amplified polymorphic DNA.

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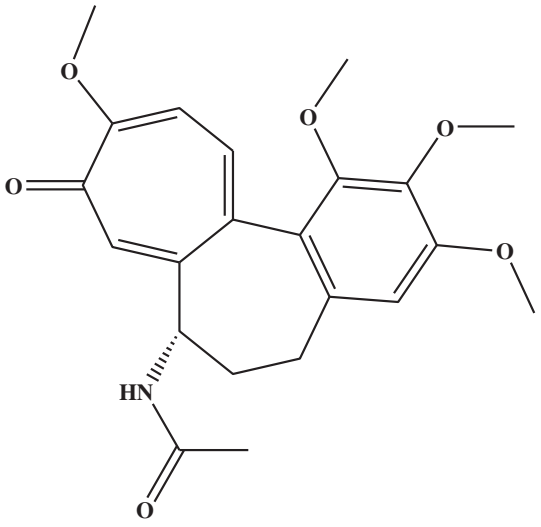
http://dx.doi.org/10.1016/j.fitote.2013.06.009

The conventional method of propagation through tubers is slow with poor multiplication ratio [9]. Its production is seasonal having susceptibility toward many pests [13]. The poor propagation coupled with over exploitation by the local population as well as pharmaceutical companies is the main factor responsible for its diminishing population size [26].

Micropropagation provides an alternate and effective means for rapid multiplication of species by the continuous production to meet the demand [34,35]. Several efforts have been made to propagate *G. superba* in vitro on various culture media as well as utilizing different regeneration pathways [4,5,26,28,32].

Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span [12]. However, micropropagation protocol is severely hindered due to incidences of somaclonal variations [11]. Somaclonal variation mostly occurs as response to the stress imposed on the plant in culture conditions and is manifested in the form of DNA methylations, chromosome rearrangements, and point mutations [14,41]. The application of sub- and supra-optimal levels of growth regulators and the recurrent subculture for indefinite period hinder maintenance of genetic fidelity in the tissue cultured clones [15]. It is, therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility. Molecular techniques are at present powerful and valuable tools used in the analysis of genetic fidelity of in vitro propagated plants. In comparison to various morphological, cytological, and protein markers used for the detection of variations in tissue-cultured raised plantlets, polymerase chain reaction (PCR) techniques randomly amplified polymorphic DNA (RAPD) [31] and inter simple sequence repeat (ISSR) [37] markers have been favored because of their simplicity, cost-effectiveness, stability, sensitivity, highly reproducible and reliability [23]. The use of two types of markers has been successfully applied to amplify different regions of the genome allowing better analysis of genetic stability in several micropropagated crops, such as *Musa* sp. [30]; *Amorphophallus albus* [6]; *Saussurea involucrata* [36]; *Bacopa monnieri* [1]; *Pogostemon cablin* [21]; *Sapindus trifoliatus* [2]; *Citrus jambhiri* [24]; and *Malus domestica* [20].



Colchicine ($C_{22}H_{25}NO_6$)

Fig. 1. Colchicine ($C_{22}H_{25}NO_6$).

To our knowledge, there has been no report on genetic stability analysis across the micropropagated plants, along with the donor mother plant in *G. superba* plantlets. So, the present study was conducted to screen tissue culture induced genetic variations (if any) in *G. superba* employing RAPD and ISSR–PCR assay.

2. Materials and methods

2.1. Plant material

The non-dormant tubers (1–1.5 cm) of *G. superba* plants collected from Ch. Devi Lal herbal garden, Chuharpur, Yamuna Nagar, Haryana (India) served as a source of explants for micropropagation. Highest shoot regeneration frequency was obtained on multiplication medium consisting of MS salts and vitamins [18] supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l), 3% sucrose and 0.4% agar [32] (Fig. 2a). The multiplied shoots were rooted, acclimatized and hardened following the standardized protocol described by Yadav et al. [33] (Fig. 2b).

The micropropagated plants were phenotypically similar to the mother plant and no morphological variations were observed among the regenerated clones. After 100 days of hardening all the micropropagated plants were randomly selected and their RAPD and ISSR profiles were compared with the mother plant.

2.2. DNA extraction and PCR amplification

Total genomic DNA was extracted using the method of Doyle and Doyle [39] from leaf samples. The quality of extracted DNA after RNase treatment was assessed on 0.8% agarose gel and finally the DNA was quantified using spectrophotometer (Optigen 2020 plus). The DNA samples were diluted to 25 ng μl^{-1} with TE (Tris–EDTA) buffer before use. Samples were stored for further study at 4 °C.

For RAPD analysis 50 primers (40 primers from set # 1 and 10 primers from set # 2) obtained from the University of British Columbia (UBC, Vancouver, Canada) were tested, and 10 were selected for carrying out the clonal fidelity assay depending on band reproducibility and clarity. The PCR reactions were performed in a 25 μl reaction mixture containing 1× assay buffer, 0.5 units of *Taq* DNA polymerase, 200 μM of each dNTPs (Bangalore Genei), 0.2 μM primers and 50 ng of template DNA. The PCR reactions were carried out in DNA thermal cycler (Model-CGI-96, Corbett Research, Australia) using a single primer in each reaction. The PCR reactions were repeated thrice for each primer to ensure the reproducibility of RAPD results. Only highly reproducible and polymorphic primers were included in the study. The PCR amplification conditions for RAPD consisted of initial extended step of denaturation at 94 °C for 4 min followed by 44 cycles of denaturation at 94 °C for 1 min, primer annealing at 37 °C for 1 min and elongation at 72 °C for 2 min followed by a final step of extension at 72 °C for 4 min. The PCR reaction products were mixed with 4 μl of 6× DNA loading buffer and fractionated on 1.2% agarose for RAPD containing 0.5 μg μl^{-1} ethidium bromide. Gels were electrophoresed until the