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Single primer amplification reaction (SPAR) methods reveal subsequent increase in genetic variations in micropropagated plants of *Nepenthes khasiana* Hook. f. maintained for three consecutive regenerations

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ABSTRACT

The genetic fidelity of *in vitro*-raised plants of three successive regenerations of *Nepenthes khasiana* Hook. f. was assessed using three different single primer amplification reaction (SPAR) methods, viz., random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and direct amplification of minisatellite DNA region (DAMD) markers. Out of 80 RAPD primers screened, 14 primers reflected a genetic variation of 4.1% in the first regeneration which was increased to 9.4% in the third regeneration. In the case of ISSR, out of 36 primers screened for assessment of genetic homogeneity of the regenerated plantlets, 12 primers showed an increase of genetic variation from 4.3% to 10% from the first to the third regenerations. In DAMD profiling, 15 primers were used for the evaluation of genetic fidelity where 8.47% of polymorphism was observed in the first regeneration which was increased to 13.33% in the third regeneration. The cumulative analysis reflected a genetic variation of 5.65% in the first regeneration which increased subsequently to 7.77% in the second regeneration and 10.87% in the third regeneration. The present study demonstrates SPAR technique to be an efficient tool for the assessment of clonal fidelity of *in vitro*-raised plants.

1. Introduction

Nepenthes khasiana Hook. f., the only representative of the genus *Nepenthes* in India belongs to the monotypic family Nepenthaceae and is a rare and endangered insectivorous plant found in Northeast India. This species captures insects with the help of curious and attractive pitchers and digests the proteins of trapped insects thereby supplementing nitrogenous salts. The species is of great botanical and horticultural interest (Khoshbakht and Hammer, 2007; Mukerjee et al., 1984). The fluid of the unopened pitcher of *N. khasiana* is used by local inhabitants as an eye drop for redness, itching, to cure cataract and night blindness and is also taken for stomach troubles, diabetes, leprosy and for female diseases (Joseph and Joseph, 1986; Kumar et al., 1980; Rao et al., 1969). Habitat destruction, deforestation, urban development, developmental projects, road laying and modern agriculture, and fragmentation of large contiguous populations into isolated small and scattered ones have rendered the species increasingly vulnerable to environmental stochasticity, which would ultimately lead to its extinction. The plant is also being collected and exported by local plant collectors to other states of India on account of the fascinating beauty of its pitcher (Bhau et al., 2009). The species has been classified as a threatened species and is included in the list of rare and threatened taxa of India (Jain and Baishya, 1977; Jain and Sastri, 1980).

Plant tissue culture techniques have been successfully applied for rapid clonal multiplication and conservation of many rare and endangered plant species (Tandon and Kumaria, 1998). *In vitro* multiplication for large-scale propagation of *N. khasiana* has been achieved using seeds as well as explants in order to conserve this pitcher plant of India (Latha and Seeni, 1994; Nongrum et al., 2009; Rathore et al., 1991; Tandon and Rathore, 1994). For large-scale production, efficiency of propagation methods is of prime importance, but perhaps even more important is the genetic stability of *in vitro* regenerated plantlets (Haisel et al., 2001). Many of the regenerated plantlets may not be the clonal copies of their donor genotype when passaged through *in vitro* cultures. The occurrence of cryptic genetic defects arising due to somaclonal variations in the regenerants can seriously limit the broader utility of the micropropagation system (Salvi et al., 2001). Therefore, it is of paramount importance to monitor the genetic uniformity in the micropropagated plants for the commercial utilization of true-to-type plants of the desired genotype.

Of the various DNA-based molecular markers, random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR)

Abbreviations: KN, kinetin; BAP, 6-benzylaminopurine; NAA, α-napthaleneacetic acid; MS, Murashige and Skoog medium; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeats; DAMD, direct amplification of minisatellite DNA regions; SPAR, single primer amplification reaction; TBE, Tris–borate–EDTA.

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are very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample and do not need any prior sequence information to design the primer. They are, thus, widely used for assessment of the genetic fidelity of in vitro raised clones as well as genetic diversity studies. In the recent years, the PCR-based single primer amplification reaction (SPAR) methods which include (a) direct amplification of minisatellite DNA regions (DAMD) (Heath et al., 1993); (b) inter simple sequence repeat (ISSR) (Gupta et al., 1994) and (c) random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) are gaining prominence as effective tools for genetic diversity studies in plants and they collectively provide a comprehensive description of the nature and extent of the diversity (Bhattacharya et al., 2005; Ranade et al., 2009). This technique would be more precise for the establishment of genetic fidelity in the micropropagated plants before they are transferred to the field for conservation. However, little information on studies of genetic fidelity of in vitro-raised plants using SPAR approach can be traced in the literatures. In the present study, we attempt to assess the genetic stability of *in vitro*-raised plants of *N. khasiana* using three different SPAR methods.

2. Materials and methods

2.1. Plant material and culture conditions

Axillary cultures of *N. khasiana* were established in vitro through nodal explants collected from Jarain, Meghalaya, Northeast India following the protocol described by Devi et al. (2013). Single-nodal segments (2–3 cm) were thoroughly washed under running water for 30 min to remove any adherent particles, immersed in 5% (v/v) laboratory detergent (Labolene, Qualigens, India) for 20 min, and rinsed under tap water before finally treating with fungicide (1% Bavistin) for 1 h. These were then surface sterilized with 0.2% $HgCl_2$ (w/v) solution for 8 min and rinsed 4–5 times with sterilized distilled water. The explants (~1.0 cm) were finally excised aseptically and cultured in shoot induction medium. The nutrient medium used consisted of MS salts and vitamins with 3% (w/v) sucrose (Himedia, India) (Murashige and Skoog, 1962). Activated charcoal (0.05% w/v) and ascorbic acid (50 mg/l) were also incorporated in the medium. The medium was solidified with 0.8% (w/v) agar (Himedia, India) and the pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The explants were cultured in half-strength MS supplemented with 2.5 mg/l kinetin (KN) and 2.0 mg/l 6-benzyl aminopurine (BAP). The elongated shoots were transferred to half strength MS medium supplemented with 2.0 mg/l α-napthaleneacetic acid (NAA) for rooting. The second and third regenerations were raised using 6 month old nodal stem segments of the first and the second regenerations respectively. All the cultures were maintained at 25 ± 2 °C under a 14 h photoperiod with a photosynthetic photon flux density (PPFD) of 60.2 μmol m^{-2} s^{-1} supplied by cool white fluorescent lamps (40 W, Philips, India) with 65–70% RH.

2.2. DNA extraction

Leaf material for DNA extraction was collected from the donor plant, as well as from *in vitro* propagated plants of the three consecutive regenerations (Figs. 1A–F). Frozen leaves were ground and powdered in a pre-chilled mortar using liquid nitrogen, and the DNA was then extracted using modified CTAB method (Porebski et al., 1997). The DNA extracted from the plant material, purified for protein fraction, treated with RNase A, was re-precipitated with pre-chilled absolute ethanol and subsequently dissolved in Tris–EDTA (TE) buffer. The quality of DNA was checked by electrophoresis on 0.8% agarose gel and the quantification was done with Lambda 35 spectrometer (PerkinElmer, USA).

2.3. Amplification reactions with RAPD, ISSR and DAMD primers

RAPD analysis was performed following the method described by Williams et al. (1990). Polymerase Chain Reactions were carried in a total volume of 25 μl containing 30 ng template DNA, 200 μM each dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 0.6 U Taq polymerase (Bangalore Genei, India) and 5 pmol of primers (Operon Technologies, USA). Initially, primers from four kits (A, C, H and K) comprising 20 decamer random primers per kit were screened for RAPD reactions with selected *N. khasiana* DNA templates. Based on this screening, primers that resulted in well-separated bands on agarose gels were selected for the amplification of all the three consecutive regenerations and the donor plant. PCR was performed in a Thermal Cycler (Applied Biosystems, USA) with a program consisting of pre-PCR cycle at 95 °C for 4 min and 30 s; 34 °C for 1 min and 72 °C for 2 min followed by initial denaturation at 94 °C for 1 min and 40 cycles of 1 min at 94 °C, 1 min at 38 °C, 2 min at 72 °C and a cycle of final extension at 72 °C for 10 min.

A set of 36 ISSR primers was procured from University of British Columbia, Canada. DNA amplification was carried out according to Gupta et al. (1994). PCR amplification of 50 ng DNA was performed 40 ng template DNA, 200 μM each dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 0.6 U Taq polymerase (Bangalore Genei, India) and 10 pmol of primers (Metabion, Germany). After initial denaturation at 94 °C for 3 min, each cycle consisted of 1 min denaturation at 94 °C, 1 min of annealing temperature 42–58 °C (depending on the primer's Tm and/or according to GC contents), at 52 °C, 2 min extension at 72 °C along with 10 min extension at 72 °C at the end was carried out 40 times.

The DAMD primers were custom synthesized from Metabion, Germany. DNA amplification was carried out according to Zhou et al. (1997). The reaction mixture contained 40 ng template DNA, 200 μM each dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 0.6 U Taq polymerase (Bangalore Genei, India) and 10 pmol of primers. DNA amplification was performed by initial denaturation at 94 °C for 2 min and 40 cycles of 1 min at 92 °C, 2 min at 55 °C, 2 min at 72 °C and a cycle of final extension at 72 °C for 10 min.

2.4. Gel electrophoresis

Amplification products were separated by electrophoresis in 1.2% (RAPD) and 1.5% (ISSR and DAMD) agarose gel in 1× TBE buffer stained with ethidium bromide under 70 V constant power supply for 3 h and photographed under UV with Gel logic 100 imaging system (Biosteps, Germany).

2.5. Data scoring and analysis

Only clear and well separated amplicons were scored across all samples. These bands were scored independently as either present (1) or absent (0). The data were scored individually, first for all the primers in a SPAR method and subsequently the data sets for all the three methods used. A dendrogram was generated by cluster analysis using the UPGMA method based on Jaccard's coefficient. Data generated by the three markers were analyzed for the three successive regenerations. The cumulative analysis was also carried out for the three molecular markers commonly regarded as SPAR for all the three successive regenerations.

3. Results and discussion

A total of 136 primers were screened and 41 primers were finally selected for further profiling (Table 1). Comparison of three different SPAR methods and the extent of polymorphism in the three consecutive regenerations are represented in Table 2.