Mitotic Chromosome Studies in *Nepenthes khasiana*,
An Endemic Insectivorous Plant of Northeast India

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**Summary** Chromosome counts were carried out in root tip cells of *Nepenthes khasiana* (Nepenthaceae), a threatened insectivorous plant of Northeast India. *N. khasiana* has become threatened in its natural habitat due to overexploitation for its medicinal uses as well as its ornamental importance. Plantlets of *Nepenthes khasiana* collected from Jarein, Meghalaya were cytologically analyzed. All the root tip cells analyzed showed the chromosome number of 2n=80 without any variations. Karyomorphological studies were not plausible in this species due to the relatively small size of the chromosomes.

**Key words** *Nepenthes khasiana*, Mitosis, Insectivorous, Polyploidy, Karyotype.

The genus *Nepenthes* belonging to the family Nepenthaceae is one of the largest genus among the insectivorous plants. It comprises of about 134 species (McPherson 2009) of which only one species is found in India (Bordoloi 1977). *Nepenthes khasiana* Hook. f. is the only species found in India and occurs as an endemic species of Meghalaya. It is believed that the species represents ancient endemic remnants of older flora which usually occur in land masses of geological antiquity (Paleoendemics), (Bramwell 1972). In India, it is usually found growing from the west Khasi Hills to the east Khasi Hills, in the Jaintia Hills, and in the east to west and south Garo Hills from 1000 to 1500 m altitude (Mao and Kharbuli 2002). It is a climbing undershrub which ranges from a few centimeters to several meters in height (Bordoloi 1977). The midrib of its leaves extends from the tip of the leaf which modify into showy and brightly coloured pitchers to trap a wide group of insects so as to compensate nitrogen and energy deficiency in the soil (Kitching and Schofield 1986).

The population of *N. khasiana* has dwindled in the last few decades due to multifarious anthropogenic activities such as deforestation, jhum cultivation, overexploitation and forest fires (Jain and Sastry 1980). Consequently, it has become threatened in its natural habitat and has been regarded as an endangered plant in Appendix 1 of CITES. The plant is also being collected, regularly, by local plant collectors because of its fascinating pitcher. *N. khasiana* is often purchased from the markets and hybridized to produce a diversity of pitcher characters (Mao and Kharbuli 2002). Therefore, the species is of great botanical and horticultural interest (Khoshbakht and Hammer 2007, Mukerjee et al. 1984).

Although many researchers have worked on *Nepenthes* on different aspects such as enzymes present in the digestive fluid (Nakayama and Amagase 1968, Tokes et al. 1974, Rottloff et al. 2011); development of the pitchers (Owen and Lennon 1999); insect trapping mechanism (Salmon 1993, Moran 1996); taxonomy (Check and Jebb 2009); multiplication and micropropagation (Latha
and Seei 1990, Rathore et al. 1991, Nongrum et al. 2008); and recently a few concerted efforts for molecular characterization of genomic DNA have been made (Meimberg et al. 2005, Eilenberg et al. 2006, Bhau et al. 2009, Nongrum et al. 2012). However, there is still a lack of basic information of the genetics such as chromosome number. Although certain passing remarks about the chromosome counts were made by a few researchers (2n=80; Heubl and Wistuba 1997), no convincing information on chromosome biology can be traced in the published literature. Therefore, an approach to determine the somatic chromosome number of Indian representatives of *Nepenthes* has been made in the present investigation.

### Materials and methods

The plant materials used in present investigation were collected from Jarain, Meghalayas, Northeast India. The plants were grown in greenhouse conditions at the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong. Plants were raised in earthen pots for obtaining actively growing root tips. The collection time of root tips was around 9 a.m. The root tips of about 0.5–1.0 cm long were excised and pretreated with 0.002 M 8-hydroxyquinoline solution for 3 h at room temperature before they were fixed in Carnoy’s fluid consisting of 3:1 of propanol and propanoic acid for 24 h at room temperature. The rootstock were preserved after fixation in 70% (v/v) ethanol and stored in a refrigerator till utilized for squash preparations. The fixed root tips were hydrolyzed in 5N HCl a for 1 h at room temperature and were subsequently stained in leuco-basic fuschin stain for 45 min under dark conditions. The stained root tips were squashed in a drop of 1% propiono-carmine under the cover glass. The cells were flattened by taping followed by removal of excess stain with Whatman filter paper pieces. Micro-photographs were taken using a Jenoptik CCD camera (Germany) attached to a Labomed LX 400 fluorescent microscope at 100X magnification. At least 5 cells with countable chromosomes from each slide were used for the determination of chromosome counts.

### Results and discussion

Chromosome complements of *N. khasiana* were studied to ascertain the somatic chromosome number in various plant collections. Somatic chromosome number has been unambiguously recorded as 2n=80 in all the cells studied (Figs. 1, 2) with no evidence of any numerical variations whatsoever. The chromosomes are characterized by their small size and it was not practicable to determine the position of the centromere(s) convincingly. Therefore, the study has been focused on chromosome counts alone.

Documentation of chromosome numbers in the genus *Nepenthes* was probably first carried out by Heubl and Wistuba (1997) where they have reported a chromosome number of 2n=80 in about 14 species of *Nepenthes* including *Nepenthes madagascariensis*, *N. pervillei*, *N. distillatoria*, *N. khasiana*, *N. rafflesiana*, *N. truncata*, *N. stenophylla*, *N. gracilis*, *N. eymai*, *N. thorelii*, *N. veitchii*, *N. albomarginata*, *N. reinwardtiana*, *N. tentaculata*. Members of Nepenthaceae are known for remarkable uniformity in chromosome numbers of 2n=80 without any indication of numerical variations. Our studies are in agreement with these reports. However, Kondo (1969) reported a deviant chromosome number of 2n=78 in *N. rafflesiana* and *N. thorelii*. Thus, there is good evidence that *Nepenthes* could be a dibasic genus with x=5 or 10. However, such observations need to be substantiated with information on greater numbers of species representing the entire genetic spectrum of the genus.

Chromosome size, condensing behaviour of chromatin and interphase nuclei are considered to be similar to that of Droseraceae (Heubl and Wistuba 1997). In view of the known phylogenetic background, one can assume that most members of Nepenthaceae are palaeopolyploids with the
basic number \(x=5\) or \(10\). The loss of taxa with lower ploidy levels, the high chromosome number, the palaeotropic distribution, uniformity in many characters and the reduced genetic variability all support the assumption that in *Nepenthes*, diploid and lower polyploids have already gone extinct (Heubl and Wistuba 1997). Based on our study, *N. khasiana*, could be regarded as a polyploidy taxa (8\(x\) or 16\(x\)), and accordingly the basic chromosome number of \(x=5\) or \(10\). Chromosome counts provide indispensable information on genetic discontinuities within and among species and they contribute to our understanding of phylogenetic relationships at all taxonomic levels (Semple *et al.* 1989). Due to the lack of chromosome information on many other species of the genus as well as difficulties in karyotype studies of the species, it is premature to predict the cytogenetical mechanisms of evolution in the genus. Therefore, further cytogenetical investigations related to meiotic analysis of species and their hybrid derivatives are essential to elucidate taxonomic and phylogenetic relationships among the species of the genus *Nepenthes*.

Acknowledgements

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References

In vitro propagation and assessment of clonal fidelity of *Nepenthes khasiana* Hook. f.: a medicinal insectivorous plant of India

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Abstract An efficient in vitro protocol for large-scale multiplication of *Nepenthes khasiana*, a threatened insectivorous plant of India, has been developed from nodal stem segments. The highest shoot proliferation of 19.16 ± 0.23 shoots/explant was recorded in half-strength Murashige and Skoog (MS) medium supplemented with 2.5 mg/l kinetin, 2.0 mg/l 6-benzyl aminopurine, 3% sucrose and 0.8% agar. The best rooting was achieved in half-strength MS medium supplemented with 2.0 mg/l α-naphthalene acetic acid with an average of 9.04 ± 0.46 roots/shoot. The plantlets were successfully transferred to the greenhouse with survival rate of 92%, exhibiting normal development. Cytological and random amplified polymorphic DNA (RAPD) analyses were carried out to assess the genetic integrity of the regenerated plantlets. Cytological analysis revealed no change in chromosome number with cells studied showing 2n = 80. Of the 80 primers screened for RAPD analysis, 14 primers resulted in clear and scorable bands. A total of 72 amplification products were obtained out of which only 4.1% bands were polymorphic. Cluster analysis of the RAPD profile revealed an average similarity coefficient ranging from 0.98 to 1.0, thus suggesting genetic stability in the micropropagated plants of *N. khasiana*.

Keywords *Nepenthes khasiana* • Multiple shoots • Nodal segments • Genetic stability • RAPD analysis

Introduction

*Nepenthes khasiana* Hook. f. is the only insectivorous pitcher plant found in India. It belongs to the monogeneric family Nepenthaceae with polyploid chromosome number of 2n = 80 (Devi et al. 2012). The plant species is endemic to Meghalaya and is found growing from West Khasi Hills to East Khasi Hills, Jaintia Hills, East to West and South Garo Hills from 1,000 to 1,500 m altitude (Mao and Kharbuli 2002). The leaves of *N. khasiana* modify into brightly coloured pitchers that develop at the tip of the leaves to trap a wide group of insects so as to compensate nitrogen and energy deficiency in the soil (Kitching and Schofield 1986). The plant is traditionally used by different indigenous communities of Meghalaya for treatment of various ailments viz. cataract, night blindness, various skin diseases, diabetes, urinary troubles, cysts, vaginal tumours and leprosy (Bordoloi 1977). The local herbalists of Khasi and Jaintia hills prescribe the fluid of the pitcher for the effective treatment of diabetes and painful urination (Devi and Venugopal 2006). Unsustainable harvest due to phenomenal increase of unscientific prescription by the local practitioners has led to rapid depletion of the species in its natural habitat. The species is also in great demand for its ornamental value due to its curious pitcher and has thus led to its further exploitation. Also, the rampant coal mining in Jaintia Hills of Meghalaya drastically affects the regeneration of this species (Prasad and Jeeva 2009). The conservation of *N. khasiana* is imperative because it is on the verge of extinction. The plant has been listed as an endangered plant in Appendix-I of CITES (Convention on

Under natural conditions, seeds of *N. khasiana* take 223 days to germinate and the percentage of germination is also very low (Bordoloi 1977). In vitro techniques are considered as reliable methods for the rapid propagation and conservation of several rare and endangered plants (Tandon and Kumaria 1998). In vitro multiplication for large-scale propagation of *N. khasiana* has been achieved using seeds to conserve this rare and unique pitcher plant of India (Nongrum et al. 2008). Although there are reports of plantlet regeneration in *N. khasiana* by some workers (Rathore et al. 1991; Tandon and Rathore 1994; Lahiri and Sen 1994; Bahadur et al. 2008) through enhanced axillary branching, the number of shoots initiated in the explants is relatively low. Also, the sustainability of the tissue culture techniques depends upon the production of true-to-type plants and maintenance of the genetic integrity of in vitro raised plants with regard to the explant source so that the advantages in the use of elite genotypes over natural seedlings is maintained. Axillary branching is considered to be one of the methods which are least susceptible to somaclonal variations. However, genetic stability cannot be guaranteed in the tissue culture-raised plants as there are reports of genetic variations in micropropagated plants (Peyvandi et al. 2009). Therefore, periodic monitoring of the degree of genetic stability of in vitro raised plants is of utmost importance for commercial utilization of the technique for large-scale production of true-to-type plants of the desired genotype (Larkin and Scowcroft 1981).

Molecular markers along with the cytological studies can be used to assess the genetic homogeneity of the regenerated plants. Among the different molecular markers, polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD) marker is extensively used to analyse genetic stability in tissue culture-derived plants (Lattoo et al. 2006; Mallon et al. 2010; Swarna and Ravindhran 2012). RAPD is technically simple, quick to perform, requires small amounts of DNA, and no prior information about the genome is required (Williams et al. 1990). Although there are reports on the study of genetic diversity among the different populations of *N. khasiana* (Bhau et al. 2009; Nongrum et al. 2012), there are no reports available yet on analysis of genetic stability in micropropagated plants of *N. khasiana*. Therefore, the present study attempts to develop an efficient and reproducible method for the mass propagation of *N. khasiana* and to assess the genetic stability in the micropropagated plants using cytological as well as molecular approaches.

### Materials and methods

#### Plant material, culture media and growth conditions for multiple shoot induction

Single-nodal segments (2–3 cm) collected from the two-years-old donor plant were thoroughly washed under running water for 30 min to remove any adherent particles, immersed in 5 % (v/v) laboratory detergent (Laboilene, Qualigens, India) for 20 min, rinsed under tap water and kept for 1 h in a fungicide (1 % Bavistin). These were then surface sterilized with 0.2 % HgCl₂ (w/v) solution for 8 min and rinsed 4–5 times with sterilized distilled water. Finally, the explants (~1.0 cm) were excised aseptically and cultured in shoot induction medium. The nutrient medium used in all the experiments consisted of MS (Murashige and Skoog 1962) salts and vitamins with 3 % (w/v) sucrose (Himedia, India). Activated charcoal (0.05 % w/v) and ascorbic acid (50 mg/l) were also added 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 full strength, half-strength and one-fourth strength MS medium supplemented with kinetin (KN; 1.0–3.0 mg/l) and 6-benzylaminopurine (BAP; 1.0–3.0 mg/l), singly and in combination. The percentage of explants producing shoots and the number of differentiated shoots per explant were recorded after 8 weeks of culture. All the cultures were maintained at 25 ± 2 °C under 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.

### Rooting and acclimatization

The elongated shoots were transferred to half-strength MS medium supplemented with various concentrations (0.5–2.5 mg/l) of α-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA). The cultures were maintained under similar physical culture conditions as described earlier. Plantlets with well-developed roots (2–3 cm) and shoots (7–8 cm) were washed with sterile water to remove any traces of agar from the roots before being transplanted into thermostoil pots containing different substrata viz., garden soil with fine stone particles in the ratio 1:1; sand with charcoal and garden soil in the ratio 1:1:1 and garden soil with sand in the ratio of 1:1(v/v). The pots and plantlets were covered with perforated plastic bags to ensure high humidity. The plastic bags were removed after 2 weeks and the plants were transferred into a greenhouse. The survivability of the transferred plantlets was recorded after 8 weeks of transfer.
### Statistical analysis

All the experiments were performed with a minimum of 20 replicates for each treatment and each experiment was repeated three times. In shoot multiplication, the percentage of response of explants, mean number of shoots per explant and shoot length were measured. For root induction, mean number of roots and root length were recorded. The data were collected after 8 weeks for shoot multiplication and 4 weeks for rooting experiments. Data were analysed statistically using analysis of variance (ANOVA) to detect significant differences between means and the means were compared using Tukey’s test at 5% probability level.

### Cytological analysis

Mitotic slides for cytological studies were prepared following the method of Devi et al. (2012). Actively growing root tips (0.5-1.0 cm) excised from five randomly selected 7-month-old micropropagated plants and the mother plant were pretreated with 0.002 M 8-hydroxyquinoline solution for 3 h at room temperature. The root tips were then fixed for 24 h at room temperature in Carnoy’s fluid consisting of 3:1 propanol and propanoic acid. The fixed root tips were hydrolysed in 5 N HCl for 1 h at room temperature and were subsequently stained in leuco-basic fuchsin stain for 45 min in dark condition. The stained root tips were squashed in a drop of 1% propiono-carmine under the cover glass. The micro-photographs were taken using Jenoptik CCD camera (Germany) attached to Labomed LX 400 fluorescent microscope at x100 magnification. At least ten cells with countable chromosomes from each slide were used for the determination of chromosome counts.

### DNA extraction and RAPD analysis

Total genomic DNA was extracted from fresh leaves of the mother plant and eight randomly selected in vitro regenerated plants using modified CTAB method (Porebski et al. 1997). The DNA quality was checked by electrophoresis on 0.8% agarose gel and the quantification was done with Lambda 35 spectrometer (PerkinElmer, USA).

Random amplified polymorphic DNA (RAPD) analysis was performed following the method described by Williams et al. (1990). PCR were carried in a total volume of 25 μl containing 30 ng template DNA, 200 μM each dNTPs, 1.5 mM MgCl2, 1X PCR buffer, 0.6 U Taq polymerase (Bangalore Genei, India) and 5 pmol of primers (Operon Technologies, USA). PCR was performed in a Thermal Cycler (Applied Biosystem, USA) with a programme 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. Amplification products were separated by electrophoresis using 1.2% agarose gel in 1X Tris borate-EDTA (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). A total of 80 decamer oligonucleotide primers from OPA, OPC, OPH and OPK series were screened for amplification of RAPD fragments.

<p>| Table 1: Effect of BAP and KN on multiple shoot formation from nodal explants of <em>N. khassiana</em> cultured in 1/2 MS medium |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>KN (mg/l)</th>
<th>BAP (mg/l)</th>
<th>% of response</th>
<th>Average shoot number</th>
<th>Mean shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>–</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>1.5</td>
<td>–</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>2.0</td>
<td>–</td>
<td>33.33 ± 0.00</td>
<td>1.87 ± 0.65</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>2.5</td>
<td>–</td>
<td>50.21 ± 0.00</td>
<td>4.04 ± 0.72</td>
<td>0.2 ± 0.15</td>
</tr>
<tr>
<td>3.0</td>
<td>–</td>
<td>41.66 ± 0.00</td>
<td>2.08 ± 0.56</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>–</td>
<td>1.0</td>
<td>58.33 ± 0.00</td>
<td>2.37 ± 0.42</td>
<td>2.10 ± 0.04</td>
</tr>
<tr>
<td>–</td>
<td>1.5</td>
<td>66.67 ± 0.00</td>
<td>4.25 ± 0.63</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>–</td>
<td>2.0</td>
<td>75.13 ± 0.00</td>
<td>5.04 ± 0.62</td>
<td>0.81 ± 0.12</td>
</tr>
<tr>
<td>–</td>
<td>2.5</td>
<td>75.55 ± 0.00</td>
<td>3.54 ± 0.44</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>–</td>
<td>3.0</td>
<td>70.12 ± 0.00</td>
<td>2.50 ± 0.34</td>
<td>1.32 ± 0.07</td>
</tr>
<tr>
<td>Means followed by the same letters within columns are not significantly different at the 5% level according to Tukeys test.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2.5</td>
<td>1.0</td>
<td>66.24 ± 0.00</td>
<td>9.12 ± 0.25</td>
<td>1.54 ± 0.07</td>
</tr>
<tr>
<td>2.5</td>
<td>1.5</td>
<td>79.16 ± 0.00</td>
<td>13.20 ± 0.20</td>
<td>3.00 ± 0.03</td>
</tr>
<tr>
<td>2.5</td>
<td>2.0</td>
<td>91.68 ± 0.00</td>
<td>19.16 ± 0.23</td>
<td>3.04 ± 0.00</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>83.72 ± 0.00</td>
<td>14.41 ± 0.25</td>
<td>2.86 ± 0.11</td>
</tr>
<tr>
<td>2.5</td>
<td>3.0</td>
<td>70.00 ± 0.00</td>
<td>11.29 ± 0.28</td>
<td>2.40 ± 0.10</td>
</tr>
</tbody>
</table>

SE standard error
Results and discussion

Multiple shoot induction

Single shoot emerged within 2 weeks of culture from the nodal stem segments in half-strength MS medium treated with KN and BAP, either alone or in combination. The development of multiple shoots was observed after the single shoots were subcultured in the media supplemented with the same concentrations of KN and BAP. Of the different concentrations of KN (1.0–3.0 mg/l) used singly, the maximum number of shoots with an average of only 4.04 ± 0.72 shoots per explant were initiated at 2.5 mg/l KN in the medium (Table 1). However, with 2.0 mg/l BAP in the medium, the number of shoot was increased to 5.04 ± 0.62 shoots per explant. In the present study, it was also observed that BAP (1.0–3.0 mg/l) when used in combination with the optimal concentration of KN (2.5 mg/l) significantly enhanced the shooting frequency. The medium supplemented with 2.5 mg/l KN and 2.0 mg/l BAP was proven to be the best for shoot induction with maximum response of explants (91.68 %) as well as the
Table 2 Rooting of in vitro regenerated shoots of *N. khasiana* in 1/2 MS medium supplemented with NAA and IBA

<table>
<thead>
<tr>
<th>NAA (mg/l)</th>
<th>IBA (mg/l)</th>
<th>% of response</th>
<th>Average root number</th>
<th>Mean root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>–</td>
<td>54.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.66 ± 0.52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.07 ± 0.29&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>–</td>
<td>62.50&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.41 ± 0.57&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.00 ± 0.32&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5</td>
<td>–</td>
<td>79.18&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>4.41 ± 0.51&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.39 ± 0.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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<td>–</td>
<td>95.54&lt;sup&gt;de&lt;/sup&gt;</td>
<td>9.04 ± 0.46&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.58 ± 0.16&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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<td>–</td>
<td>70.86&lt;sup&gt;de&lt;/sup&gt;</td>
<td>5.12 ± 0.70&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.10 ± 0.40&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>–</td>
<td>0.5</td>
<td>33.5&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>1.50 ± 0.49&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.37 ± 0.11&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>–</td>
<td>1.0</td>
<td>41.12&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>1.70 ± 0.43&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.91 ± 0.22&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>–</td>
<td>1.5</td>
<td>50.00&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.25 ± 0.68&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.04 ± 0.50&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>–</td>
<td>2.0</td>
<td>62.64&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.66 ± 0.44&lt;sup&gt;df&lt;/sup&gt;</td>
<td>1.21 ± 0.19&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>–</td>
<td>2.5</td>
<td>45.74&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.04 ± 0.47&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>1.39 ± 0.31&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means followed by the same letters within columns are not significantly different at the 5 % level according to Tukey's test. Data recorded after 4 weeks of culture. SE standard error

maximal number of shoots (19.16 ± 0.23 shoots per explant) and shoot length (3.04 ± 0.00 cm) (Fig. 1a, b; Table 1). KN and BAP had a synergistic effect on multiple shoot induction in *N. khasiana*. Similar stimulatory effect of cytokinins on shoot multiplication has been well documented in other plant species (Babu et al. 2003; Ahmed et al. 2007; Tandon et al. 2007; Kumar et al. 2010; Dang et al. 2011). The number of shoots per explant was found to decrease with an increase in the levels of KN and BAP in the medium suggesting the inhibitory effect of cytokinin at higher concentrations, confirming the results of Swarna and Ravindhran (2012). This may be due to the supra-optimal concentrations of the growth regulators which are not desirable for the growth of the plants (Sharma and Tandon 1986). Addition of activated charcoal (0.05 % w/v) and ascorbic acid (50 mg/l) reduced leaching from the cultured explants. This may be attributed to their ability to adsorb phenolic compounds produced by the explants. In the present study, the reduced strength of medium was found to be suitable for shoot multiplication from nodal explants as it had been reported that a low concentration of nutrients is required for growth of *N. khasiana* (Kitching and Schofield 1986).

Rooting and acclimatization

The effect of different auxins on root induction of shoots has been presented in Table 2. In the present study, NAA was found to be more effective than IBA for root induction. A significant response on root induction and pitcher development was observed within 4 weeks of culture in the medium supplemented with NAA (Fig. 1c-e). The regenerated shoots cultured in half-strength MS supplemented with 2.0 mg/l NAA developed maximum frequency of 95.54 % with an average of 9.04 ± 0.46 roots/shoot (Table 2). However, the addition of IBA in the medium did not have any significant effect on root induction. Earlier reports have also shown the effectiveness of NAA in root initiation in *N. khasiana* (Rathore et al. 1991; Tandon and Rathore 1994). Similarly, the efficiency of NAA on rooting was reported in several plant species (Ceasar et al. 2010; Swarna and Ravindhran 2012).

Of the different potting mixtures tested, garden soil with sand in the ratio of 1:1(v/v) was found to be best suited for the hardening of the complete plantlets (Fig. 1f). The compost used was suitable for growth of plantlets as it might have facilitated proper drainage and aeration for root respiration. Use of polythene bags was beneficial as it could retain moisture for the growth of the plants. These plantlets had a healthy root system and leaves with well-developed pitchers ensuring proper growth. The plantlets were established in the glass house in about 3–4 months with 92 % of survival rate. The regenerated plants were transplanted to soil successfully and were observed to be morphologically similar to the parental plants.

Cytological analysis

Cytogenetic analysis showed that there was no change in chromosome number of the regenerated plants. All the cells
Fig. 3 RAPD profiles of *Nepenthes khasiana* with primers a OPK10, b OPK19. Lane M 500 bp ladder, Lane 1 mother plant, Lanes 2-9 micropropagated plants

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer code</th>
<th>Primer sequence (5'→3')</th>
<th>No. of scorable bands per primer</th>
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<th>% polymorphic bands</th>
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</tbody>
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from the root tips of the mother plant and the micropropagated plants exhibited chromosome number of 2n = 80 (Fig. 2). Due to the very small size of the chromosomes, the study has been focused on the chromosome count only. Mitotic irregularities underlie the occurrence of chromosomal variations in plant tissue culture (Larkin and Scowcroft 1981). Therefore, cytogenetic studies can provide information about abnormal mitosis or changes in ploidy levels (Radic et al. 2005). Unequal chromosome distribution, involving the distribution of replicated chromosomes unequally into only one daughter cell, results in polyploidization in tissue culture (Lee and Phillips 1988). Cytological approach has been successfully used to determine the genetic stability/variations in micropropagated plants (Pandey et al. 1992; Mallon et al. 2010; Hao and Deng 2002).

**RAPD analysis**

A total of 80 primers were screened for assessment of genetic homogeneity of the regenerated plantlets and among them 14 primers resulted in clear, unambiguous, consistently reproducible uniform and scorable bands. Of the 72 amplification products, only 4.1% bands were polymorphic, while the rest were monomorphic. The number of bands varied from 2 (OPH-1) to 9 (OPK-17) (Fig. 3a, b; Table 3). Amongst the 14 amplified primers, 11 primers produced monomorphic bands and 3 primers produced polymorphic bands. A dendrogram (Fig. 4) was generated by cluster analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method based on Jaccard's coefficient which indicated that genetic similarity among the micropropagated plants ranged from 0.98 to
Introduction

The present investigation describes an efficient and simple method for the micropropagation of *N. khasiana* which can be effectively used for its conservation through mass propagation and also reports, for the first time, the genetic stability of the micropropagated plants of *N. khasiana*.

Author contribution

S. P. Devi carried out the experiment, analysed the data and drafted the manuscript. S. Kumaria, S. R. Rao and P. Tandon supervised the work. S. Kumaria edited the manuscript. All authors read and approved the final version of this manuscript.

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References


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 consecutive 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% in the first regeneration which increased to 9.4% in the second regeneration. In the case of ISSR, out of 36 primers screened for assessment of genetic homogeneity of the micropropagated plants, 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 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.

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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; Mukherjee 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 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 Saxen, 1980).

Plant tissue culture techniques have been successfully applied for rapid clonal multiplications 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 the pitcher plant of India (Latha and Sen, 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 plants (Hassel 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)
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 for 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

Auxiliary cultures of N. khasiana were established in vitro through nodal explants collected from Jarain, Meghalaya, Northeast India following the protocol described by Dev 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% Bauton) for 1 h. These were then surface sterilized with 0.2% HgCl2 (w/v) solution for 8 min and rinsed 4–5 times with sterilized distilled water. The explants (~1 cm) were finally excised aseptically and cultured in shoot inducing medium. The nutrient medium used consisted of MS salts and vitamins with 33 (w/v) sucrose (Himedia, India) (Murashige and Skoog, 1962). Activated charcoal (0.058% w/v) and acetic acid (50 mg/l) were also incorporated in the medium. The solidified 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 (KK) and 2.0 mg/l 6-benzylaminopurine (BAP). The elongated shoots were transferred to half strength MS medium supplemented with 2.0 mg/l α-naphthaleneacetic 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 602 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-purified 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 Reaction reactions were carried in a total volume of 25 μl containing 30 ng template DNA, 200 μM each dNTPs, 1.5 mM MgCl2, 1 × PCR buffer, 0.6 U Taq polymerase (Bangalore Gene, India) and 5 pmol of primers (Operon Technologies, USA). Initially, primers from four loci (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 MgCl2, 1 × PCR buffer, 0.6 U Taq polymerase (Bangalore Gene, India) and 10 pmol of primers (MetaHrom, 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 MetaHrom, 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 MgCl2, 1 × PCR buffer, 0.6 U Taq polymerase (Bangalore Gene, 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 ampicons 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 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.
3.1. SPAR analysis for the first regeneration

A total of 80 primers were used for initial screening out of which 14 RAPD primers resulted in 72 clear, well-separated and reproducible fragments of which 9 fragments were polymorphic (4.1%) with an average of 0.21 polymorphic bands per primer (Fig. 2A). The genetic distance recorded using Jaccard’s coefficients of similarity ranged from 0.38 to 1.00. In the case of ISSR profiling, 12 primers generated a total of 46 fragments of which 2 bands were polymorphic (4.3%) with an average polymorphic band of 0.16 per primer (Fig. 2B) and showed a genetic distance of 0.96-1.00. Out of 20 DAMD primers screened, 15 primers resulted in 59 clear and scorable bands of which 5 bands were polymorphic (8.4%) with an average of 0.21 polymorphic bands per primer (Fig. 2C). The genetic distance recorded using Jaccard’s coefficients of similarity ranged from 0.98 to 1.00. Out of 177 fragments produced collectively in the first regeneration, 10 fragments were polymorphic (5.65%) with an average polymorphic band of 0.24 fragments per primer. The cumulative data were also used to compute pairwise distances by Jaccard’s coefficient which showed a distance range of 0.98–0.99 with an average value of 0.98 among the micropropagated plants and the mother plant (Fig. 3A).

3.2. SPAR analysis for the second regeneration

Fourteen RAPD primers resulted in 72 clear, well-separated and reproducible fragments of which 5 fragments were polymorphic (6.9%) exhibiting an average polymorphic band of 0.28 per primer (Fig. 2A). The genetic distance recorded using Jaccard’s coefficients of similarity ranged from 0.96 to 1.00. In the case of ISSR profiling, 12 primers generated a total of 40 fragments of which 3 bands were polymorphic (6.0%) with an average polymorphic band of 0.25 per primer (Fig. 2B) and showed a genetic distance of 0.95-1.00. Out of 20 DAMD primers screened, 15 primers resulted in 59 clear and scorable bands of which 6 bands were polymorphic (10.10%) with an average of 0.40 polymorphic bands per primer (Fig. 2C). The genetic distance recorded using Jaccard’s coefficients of similarity ranged from 0.92 to 1.00. Out of 180 fragments produced collectively in the second regeneration, 10 fragments were polymorphic (5.56%) with an average polymorphic band of 0.23 fragments per primer.
Table 1 Details of RAPD, ISSR and DAMD used in the present study.

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
</table>

**RAPD**

1. OPH-1
2. OPH-5
3. OPH-8
4. OPH-11
5. OPH-10
6. OPH-11
7. OPH-12
8. OPH-13
9. OPH-14
10. OPH-17
11. OPH-18
12. OPH-19
13. OPH-7
14. OPH-11

**ISSR**

1. N1
2. N2
3. N3
4. N4
5. N5
6. N6
7. N7
8. N8
9. N9
10. N10
11. N11
12. N12

**DAMD**

1. URH1F
2. URH2F
3. URH3R
4. URH4R
5. URH5F
6. URH6R
7. URH7R
8. URH8R
9. URH9R
10. URH10F
11. URH11F
12. URH12F
13. URH13F
14. URH14F
15. URH15F

4.39 fragments per primer. The cumulative data were also used to compute pairwise distances by Jaccard's coefficient which showed a distance range of 0.96–1.00 with an average value of 0.98 among the micropropagated plants and the mother plant (Fig. 3B).

3.3. SPAR analysis for the third regeneration

Out of 74 scorable bands produced in the case of RAPD, 7 bands were polymorphic (9.4%) with an average of 0.5 polymorphic bands per primer (Fig. 2A). The genetic distance among the micropropagated plants and the mother plant was 0.96–1.00. 12 ISSR primers generated a total of 50 fragments of which 5 fragments were polymorphic (10.0%) with an average of 0.41 polymorphic bands per primer (Fig. 2B). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.92 to 1.00. In the case of DAMD, 15 primers produced a total of 60 scorable bands of which 8 bands were polymorphic (13.33%) with an average of 0.53 polymorphic bands per primer (Fig. 2C). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.95 to 1.00 (Fig. 3C).
4. Discussion

Somatic variation is one of the most serious drawbacks in propagation of true-to-type plants due to their unpredictable nature (Rahman and Rajora, 2001). The phenotypic and genetic variations may occur during in vitro propagation and subsequently may give rise to somaclonal variants (Keppler et al., 2000). The variations generated during tissue cultures are generally the consequences of chromosomal rearrangements and single gene mutations (Phillips et al., 1994). These may also be caused by the activation of transposable elements (Hirochika et al., 1996), DNA hypomethylation (Jaligot et al., 2000; Keyre et al., 2006; Lukens and Zhu, 2007), genome adaptation to different regulatory microenvironments (Boqan et al., 1996) and the presence of hot spots (Lamceren et al., 2000). The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martins et al., 2004).

Genetic variations induced in tissue cultured plants are most likely to be reflected in the banding profiles developed by different marker systems (Phillips et al., 1994). The use of more than one DNA fingerprinting techniques generates discrete PCR profiles from different genomic regions that do not always overlap between them, and, therefore ensuring a much wider coverage of the genome being analyzed (Palambo and Damiano, 2002). Therefore, the present investigation deals with three different single primer based PCR amplification methods to analyze genetic variation in micropropagated plants of N. khasiana maintained for the three consecutive regenerations. SPAR techniques have been used to analyze intra- as well as inter-species genetic diversity in Oryza (Wu et al., 1997), Piper (Verma et al., 2004), mulberry (Bhattcharya et al., 2005), Murmaya species (Ranaled et al., 2006), Jatropha (Kumar et al., 2011; Ranade et al., 2008), Sapiindus (Mahar et al., 2011), Mentiski species (Liarma et al., 2012) and Vanda (Manners et al., 2013).

In the present study, DAMD revealed higher percentage of polymorphism in comparison to RAPD and ISSR (Table 2), confirming DAMD to be the best suited marker system for determining the genetic variation in regenerated plants of N. khasiana. The three SPAR methods collectively as well as individually revealed an increase of genetic variation among the morphologically similar regenerants and the donor mother plant from the first regeneration to the third regeneration (Table 2). In the present investigation, cumulative data set showed that genetic variation was increased from 5.65% (first regeneration) to 10.87% (third regeneration). This may be due to an increase in duration of the regenerants under tissue culture conditions being exposed to various factors which induce somaclonal variations. Also, the genetic variations occur due to accumulation of mutation by factors such as in vitro process and its duration, in vitro stress induced by biochemicals, or other nutritional conditions, all of which are known to induce somaclonal variation (Devaramath et al., 2002). In the present study, during the initiation of cultures, mercuric chloride was used to surface sterilize the primary explants which is known to cause oxidative stress (Patra et al., 2001). Moreover, the culture medium was also incorporated with essential nutrients and the plant growth regulators, which might be involved in resulting oxidative stress, for triggering the growth and development of the plants. High levels of oxidative stress cause DNA damage, including microsatellite instability in tissue culture raised plants (Jackson et al., 1998). Genome instability in the phenotypically normal regenerants, as in this case, implies that the culture-induced genomic changes largely occurred at non-coding regions which imposed little effect on gene expression (Guo et al., 2006). Polymorphism in the DNA profiles while analyzing for genetic fidelity has been reported in Codonopsis lanceolata (Guo et al., 2006), Dactyospermum ovahfblium (Chandrika et al., 2008) and Spilanthes calva (Razaq et al., 2012) using PCR-based molecular markers. Confirmatory results using RAPD and ISSR markers for testing the clonal fidelity have also been reported in other plant species (Alsadehk and Singh, 2009; Steedhe et al., 2007).

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5. Conclusion

SPAR techniques revealed an increase of genetic variation, although relatively low, from first to third regenerations of the micropropagated plants of N. khasiana. This variation may further increase of the regenerants are cultured for a longer period of time. Micropropagation
Fig. 3. UPGMA dendrogram generated for cumulative band data from the three SPAR methods (RAPD, DAMD and ISSR) illustrating coefficient similarities among regenerated plants and the mother plant (first regeneration, A, second regeneration, B, third regeneration, C).
using axillary bud proliferation is considered to be one of the methods which gives rise to genetically uniform and true-to-type plants. However, the present investigation clearly shows that this may not always be the case which further supports the need for testing macropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the macropropagation protocol for its large scale production.

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References


Kumar Y.J.C, Handasan S., Rao R.R., 1980 Ethnobotanical notes on certain medicinal plants from the present investigation clearly shows that this may not always be the case which further supports the need for testing macropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the macropropagation protocol for its large scale production.

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