ASSESSMENT OF GENETIC VARIATIONS IN MICROPROPAGATED PLANTS OF *NEPENTHES KHASIANA* HOOK. F., AN ENDEMIC INSECTIVOROUS PLANT OF MEGHALAYA

ABSTRACT

BY

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SUBMITTED IN FULFILLMENT OF THE REQUIREMENT OF THE

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BOT.

Khasi

Abstract

Nepenthes khasiana Hook f. is the only representative member of the genus *Nepenthes* found in India. It is endemic to the state of Meghalaya located in Northeast India. The species is in great demand for its ornamental value on account of the fascinating beauty of the pitchers. It also has some ethno-medicinal values. The local herbalists prescribe the fluid of the pitcher for the treatment of diabetes and painful urination. The unopened pitcher with its content is made into a paste and applied for various skin diseases, leprosy, sometimes it is taken to ease urinary troubles and blockages.

The advent of biotechnological approaches has opened up newer areas for genetic improvement and micropropagation. Micropropagation methods allow rapid propagation of a large number of plants in short period of time in a limited space thereby protecting the biological, physiological and genetic specifications of the plant which are usually affected by environmental restrictions. However, the broader utility of any micropropagation system may be limited due to occurrence of cryptic genetic changes and development of somaclones. In a micropropagation programme, it is of paramount importance to produce true-to-type planting materials as somaclonal variations of any kind, if induced, may lead to loss of the chief characteristics of the parent rootstocks. Occurrence of somaclonal variations in tissue culture is a common phenomenon which makes it mandatory to check the genetic stability of *in vitro*-raised plants. In spite of several protocols for tissue culture being reported, the regeneration efficiency has been

shown to be influenced by explant type, genotype and also the morphogenetic response varying within the same explant. In the present study, *in vitro* mass multiplication of *N. khasiana* was successfully attempted through enhanced axillary branching for production of quality planting material at an accelerated pace within a short period of time. The half-strength MS (Murashige and Skoog) medium supplemented with 2.5 mg/l kinetin (KN) and 2.0 mg/l 6-benzyl aminopurine (BAP) was proven to be the best for shoot induction with maximum response of explants (91.68 %) as well as the maximum number of shoots per explant (19.16) with BFC index of 17.48. The regenerated shoots were successfully rooted in half-strength MS medium supplemented with 2.0 mg/l α-naphthalene acetic acid (NAA) with maximum response of 95.54% with an average of 9.04 roots per shoot. The ultimate success of micropropagation on a commercial scale depends on the ability of the tissue culture-raised plants to acclimatize in the natural conditions. 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 showing the survival rate of 91.66%.

The plantlets of the second and the third regenerations were raised using the explants collected from the 8-months-old rooted plants in culture belonging to the first and the second regenerations respectively. Assessment of genetic fidelity among the micropropagated plantlets of the three consecutive regenerations was carried out using various approaches. First and foremost, the morphology of the plantlets of the three consecutive regenerations was compared considering several growth parameters and it was observed that there was no difference in the external morphology of the micropropagated plantlets. However, lack of any phenotypic variation among regenerants does not necessarily imply a concomitant lack of genetic changes and it is, therefore, important to assay the outcomes of *in vitro*-raised plantlets at the genotypic level.

Somatic chromosome number as 2n=80 has been confirmed with no evidence of any numerical variations in *N. khasiana*. The position of the centromere(s) could not be determined due to very small sized chromosomes. Therefore, variations only in the number of chromosomes have been studied in the micropropagated plantlets. All the cells analyzed from the mother plant showed normal somatic chromosome number of 2n=80 unambiguously. However, from the plantlets of the first regeneration showed normal somatic chromosome number of 2n=80 in 76.66% of the cells while the remaining 23.33% cells showed deviant chromosome numbers of 2n=76, 78. In case of the plantlets of the second regeneration, 66.66% cells showed normal somatic chromosome number as 2n=80 while the remaining 33.33% cells showed deviant chromosome numbers of 2n=70, 76, 78, 84, 86. In the plantlets of the third regeneration, cells showing normal somatic chromosome number of 2n=80 was decreased to 60% and deviant chromosome complements of 2n=70; 76; 84; 86 were observed in 40% cells analyzed.

The repetitive sequences located in the heterochromatin regions of the nuclear genome are also reported to influence chromosomal instability in tissue culture-raised plants. Therefore, in the present study, an attempt was made to observe if there was any change in the copy number of the heterochromatin repetitive sequences of the *in vitro*-raised plants of *N. khasiana* using base-specific fluorochromes *viz.*, chromomycin A3 (CMA), 4-6-diamidino-2-phenylindole (DAPI). A total of 30 cells were analyzed in both the mother plant and the plantlets of the three subsequent regenerations. In the mother plant, the number of $DAPI^+$ sites recorded was 5.33±0.73 with the range of 2-8. In case of the plantlets of the first regeneration, the number of $DAPI^+$ sites was 5.74±0.47 with the range of 2-8 which was found to be increased to 6.61±0.39 with the range of 5-12 and 6.74±0.57 with the range of 3-12 in the plantlets of the second and the third regenerations respectively. The number of CMA^+ sites observed in the mother plant was 5.11±0.47 with the range of 2-6. In the plantlets of the first regeneration, the number of CMA^+ sites observed was 5.00±0.30 with the range of 4-9 which was found to be decreased to 4.63±0.45 with the range of 1-8, and 4.16±0.47 with the range of 0-8 in the plantlets of the second and the third regenerations respectively. These results indicated that there was an increase in the number of AT base pairs and a corresponding decrease in the number of GC base pairs with the increase in the regeneration stages of the micropropagated plants in culture.

Genetic fidelity among the micropropagated plantlets of *N. khasiana* was also analyzed with the help of single primer amplification reactions (SPAR) methods using the molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and directed amplification of minisatellite DNA (DAMD). A total of 136 primers for RAPD, ISSR and DAMD were screened out of which 41 primers were finally selected for further profiling. Fourteen RAPD primers resulted in 74 clear, well-separated and reproducible bands out of which 10 bands were polymorphic exhibiting 13.51% polymorphism across all the plantlets of the three regenerations. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.94 to 1.00 among the micropropagated plantlets and the mother plant. In case of ISSR, twelve primers generated a total of 50 distinct and scorable bands of which 7 bands were polymorphic exhibiting 14% polymorphism. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.94 to 1.00 among the micropropagated plantlets and the mother plant. In DAMD profiling, 15 primers resulted in 60 uniform and scorable bands out of which 10 bands were polymorphic showing 16.66% polymorphism. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.91 to 1.00 among the micropropagated plantlets and the mother plant. The cumulative analysis commonly regarded as SPAR was also carried out for the three molecular markers in which a total of 184 fragments were produced collectively of which 27 fragments were polymorphic indicating 14.67%. The cumulative data were also used to compute pairwise distances by Jaccard's coefficient which showed a distance range of 0.95-1.00 among the micropropagated plantlets and the mother plant.

Using RAPD marker, 4.1% polymorphism was observed in the plantlets of the first regeneration which was increased to 6.9% and 9.4% in the plantlets of the second and the third regenerations respectively. In case of ISSR, 4.3% polymorphism was observed in the plantlets of the first regeneration which was increased to 6.1% and 10% in the plantlets of the second and the third regenerations respectively. DAMD marker detected 8.47% polymorphism in the plantlets of the first regeneration which was increased to 10.10% and 13.33% in the plantlets of the second and the third regenerations respectively. However, collective data regarded as SPAR approach detected polymorphism of 4.65% in the plantlets of the first regeneration which was subsequently increased to 7.77% and 10.87% in the plantlets of the second and the third regenerations respectively.

In conclusion, from the present study it was observed that there was a subsequent increase in genetic variation from the plantlets of the first regeneration to the plantlets of the third regeneration although no perceptible difference in the general morphology of the regenerated plantlets of the three subsequent regenerations relative to their mother plant was observed. The efficient protocol described in the present study for the micropropagation of *N. khasiana* through axillary bud multiplication facilitates the rapid propagation of this rare plant species. However, in the context of providing quality planting material of *N. khasiana* germplasm it is particularly important to assess the genetic stability of the *in vitro*-raised plants. Micropropagation using axillary bud proliferation is considered to be one of the safest methods which give rise to genetically uniform and true-to-type plants. The present investigation clearly shows that this may not always be the case, which further supports the need for testing micropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the micropropagation protocol for its large scale production.

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Botany

Dedicated to My Mother

NORTH–EASTERN HILL UNIVERSITY

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DECLARATION

I do hereby declare that the thesis entitled "**Assessment of genetic variations in micropropagated plants of *Nepenthes khasiana* Hook. f., an endemic insectivorous plant of Meghalaya**" is a record of original and independent research work carried out by me in the Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong under the supervision of Prof. Suman Kumaria, Prof. Pramod Tandon and Prof. S. Rama Rao* (*Department of Biotechnology and Bioinformatics). The work done is original and no part of the thesis has been submitted for any degree or diploma of any university.

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We certify that the thesis entitled "**Assessment of genetic variations in micropropagated plants of *Nepenthes khasiana* Hook. f., an endemic insectivorous plant of Meghalaya**" submitted by Ms. Soibam Purnima Devi for the degree of Doctor of Philosophy in Botany Department of the North-Eastern Hill University, Shillong, embodies the record of original investigation carried out by her under our supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph. D. degree.

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Chapter 1

General Introduction

1.1 Insectivorous plants

Nature has endowed the earth with diverse forms of plants ranging from the simplest cryptogams to complex angiosperms. In cryptogams, the plant body is called thallus in which there is no differentiation into root, stem and leaves. These plants, nevertheless, perform all the physiological functions required for their growth and development. Angiosperms, on the other hand, represent the most complex and advanced group of plants in which different parts of the plant perform various functions. Extreme genome size reductions with evolution have been reported in the angiosperm family tree over the period of time. It has been suggested that the compressed architecture of the genome of carnivorous plant, *Utricularia gibba*, indicates that a small fraction of intergenic DNA, with few or no active retrotransposons, is sufficient to regulate and integrate all the processes required for the development and reproduction of a complex organism (Ibarra-Laclette *et al.* 2013). In course of evolution, some of the plants have acquired special characteristics in order to adapt to the harsh environmental conditions. Plants inhabiting the nutrient limited environments are specialized for trapping a wide group of insects in order to supplement a part of their nutrition and hence are popularly known as insectivorous plants. This insects-trapping mechanism might have evolved as an adaptation to grow in nutrient deficient acidic soils, so as to provide a complemental source of nutrients, especially nitrogen (Givnish *et al.* 1984; Gallie and Chang 1997). Insectivorous plants are, therefore, among the curiosities of nature being different from the normal plants in their mode of nutrition. These plants have fascinated evolutionary ecologists, botanists and horticulturists for centuries. Charles Darwin (1875) provided the evidence for carnivory in several genera for the first time. Carnivorous plants represent members of five orders including both monocotyledons and eudicotyledons: Caryophyllales, Oxalidales, Ericales, Lamiales and Poales. Over 600 species of carnivorous plants belonging to 9 families are found growing all over the world. Insectivorous plants of India belong to mainly three families: Droseraceae, Nepenthaceae and Lentibulariaceae. They have unique structural specialized organs such as pitcher-like leaves or analogous leaf arrangements to trap insects (Juniper *et al.* 1989; Barthlott *et al.* 2007).

The genus *Nepenthes* popularly known as tropical pitcher plants, belonging to the monotypic family Nepenthaceae is one of the largest genus among the insectivorous plants. It comprises about 134 species including numerous natural and many cultivated hybrids (McPherson 2009). *Nepenthes* species certainly attract and kill their prey through active production of attractive colours, sugary nectar, and even sweet scents. The plants primarily gain nitrogen and phosphorus through the trapped insects to supplement their nutrient requirements for growth, given that these soil nutrients are typically lacking. The most frequent prey belongs to abundant and diverse group of arthropods, with ants and other insects. Most of its species are characterized by an ontogenetic pitcher dimorphism with young rosette, self-supporting plants exhibiting terrestrial pitchers of the "lower" type and older climbing plants exhibiting aerial pitchers of the "upper" type (Cheek and Jebb 2001; Di Giusto *et al.* 2009).

Indo-Malaysia is considered as the center of evolution of the genus *Nepenthes*. The genus is mostly distributed in the Malay Archipelago with the greatest diversity in Borneo and Sumatra and the Philippines with many endemic species. It is also found in Madagascar (*N. madagascariensis* and *N. masoalensis*), the Seychelles (*N. pervillei*), Sri Lanka (*N. distillatoria*), India (*N. khasiana*), Australia (*N. mirabilis, N. rowanae,* and *N. tenax*) and New Caledonia (*N. vieillardii*) in the Southeast. *N. mirabilis* is the most widely distributed species in the genus, ranging from Indo-China to Australia (Jebb and Cheek 1997; Barthlott *et al.* 2007; McPherson 2009). Many of the species occur in hot and humid lowland areas, but most of the species are found growing in the tropical regions receiving warm and humid climate. *Nepenthes* species usually grow in acidic soils composed of peat, white sand, sandstone, or volcanic soils. A few species thrive in soils with high heavy metals (*N. rajah*) and in sandy beaches (*N. albomarginata*) (Barthlott *et al.* 2007).

The name *Nepenthes* was formally published as a generic name in 1753 in Linnaeus's famous *Species Plantarum*, which established botanical nomenclature as it exists today. "Nepenthe" literally means "without grief" and, in Greek mythology, is a drug that quells all sorrows with forgetfulness. In Homer's *Odyssey*, "*Nepenthes pharmakon*" is given to Helen by an Egyptian queen. Linnaeus (1737) explained:

"If this is not Helen's *Nepenthes*, it certainly will be for all botanists. What botanist would not be filled with admiration if, after a long journey, he should find this wonderful plant. In his astonishment past ills would be forgotten when beholding this admirable work of the Creator!" [translated from Latin by H. J. Veitch, 1897]

1.2 *Nepenthes khasiana*, an endemic insectivorous plant of Meghalaya, India

Nepenthes khasiana Hook. f. is the only representative member of the genus *Nepenthes* found in India with polyploid chromosome number of 2n=80 (Devi *et al.* 2012). The species has been named after the Khasi Hills of the state of Meghalaya, India (Fig. 1.1a, b). It is a scandent insectivorous shrub of the tropical and subtropical climatic regions. The local communities of Meghalaya call the plant by different names which mean demon-flower or the basket of the devil.

1.2.1. Distribution

The plant species has a very localized distribution. It 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 1000 to 1500 m altitude (Mao and Kharbuli 2002). It occurs in the Jarain area of Jaintia Hills and the Baghmara, Bandari, Chokpot area of Garo Hills, and few more localities, such as Nongstoin, Mukthapur, Bhagmara, Lawbah and Sonapahar in Meghalaya (Joseph and Joseph 1986). 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).

Fig. 1.1. (a) *Nepenthes khasiana* in its natural habitat, and (b) well developed pitchers

1.2.2. Habit

N. khasiana is a climbing undershrub which ranges from a few centimeters to several meters in height (Bordoloi 1977). Two heights of plant are noticeable, dwarf plant which grows on rocky or sandy pockets attaining a height of 10-15 cm only and tall plant which grows along hill streams or on moist soil strata of substantial depth, straggling up on to small trees or large shrubs with the help of tendrils and attaining a height of 15-20 m. The plant has very superficial root system penetrating only a few centimeters into the soil. The stem is cylindrical, green in colour when young and ultimately turns brown in the older parts. The leaf is very interesting and of great morphological importance as parts of it undergoes different modifications to carry out different functions. The midrib of the leaf extends from the tip which modify into showy and brightly coloured pitchers to catch insects so as to balance the limited nutrients acquired from the soil (Kitching and Schofield 1986). Flowering season is from June to October. The plant is dioecious bearing male and female flowers on separate plants. The inflorescence is a raceme consisting of 2-flowered cymes approximately 25-60 cm long (Joseph and Joseph 1986). The male inflorescence is twice as long and denser compared to the female inflorescence (Fig. 1.2a, b). Fruits are elongated capsules ranging from 20 to 25 mm long (Fig. 1.2b).

Fig. 1.2. (a) Male inflorescence, and (b) female inflorescence and mature capsules of *N. khasiana.*

1.2.3. Economic importance

The species is of ethno-medicinal importance. It is traditionally used by different indigenous communities of Meghalaya for treatment of various ailments (Bordoloi 1977). The fluid of the unopened pitcher is used by local Khasis and Garos as an eye drop for redness, itching, cataract, night blindness and is also taken for stomach ailments and female diseases (Rao *et al.* 1969; Kumar *et al.* 1980; Joseph and Joseph 1986). The unopened pitcher with its contents is made into a paste and applied for various skin diseases, including leprosy. The local herbalists of Khasi and Jaintia Hills prescribe the fluid of the pitcher effectively for the treatment of diabetes and painful urination (Rao *et al.* 1969; Kharkongar and Joseph 1981; Devi and Venugopal 2006). Pitcher extract of *N. khasiana* has been reported to reduce the level of glucose and lipid significantly in rats confirming the traditional use of this plant in the treatment of diabetes (Shil *et al.* 2010). Staining properties of plumbagin, a kind of chemical naphthoquinone present in the leaves of the genus *Nepenthes* has also been studied (Cannon *et al.* 1980). Naphthoquinones are allelopathic substances and exhibit high biological activities such as insecticidal, molluscidal, antifeedant and antifungal activities (Harbone 1982; Reynolds 1987; Thomson 1987; Jayaram and Prasad 2005). In addition to its ethno-medicinal values, *N. khasiana* is also in great demand for its ornamental value on account of the fascinating beauty of the pitchers. The plant is, therefore, being collected from the wild and sold at the rate of Rs. 40-50 per plant in the markets of Meghalaya (Mao and Kharbuli 2002).

1.2.4. Status in the natural habitat

The majority of *N. khasiana* habitats have been destroyed, and remaining populations have declined severely as a result of unsustainable poaching and indiscriminate collection even by the students of Botany (Tandon *et al.* 2009). Unsustainable harvests due to phenomenal increase of prescription by the local medical practitioners have also led to rapid depletion of the species in its natural habitat. The species is also reported to be exported by local plant collectors to other states of India and has, thus, led to its further exploitation (Bhau *et al.* 2009). The rampant coal mining in Jaintia Hills of Meghalaya has drastically affected the regeneration of *N. khasiana* in nature (Prasad and Jeeva 2009). Habitat destruction, deforestation, urban development, developmental projects and modern agriculture, fragmentation of large contiguous populations into isolated small and scattered ones have rendered the species increasingly vulnerable to environmental stochasticity, which, if unchecked, would ultimately lead to its extinction. At present, *N. khasiana* has become threatened in its natural habitat.

1.2.5. Conservation strategies

In an attempt to protect the existing stands of *N. khasiana* in the wild, the Government of India banned its export during the 1970s. *N. khasiana* is also included in the Appendix-I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and Negative List of Exports of the Government of India (Ziemer 2010). The potential for long term survival of this species in the wild is uncertain, but will surely depend upon the continuing efforts of the local communities to preserve its habitats. It is of paramount importance that all lineages of *N. khasiana* are retained in cultivation and propagated to preserve the reproductive potential of this species. Under natural conditions, *N. khasiana* propagates mostly through seedlings produced from the rhizomatous basal portion of the stem as the seeds have been reported to take around 223 days to germinate and the percentage of germination is also very low in nature (Bordoloi 1977). However, propagation using *in vitro* seed germination is possible to obtain a large number of plants for the conservation of this rare, unique endemic pitcher plant of India (Nongrum *et al.* 2008).

1.3 Plant tissue culture and assessment of genetic stability in regenerated plants

Clonal multiplication can be accomplished, in nature, using vegetative propagation so that the desirable characteristics of the parent can be preserved in the offspring. However, vegetative propagation methods of some of the species may be cumbersome, season-dependent, and cost-intensive. The regeneration potential of vegetative propagules also declines with increase in age of the mother plant. Therefore, *in vitro* propagation has emerged as a powerful technique for large-scale propagation of commercially important plants. Plant tissue culture is recognized as one of the key areas of biotechnology because of its potential to regenerate elite and conserve valuable plant genetic resources. Plant tissue culture techniques have been successfully applied for rapid clonal multiplication of many rare and endangered plant species (Tandon and Kumaria 1998). Clonal multiplication has five major advantages over conventional methods of plant propagation: (i) can be used to multiply the elite clones of recalcitrant species; (ii) enables to multiply the plants irrespective of the season; (iii) pathogen-free plants can be propagated; (iv) plant materials such as restorer lines, male sterile and fertility maintainer can be cloned; and (v) allows the propagation of a large number of plants in a short period of time in a limited space (Rani *et al.* 1995, 2000; Rani and Raina 1998, 2000). For large-scale production of a plant species, efficiency of propagation methods is of prime importance, but perhaps even more important is the genetic stability of the *in vitro* regenerated plantlets (Haisel *et al.* 2001). Enhanced axillary branching and somatic embryogenesis are considered to give rise to genetically uniform and true-to-type plants, as the organized meristems are considered to be least susceptible to genetic modifications under *in vitro* conditions (Vasil 1985; Shenoy and Vasil 1992). However, genetic stability cannot be guaranteed in the tissue culture-raised plants as there are reports of genetic variations in micropropagated plants (Feyissa *et al.* 2007; Peyvandi *et al.* 2009). Many of the regenerated plantlets may not be the clonal copies of their donor genotype when passaged through *in vitro* cultures due to a phenomenon known as somaclonal variation. Culture environment, explant source, ploidy level and duration of *in vitro* culture are the primary factors inducing somaclonal variations (Rani and Raina 2000). These variations may appear due to cell cycle disturbances caused by exogenously supplied growth regulators, accumulation of mutations over a period of time, alteration in DNA methylation patterns, DNA damage and mutation, alteration of cell's ability to repair damaged and mutated DNA (Peschke and Phillips 1992; Phillips *et al.* 1994; Rodrigues *et al.* 1998; Leroy *et al.* 2000). Such occurrence of cryptic genetic defects in the tissue culture-raised plants can seriously limit the broader utility of the micropropagation system (Salvi *et al.* 2001). Somaclonal variations may occur in *in vitro*-raised plants which require clonal uniformity, as in the horticulture and forestry where tissue culture techniques are widely employed for rapid propagation of elite genotypes. The risks of genetic changes induced by tissue culture and the importance of assessing the genetic stability of the micropropagated plants at regular intervals must be considered to minimize such defects at later stages (Panda *et al.* 2007; Chandrika and Rai 2009). Therefore, it is of paramount importance to monitor the genetic uniformity of the *in vitro*-raised plants. for utilization of the techniques in large scale production of true-to-type plants of the desired genotype and also, to ascertain the suitability of a particular micropropagation protocol developed for a particular species, where commercial success in micropropagation depends solely on the maintenance of clonal uniformity (Larkins and Scowcroft 1981; Heinz and Schmidt 1995).

In vitro multiplication of *Nepenthes khasiana* has been successfully attempted for its propagation at an accelerated pace within a short period of time (Rathore *et al.* 1991; Tandon and Rathore 1994). However, assessment of genetic fidelity of these micropropagated plantlets of *N. khasiana* has not been carried out so far.

1.4 Objectives

Considering the importance of genetic uniformity in the tissue culture-raised plants for production of desired genotypes, the main objective of the present study was to assess the genetic variations in the micropropagated plants of *Nepenthes khasiana*. For achieving this objective, the study was divided into the following:

a. Micropropagation of *N. khasiana* using nodal segments.
b. Cytological evaluation of the germplasm and the regenerated plantlets to determine variability, if any.
c. Use of appropriate PCR-based molecular markers to define the genetic variations observed.

Chapter 2

Review of literature

2.1 Micropropagation and genetic variations

Plant Biotechnology has become increasingly important at the global level, as it offers opportunities to increase sustainability, profitability and international competitiveness in agriculture and forestry. One of the most extensively exploited components of Biotechnology has been the rapid clonal multiplication or micropropagation of selected genotypes to meet the growing demands for elite planting material in the current century. The concept of micropropagation using *in vitro* culture techniques originated from the pioneering research of Gottlieb Haberlandt (1902) who attempted to culture isolated plant cells for the first time in the history of Plant Tissue Culture.

Micropropagation consists of three types of vegetative propagation *viz.*, i) somatic embryogenesis in which structures containing a shoot and root connected by a close vascular system are formed, ii) adventitious shoot production comprising *de novo* meristem formation from callus tissue or directly from the organized tissues, and iii) axillary shoot production where axillary buds and meristems give rise to shoots that are excised and used to give additional such shoots. Micropropagation provides an alternative means of plant propagation and conservation of germplasm of several plant species (Cervelli 1987; Arumugam and Bhojwani 1990; Rathore *et al.* 1991; Prance 1997; Feijoo and Iglesias 1998; Lynch 1999). It has the advantage of rapid mass propagation of valuable genotypes in a limited space, especially in case of plants which are rare, threatened or critically endangered. Planting material of ornamental plants is in great demand for commercial production as well as for domestic gardens and landscaping. The better quality planting material is a basic need of growers for the enhancement of production. Chebet *et al.* (2003) reported the use of biotechnological approaches to improve horticultural crop production. It has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry. However, this technique is very expensive and if the explants used are infected, all the clones produced are vulnerable to the same infections since micropropagation results in monocultures. Moreover, all plants can not be successfully micropropagated as some of the plants produce secondary metabolites that kill the explants. The major limitation in the use of micropropagation is the inclusion of genetic variations in the *in vitro*-raised plants. Therefore, the most crucial concern has been the maintenance of the genetic integrity of micropropagated plants with regard to the explant source, so that the advantages in the use of elite genotypes over natural seedlings are maintained.

The sustainability of the micropropagation process depends upon the production of true-to-type plants and maintenance of the genetic integrity of micropropagules. Tissue-culture technique involves the application of plant growth regulators which are known to be associated with genetic instability in plants, a phenomenon called somaclonal variation (Karp 1989; Cullis 1992; Phillips *et al.* 1994). Although somaclonal variations might have been used as a source for variations to get superior clones, it could be also a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant off-types (Karp 1993; Cassells *et al.* 1999). Occurrences of tissue culture induced variations have been extensively reported in many plant species (Brown *et al.* 1993; Rani *et al.* 1995; Munthali *et al.* 1996; Hashmi *et al.* 1997; Chen *et al.* 1998). Such somaclonal variations are found to be associated with point mutations, chromosomal rearrangements and recombination, DNA methylation, altered sequence copy number, transposable elements, etc. (Jain 1997; Veilleux and Johnson 1998; Jain *et al.* 1998).

Somaclonal variations were first detected in sugarcane plant derived from cell cultures as early as 1969 by various researchers (Heinz and Mee 1969, 1971; Heinz *et al.* 1969). They observed considerable amount of variations in chromosome number in the cultured cells, as well as morphological and enzymatic variations among the regenerated plants. Variations were also detected by the high frequency of qualitatively segregating phenotypes observed among progeny of plants which were expected to be genetically identical (Larkin and Scowcroft 1981, 1983; Orton 1984; Ahloowahlia 1986; Larkin 1987; Sun and Zheng 1990; Peschke and Phillips 1992; Kaeppler and Phillips 1993). It was Larkin and Scowcroft (1981) who termed the variations in tissue culture-derived plants as 'somaclonal variations'. Later on, it became known that a wide array of alterations in nuclear and cytoplasmic genetic elements contributed to the observed phenotypic variations, and many of them were of epigenetic nature (Micke 1999).

The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic divisions of the cell and hence theoretically, it should not cause any variation. Ideally, clonal multiplication of genetically uniform plants is expected (Larkin 1998). The occurrence of uncontrolled and random spontaneous variations during the culture process is, therefore, an unexpected and mostly undesired phenomenon (Karp 1994). The presence of somaclonal variations in tissue culture-raised plants affects the use of tissue culture negatively and has remained a major problem. However, its usefulness in crop improvement through creation of novel variants is also well documented (Bouharmont 1994; Mehta and Angra 2000; Predieri 2001).

The causes of somaclonal variations are not always well understood and have not been fully elucidated; although studied extensively, remain largely theoretical or unknown (Skirvin *et al.* 1993, 1994). According to George (1993), variations in tissue culture could either be pre-existing or tissue culture induced. Tissue culture itself acts as a mutagenic system because cells are subjected to traumatic experiences from isolation, and may reprogramme during plant regeneration which are different from conventional method under natural conditions. Reprogramming of events can create a wide range of epigenetic variations in newly regenerated plants (Jain 2000). The literature till date indicates that these variations could range from a specific trait to the whole plant genome. By revealing mitochondrially controlled male sterility using restriction enzyme analysis of isolated mitochondrial DNA, Gengenbach and Umpeck (1982) demonstrated that somaclonal variations are not limited to nuclear DNA.

Tissue culture-derived variations can be determined at the morphological, biochemical, cytological and molecular levels using several techniques. Traditionally, species identification and assessment of variations in plants were carried out on the basis of differences in phenotypic characters because of a range of morphological characteristics present for distinguishing closely related individuals. Somaclonal variants can be easily detected based on visible morphological characters such as differences in plant stature, leaf morphology and pigmentation abnormality (Bailey 1983; Israeli *et al.* 1991; Pereira *et al.* 1996). The flower parts in the variant somaclones of *Phalaenopsis* showed significant abnormalities including colour and shape, when compared to normal flowers (Chen *et al.* 1998). Phenotypic changes associated with genetic alterations had also been reported among tissue culture-derived plants of several species such as *Allium*, *Pelargonium*, *Arachis*, *Musa* and *Saintpaulia* (Novak 1980; Cassells *et al.* 1997; Eapen *et al.* 1998; Grajal-Martin *et al.* 1998; Paek and Hahn 1999). Phenotypic off-types were detected in micropropagated bananas during acclimatization in the green house before transplanting to the field (Rodrigues *et al.* 1998). Zaid and Al Kaabi (2003) detected somaclonal variants in date palm on the basis of morphological traits such as excessive vegetative growth, leaf whitening and variegation. Variant morphologic characteristics were also observed in regenerated plants of *Heliconia bihai*, with regard to variation in plant stature, colour and shape of the leaf, pseudostem and inflorescence (Rodrigues 2008).

However, the detection by phenotypic identification of plants is cumbersome, time-consuming and is more difficult in perennial crops, where many observations have to be made until maturity. Moreover, morphological markers used for phenotypic characters are limited in number, often highly influenced by environmental factors and all the genetic changes may not be reflected in the observed phenotypic changes (Cloutier and Landry 1994). Furthermore, the detection of variants using morphological markers is often mostly feasible for fully established plants either in the field or greenhouse (Bairu *et al.* 2011).

The development of protein based markers over three decades ago greatly facilitated the understanding of existing genetic variations in plant populations. It is well known that morphological variation is a result of biochemical variation which is expressed as variation among proteins. Isozymes were one of the most widely used markers for studying genetic variations in most organisms (Weising *et al.* 2005). Isozymes are defined as structurally different molecular forms of an enzyme with the same catalytic function. The isozyme profile of individual samples could be observed after specific staining (Hadacova and Ondrej 1972; Vallejos 1983; Soltis and Soltis 1989). The discriminating property of isozymes is a function of the number of polymorphic loci that can be identified and genetically characterized in an organism (Jarret and Gawel 1995). Isozymes analysis has been used for various purposes such as to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to study population genetics and developmental biology, for characterization of plant genetic resources for management and plant breeding

(Bretting and Widrlechner 1995; Staub and Serquen 1996). Due to consistency in their expression, isozymes have been proven to be reliable genetic markers in breeding and genetic studies of plant species (Heinz 1987). Variations in somaclones can be detected by analysing clones for enzyme polymorphism. Isozymes such as peroxidase, malate dehydrogenase and superoxide dismutase have been extensively used to study variations in banana, sugarcane and beans (Bonner *et al.* 1974; Rivera 1983; Srivastava *et al.* 2005; Gonzalez *et al.* 2010). Although, analyses of isozymes patterns of specific enzymes provide a convenient method for detection of genetic changes, isozymes are limited in number and only DNA regions coding for soluble proteins can be sampled (Venkathachalam *et al.* 2007). The banding profile obtained for a particular isozyme marker may change depending on the type of tissue used for the analysis (Kumar *et al.* 2009).

2.2 Somaclonal variations at the chromosomal level

Cytological approach has also been used to study genetic abnormalities among tissue culture-derived plants (Straus 1954; Murata and Orton 1982; Rao *et al.* 1992; Maluszynska and Schweizer 1999). Changes in chromosome number and structure disfunction of mitotic spindle are the cytological characteristics commonly observed for somaclonal variations (Bayliss 1980; Lee and Phillips 1988; D'Amato 1990). Changes in chromosome number are more common, but structural chromosome changes have been observed with high frequency in some species (Joachimiak *et al.* 1993). Chromosomal abnormalities associated with translocations involving inversions and insertions or deletions are also observed frequently (Kaeppler and Kaeppler 2000). Chromosomal aberrations due to chromosomal breaks, chromatin abnormalities such as anaphase bridges and chromosome stickiness, and spindle failures resulting in disturbed and multipolar anaphases, lagging chromosomes, aneuploidy and polyploidy have been considered as mitotic abnormalities. The genetic features of explants, nutrient medium composition especially the kind and concentrations of plant growth regulators used, culture conditions as well as duration of culture influence the chromosomal aberration types and frequencies (Edallo *et al.* 1981; McCoy *et al.* 1982; Lee and Phillips 1987).

Occurrence of chromosomal variations originating in plant tissue culture was earlier reviewed for cultured cells and regenerated plants separately (Sunderland 1977; D'Amato 1977; Bayliss 1980). However, subsequent studies with chromosomal variations in cultured cells as well as regenerated plants were undertaken and several hypotheses were presented to explain the origin of these variations (Lee and Phillips 1988; Phillips *et al.* 1994). Chromosome instability is believed to be one of the most common causes of the tissue culture induced variations. Cell division takes place after the completion of DNA replication in normal cell cycle, which is presumed to be disrupted by tissue culture, resulting in chromosomal breakage which in turn causing aberrations (Phillips *et al.* 1994; Duncan 1997). Chromosomal variations have been reported in several tissue culture-derived plant species, and their progenies (Ahloowalia 1976, 1983, 1986; Duncan 1997; Roth *et al.* 1997; Gupta 1998; Kaeppler *et al.* 1998). Polyploidy observed in tissue culture-derived plants generally results from endopolyploidization or nuclear fusion. Aneuploidy may be caused by non-disjunction, aberrant spindles, lagging chromosomes, chromosome breakage that produces dicentric and acentric chromosomes (Sunderland 1977; Bayliss 1980). Hang and Bregitzer (1993) found ploidy changes to be the most prevalent cytological changes among barley regenerants, although chromosome breakage was also observed. The frequency of anueploidy in potato regenerants derived from protoplasts varied from 20% to 82% in the morphological variants (Sree Ramulu *et al.* 1983). Several aneuploid and polyploidy cells were observed in root-tip cells of long term culture of *Centaurea ragusina* due to pronounced stickiness of the chromatin matrix which resulted in abnormal typical metaphases and anaphases (Radic *et al.* 2005). Roth *et al.* (1997) found that the embryogenic cell cultures of *Abies alba* had malformed suspensor cells and lost maturation capacity, and the chromosome counts of cells showed trisomy. Most of the karyotypic stability has been studied mainly in cultures regenerated from immature embryos, however explant source and genotype may influence the karyotypic abnormalities of the cultures; and only few reports are available on the plants regenerated from the callus (Gaponenko *et al.* 1988; Ziauddin and Kasha 1990; Hang and Bregitjer 1993). Although, a wide range of chromosomal abnormalities are known to occur in tissue culture, frequency of these abnormalities is generally reduced in regenerated plants due to reduced morphogenic potential and the viability of the aberrant cultured cells (Gupta 1998).

2.3 Somaclonal variations and the repetitive sequences of heterochromatin

The degree of chromosomal instability in tissue culture-derived plants is influenced by repetitive sequences located in the heterochromatin which varies from one species to another (Gupta 1998). As a consequence of various cellular stresses, the copy number of tandemly repeated sequences in *in vitro*-raised plants is variable (Sutherland and Richards 1994). It is, therefore, expected that repeated sequence variation detected among the tissue culture regenerants may be responsible for some of the observed phenotypic variabilities among regenerants. Copy-number variability is most likely affected by mitotic recombination where either inter-chromatid unequal crossing over or intra-chromatid exchange of inverted repeats could result in the loss or gain of genetic information. The age of the callus also affects the frequency of chromosomal aberrations. In general, as the callus gets older the frequency of chromosomal instability increases. However, in maize callus it was reported that there was no aging influence on chromosomal changes (Jain 2001).

Analysis of structural chromosome changes suggested that the chromosome breakage involved specific regions of late replicating chromosome carrying the heterochromatin blocks (Lee and Phillips 1987; Benzion and Phillips 1988). Possible mechanisms causing late replication of heterochromatin include abnormal cell cycles and metabolic disturbances such as imbalance of nucleotide pools (Peschke and Phillips 1992). A high proportion of chromosomal aberrations involves chromosome with nucleolar organizing regions as reported in the case of *Crepis capillaries* and *Zea mays* (Sacristan 1971; Lee and Phillips 1987). Similarly, non random rearrangement has also been reported in many crops such as triticale, celery, oats (Brettell *et al.* 1986; Murata and Orton 1982; Johnson *et al.* 1987). The fact that most breakages were either between heterochromatic knobs and the centromere in maize, or within the centromeric heterochromatin in oat, led to the hypothesis that replication of heterochromatin occurs late in tissue culture leading to chromosome bridges and breakage events (Johnson *et al.* 1987). Hang and Bregitzer (1993) reported the involvement of heterochromatin in chromosome breakage events, further supporting this hypothesis. The chromosome breakage may create mutations directly through 'position effect' or alteration in gene expression from chromosomal rearrangement (Psechke and Phillips 1992). Csink and Henikoff (1998) proposed that centromere function is also related to the time of replication which in turn is determined by the accumulation of heterochromatin forming repeats. It is, therefore, possible that the primary cytological aberrations observed in cells and tissues are caused by modification of the heterochromatin repeats under stress conditions. The degree of chromosomal instability in tissue culture varies from one species to another. Rye has been reported to exhibit more chromosomal instability than either barley or pearlmillet, which is due to repetitive sequences located in the heterochromatin of its genome (Gupta 1998).

2.4 Somaclonal variations and PCR-based molecular markers

During the last few decades, the use of molecular markers, revealing polymorphism at the DNA level, has revolutionized the entire scenario of biological sciences. The development of molecular techniques for genetic analysis has led to a great contribution in our understanding of the structure and behavior of various plant genomes. DNA-based markers have become the most efficient tools for investigating various aspects which include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding, and diagnostics (Kumaria and Misra, 2013). A success of any genetic maintenance programme is dependent on an understanding of the amount and distribution of the genetic variations present in the gene pool. Molecular markers, based on differences in the DNA sequence, are not environmentally influenced, highly heritable and relatively easy to assay and, therefore, the same banding profiles can be expected for the same genotype.

Molecular markers are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In case of hybridization-based markers, the restriction enzyme-digested DNA is hybridized to a labeled probe, which is a DNA fragment of known sequence and DNA profiles are visualized with autoradiography. On the other hand, PCR-based markers involve amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences called primers and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as autoradiography and staining. The PCR-based markers are technically simpler, cheaper, and less labour intensive than hybridization-based markers and require very small amount of DNA. PCR is a versatile technique and its applications in research and clinical laboratories has increased tremendously after the thermostable DNA polymerase was introduced in 1988 (Saiki *et al.* 1985). PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology. The molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR) and directed amplification of minisatellite DNA (DAMD) are used frequently to investigate clonal diversity and population genetics (Tani *et al.* 1998; Esselman *et al.* 1999; Rossetto *et al.* 1999).

The concept of using variations at DNA level as genetic markers started with the RFLP technique. When the DNA of different individuals is digested with restriction enzymes, differences in size of the resulting fragments of DNA can be visualized via Southern hybridization with labeled probe (Southern 1975). The differences are due to evolutionary changes in sequence of nucleotides in the DNA of different individuals. The first documentation of RFLP came from viruses (Grodzicker *et al.* 1974) followed by a subsequent demonstration made in the human-globin gene cluster (Jeffreys 1979). Also, RFLP was one of the first techniques used to study somaclonal variations in several plant species. Generally, RFLP markers are relatively highly polymorphic, co-dominantly inherited and highly reproducible (Agarwal *et al.* 2008). Jaligot *et al.* (2002) described methylation-sensitive RFLP markers that differentiated between normal and abnormal embryogenic calli of oil palm. Although RFLP markers are useful for sampling various regions of the genome and are potentially unlimited, the technique is time consuming, costly and a large amount of plant tissue is required for analyses (Piola *et al.* 1999). Moreover, it involves the use of radioactive reagents and is technically demanding. It requires the development of cDNA or genomic DNA probes when heterologous probes are unavailable (Karp *et al.* 1996). These limitations led to the development of new set of less technically complex methods which are known as PCR-based molecular markers.

Morphological variation is known to occur at a much lower frequency than at the DNA level (Evans *et al.* 1984). Moreover, genetic changes cannot be observed at a morphological level as the structural differences in the gene product may not alter its biological activity sufficiently to result in an altered phenotype (Sabir *et al.* 1992). It is, therefore, necessary to examine for potential variations at the molecular level in order to determine locations and extent of deviance from the true-to-type plants (Cloutier and Landry 1994). At the molecular level, variations in tissue culture-derived plants arise from changes in chromosome number or structure, or from more subtle changes in the DNA (Gostimsky *et al.* 2005). These variations can be determined at an early growth stage, while still in tissue culture, prior to regeneration of complete plantlets. Presently, a number of molecular techniques are available to detect sequence variation between closely related genomes including differences between source plants and somaclones. These techniques involve the use of molecular markers which are useful in comparing the DNA from different samples for the differentiation in plants due to sequence variation by identifying random polymorphisms (Cloutier and Landry 1994). DNA extracts from the leaf part are mostly used in investigations because of the ease of acquisition and preparation (Jarret 1986). As a result of the high specificity of DNA, molecular markers are able to identify a particular fragment of DNA sequence that is associated with a part of the genome and comparisons are usually made on the basis of the presence or absence of a DNA band. Polymorphism at DNA level has been reported in many tissue culture-raised plants such as *Frageria, Triticum*, *Populus, Oryza* and *Phoenix* (Brown *et al.* 1993; Rani *et al.* 1995; Damiano *et al.* 1997; Godwin *et al.* 1997; Saker *et al.* 2000, 2005).

PCR-based markers have come up as the most desirable tools for establishing genetic uniformity of the micropropagated plants. A recent PCR-based single primer for amplification reactions (SPAR) method is used as an effective tool for genetic diversity studies in plants and provides a comprehensive description of the nature and extent of the diversity. SPAR method generate reliable markers with a high throughput for analysis, rapidity for identification, discrimination of genotypes and it does not require any prior sequence information to design the primer (Williams *et al.* 1990; Godwin *et al.* 1997). Thus, it is suitable for the assessment of genetic fidelity of the *in vitro*-raised clones. This method includes (1) RAPD (Williams *et al.* 1990), (2) ISSR (Gupta *et al.* 1994), and (3) DAMD (Heath *et al.* 1993).

RAPD involves the use of single short primers of arbitrary nucleotide sequence to reproducibly amplify segments of target genomic DNA. These short primers referred to as genetic markers are used to reveal polymorphisms among the amplification products (Wiliams *et al.* 1990). A single primer (10–15 bp) is used for amplification for initial two cycles at low stringency. Subsequently, the remaining cycles are performed at higher stringency by increasing the annealing temperature (Welsh and McClelland 1990). It is an efficient technique for rapid identification and isolation of chromosome-specific DNA fragments. The use of RAPD markers is especially beneficial to discriminate between genetically dissimilar individuals, to evaluate genetic variability within a collection and to choose the components of the core collection (Piola *et al.* 1999). Technically, RAPD has been described as the simplest PCR-based marker with arbitrary primers used for detecting DNA variations (Weising *et al.* 2005). RAPD technique has been widely used to assess genetic relationship in several plants (Royo and Itoiz 2004; Devarumath *et al.* 2007; Kumar *et al.* 2011; Nongrum *et al.* 2012). Also, this technique has been successfully employed in assessing tissue culture induced variations in many other plant species (Rani *et al.* 1995; Taylor *et al.* 1995; Hashmi *et al.* 1997; Chen *et al.* 1998; Bairu *et al.* 2006). Several investigators reported the presence of genetic variations in micropropagated plants using RAPD markers. Bindiya *et al.* (2003) reported 32% polymorphism in sixth subculture of micropropagated *Robinia pseudoacacia* plantlets. Similarly, Hofmann *et al.* (2004) reported RAPD band polymorphism in embryogenic cultures of soybean that were treated with ethyl methanesulfonate (EMS). Guo *et al.* (2006) detected 24.87% polymorphism in regenerated plants of *Codonopsis lanceolata* using RAPD markers. Nevertheless, RAPD technique has been inconclusive or ineffective in some species. RAPD markers could not detect variations in *Begonia* regenerated from leaf explants treated with nitrosomethylurea as well as X-ray-induced garlic mutants (Anastassopoulos and Keil 1996; Bouman and De Klerk 2001). This technique also has a lower reproducibility and reliability and is less informative compared to other molecular markers thereby limiting its application in some species (Mulcahy *et al.* 1993; Vos *et al.* 1995; Jones *et al.* 1997). Despite all these limitations, RAPD has remained the choice of markers to researchers because of the fact that RAPD marker is cheaper and faster than other molecular markers such as AFLP, RFLP and microsatellites (Belaj *et al.* 2003; Weising *et al.* 2005). Moreover, it has been suggested that the problem of RAPD reliability and transferability among laboratories could be minimized and eliminated by following a standard protocol, replication of amplification reactions and a conservative criterion of band selection (Belaj *et al.* 2003).

ISSR marker, on the other hand, has emerged as an alternative system with reliability and advantages of microsatellites (SSR). This technique involves amplification of genomic segments flanked by inversely oriented and closely spaced microsatellite sequences by a single primer or a pair of primers based on SSRs anchored 5' or 3' with 1-4 purine or pyramidine residues. SSRs or microsatellites are short tandem repeats of 1–4 bases of DNA ubiquitously present in eukaryote genomes (Tautz and Renz 1984). They are dispersed throughout the genome and vary in the number of repeat units. The hypervariability of ISSR marker enables detection of small differences even at the sub-species level (Wolfe and Richard 1998). Studies have indicated that ISSRs produce more reliable and reproducible bands compared to

RAPDs because of their higher annealing temperature and longer sequences (Tsumura *et al.* 1996; Nagaoka and Ogihara 1997; Qian *et al.* 2001). ISSRs have proved to be useful in detecting clonal diversity and variations existing in closely related individuals (Zietkiewiez *et al.* 1994; Wolfe and Liston 1998; Esselman *et al.* 1999; Joshi and Dhawan 2007). ISSR marker is advantageous due to its co-dominant inheritance nature, high abundance in organisms, enormous extent of allelic diversity as well as the ease of assessing microsatellite size variation using PCR with pairs of flanking primers (Li *et al.* 2002; Weising *et al.* 2005; Agarwal *et al.* 2008). For establishing genetic stability of several micropropagated plants such as trembling aspen banana, grapevine, sugarcane, London plane tree, wheat and sorghum, ISSR marker has been found immensely useful (Rahman and Rajora 2001; Hautea *et al.* 2004; Ray *et al.* 2006; Welter *et al.* 2007; Singh *et al.* 2008; Haung *et al.* 2009; Khlestkina *et al.* 2010 Zhang *et al.* 2010). Guo *et al.* (2006) observed 15.72% polymorphism in regenerated plants of *Codonopsis lanceolata* using ISSR marker. Liu and Yang (2012) used ISSR marker for assessing genetic variations in micropropagated plants of *Psidium guajava* which revealed 1.65% polymorphism among the regenerated plantlets. ISSR has been employed to detect somaclonal variations in *Artemisia amygdalina* plantlets regenerated from nodal explants (Khan *et al.* 2013). Besides, screening of somaclonal variations produced in tissue-cultured plants of several other species has been done using ISSR marker (Salvi *et al.* 2001; Varshney *et al.* 2001; Rout and Das 2002; Singh *et al.* 2002, 2004; Venkatachalam *et al.* 2007; Joshi and Dhawan 2007; Bhowmik *et al.* 2008).

Heath *et al.* (1993) reported a technique, called direct or directed amplification of minisatellite region DNA amplified using the polymerase chain reaction (DAMD-PCR) to direct the amplification of tandemly repeated region of a genome. Minisatellites are tandem repeats of 10 to 60 bp DNA sequence many of which show high levels of length differences due to variations in the number of repeat units and are widely distributed throughout the eukaryote genomes (Jeffreys *et al.* 1985). Variation in the tandem repeat copy number of minisatellite is proved to be the source of the polymorphism in several organisms (Jeffreys *et al.* 1985; Winberg *et al.* 1993). These sequences can be effective as PCR primers at relatively high stringencies yield reproducible DNA markers (Karaca and Ince 2008). It is speculated that minisatellites and the DNA sequences flanking them are involved in inversions, which results in their distribution on both strands in opposite orientations. Thus, it makes PCR possible using minisatellite core sequence as a single primer. DAMD technique has been successfully used to establish DNA fingerprints of species such as in *Triticum*, *Oryza*, *Capsicum*, *Cucumis*, *Citrus* (Bebeli *et al.* 1997; Zhou *et al.* 1997; Ince *et al.* 2009; Hu *et al.* 2011; Kumar and Nair 2013).

SPAR methods have been used together for biodiversity and biosystematic studies in plants (Verma *et al.* 2004; Bhattacharya *et al.* 2005; Ranade *et al.* 2006, 2009; Srivastava *et al.* 2007). The existing natural genetic variation at intra-specific level has been studied in *Prosopis cineraria*, *Cymbidium spp.*, *Mantisia spathulata*, *M. wengeri*, *Jatropha curcus*, *Vanda coerula* using SPAR methods (Sharma *et al.* 2010, 2011, 2012; Kumar *et al.* 2011; Manners *et al.* 2013). These methods based on different principles and experimental rationales generate different extent and nature of polymorphism that can be scored. The use of more than one DNA fingerprinting techniques which amplify different regions of the genome provides precise information regarding genetic variations in the regenerated plants (Palombi and Damiano 2002). Various molecular markers have been employed together for determination of genetic stability in micropropagated plants of several species (Venkatachalam *et al.* 2007; Bhatia *et al.* 2009; Nadha *et al.* 2011; Singh *et al.* 2012; Razaq *et al.* 2012; Phulwaria *et al.* 2013). Till date no report is available on assessment of genetic fidelity of the *in vitro* raised plants of *N. khasiana* although genetic diversity among its different populations has been studied (Bhau *et al.* 2009; Nongrum *et al.* 2012).

Chapter 3

In vitro establishment of three consecutive regenerations of *Nepenthes khasiana* plantlets using nodal segments

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3.1 Introduction

Establishment of cell, tissue and organ culture and regeneration of plantlets under *in vitro* conditions has opened up new avenues in the area of plant biotechnology. Biotechnology can directly assist plant conservation programmes through *in vitro* technologies, molecular diagnostics, and cryopreservation (Tandon and Kumaria 2005; Tandon *et al.* 2009). The ability to grow plant cells and tissues in culture and to control their development form the basis of many practical applications in agriculture, horticulture and chemical industries and is a prerequisite for genetic engineering. Plant tissue culture has become one of the fundamental tools of plant science research and is extensively employed in conservation and improvement of plant genetic resources. The tremendous potential of tissue culture technique can be utilized for large scale propagation for providing elite planting material, particularly for the species with reproduction problems and/or extremely reduced population.

Micropropagation leads to simultaneous accomplishment of rapid large scale propagation of true-to-type plants. The most crucial aspect of plant propagation is the retention of genetic integrity with respect to the mother plants; however, it is known that *in vitro* culture techniques can induce genetic variability known as somaclonal variations (Larkin and Scowcroft 1981). There are no reports that authenticate the genetic stability of the micropropagated plantlets of *N. khasiana*.The present chapter deals with the *in vitro* establishment of three consecutive regenerations of *N. khasiana* plantlets so as to determine the genetic integrity of the micropropagated plantlets over a period of time in culture.

3.2 Materials and methods

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

Single-nodal segments (2–3 cm) collected from the two-year-old mother plant 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 then rinsed under tap water before final treatment with fungicide (1% Bavistin) for 1 h. These were then surface sterilized with sodium hypochlorite and mercuric chloride at various concentrations (w/v) (0.1- 0.5%) for different time periods. The explants were then rinsed 4-5 times with sterilized distilled water to remove the traces of surface sterilants. Finally, the explants measuring ~1.0 cm were excised aseptically and cultured in shoot induction medium. The nutrient medium used in all the experiments consisted of MS salts and vitamins (Murashige and Skoog 1962) with 3% sucrose (w/v) (Himedia, India). Activated charcoal (0.05% w/v) and ascorbic acid (50 mg/l) were also incorporated in the medium (Tandon and Rathore 1994). 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 medium (Tandon and Rathore 1994) supplemented with kinetin (KN) and 6-benzylaminopurine (BAP) (1.0-3.0 mg/l), both 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 of 60.2 µmoles $m^{-2}sec^{-1}$ supplied by cool white fluorescent lamps (40 W, Philips, India) with 65-70% RH. The bud-forming capacity (BFC) was calculated based on the average number of buds and percentage of response of the explants as follows (Tandon *et al.* 2007):

BFC = (average number of buds per explants × % of explants forming buds) ÷ 100

3.2.2. Rooting and acclimatization

The elongated shoots were transferred for rooting to half-strength MS medium supplemented with α-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) at various concentrations (0.5-2.5 mg/l). 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 pots containing different substrata *viz.*, (i) garden soil, (ii) garden soil with fine stone particles in the ratio of 1:1, (iii) garden soil, sand and powdered charcoal in the ratio of 1:1:1, (iv) garden soil with sand in the ratio of 1:1, and (v) garden soil, sand and decaying litter in the ratio of 1:1:1 (v/v). The pots and plantlets were covered initially with perforated plastic bags for 2 weeks to ensure high humidity and finally transferred to the glass house. The RH of the glass house was around 70-80%. The minimum and maximum temperatures of the glass house at the time of transplantation were 18°C and 25°C respectively. The plantlets were watered twice a day and survivability of the transferred plantlets was recorded after 8 weeks of transfer.

3.2.3. Establishment of the second and the third regenerations

The 8-months old rooted plantlets in culture raised in the first regeneration as described above were used as the source of explants for raising the plantlets of the second regeneration and the same method was adopted for raising the plantlets of the third regeneration. The plantlets of the second and the third regenerations were subsequently hardened, transferred to soil and were maintained in the glass house. BFC index and the morphology of the plantlets of the three consecutive regenerations were also compared.

3.2.4. Statistical analysis

All the experiments were performed with a minimum of 24 replicates for each treatment and each experiment was repeated thrice. For recording the shoot multiplication, the percentage of response of explants, mean number of shoots per explant and shoot length were measured after 8 weeks of culture. For root induction, mean number of roots and root length were recorded after 4 weeks of culture in the rooting medium. Data were analyzed 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.

3.3 Results

3.3.1. Standardization of surface sterilization

The explants sterilized with different concentrations of sodium hypochlorite were found to be susceptible to fungal contamination within 6-7 d of culture. However, surface sterilization of explants with mercuric chloride was found to be suitable for overcoming the problem of contamination. Surface sterilization with 0.2% mercuric chloride for 8 min exposure was found to be effective in obtaining healthy shoot proliferation without contamination. Explants were contaminated when lower concentration of mercuric chloride was used and duration of exposure was reduced. On the contrary, when the concentration and duration of mercuric chloride were increased, there was no contamination, however, shoot proliferation was delayed. Also, the exposure of explants to mercuric chloride for longer duration resulted in the browning and ultimate death of the explants within 4-5 d of treatment. Hence, the optimum concentration and the duration of exposure of explants to surface sterilant were standardised.

3.3.2. Initiation of cultures and multiple shoot induction

Shoots were induced from the nodal stem segments in half-strength MS medium supplemented with KN and BAP, either singly or in combination (Table 3.1; Fig. 3.1a). However, no response was observed from the nodal explants inoculated in the control and the medium supplemented with 1.0 mg/l and 1.5 mg/l of KN. The development of multiple shoots was observed after the shoots were subcultured in the medium with the same concentrations of KN and BAP. Of the different concentrations of KN (1.0-3.0mg/l)

Table 3.1. Multiple shoot formation from nodal explants of *N. khasiana* cultured in half-strength MS medium supplemented with KN and BAP

KN (mg/l)	BAP (mg/l)	% of response	BFC	Average shoot number	Mean shoot length(cm)
Control	-	0.0^{b}	0.0^{b}	0.0±0.0^{h}	0.0±0.0^{f}
1.0	-	0.0^{b}	0.0^{b}	0.0±0.0^{h}	0.0±0.0^{f}
1.5	-	0.0^{b}	0.0^{b}	0.0±0.0^{h}	0.0±0.0^{f}
2.0	-	33.32^{c}	0.62^{b}	1.87±0.65^{h}	1.62±0.15^{c}
2.5	-	50.23^{c}	2.02^{b}	4.04±0.72^{e}	1.29±0.07^{cd}
3.0	-	41.66^{bc}	0.86^{b}	2.08±0.56^{gh}	2.05±0.00^{b}
-	1.0	58.34^{ac}	1.38^{b}	2.37±0.42^{fg}	2.10±0.04^{b}
-	1.5	66.67^{ac}	2.83^{b}	4.25±0.63^{ef}	1.13±0.04^{de}
-	2.0	75.13^{a}	3.75^{b}	5.04±0.62^{de}	0.81±0.12^{e}
-	2.5	75.52^{a}	2.67^{b}	3.54±0.44^{efg}	1.02±0.02^{de}
-	3.0	70.12^{a}	1.75^{b}	2.50±0.34^{fg}	1.32±0.07^{cd}
2.5	1.0	66.24^{a}	6.04^{b}	9.12±0.25^{cd}	1.54±0.07^{cd}
2.5	1.5	79.16^{a}	10.44^{b}	13.20±0.20^{bc}	3.00±0.03^{a}
2.5	2.0	91.68^{a}	17.48^{b}	19.16±0.23^{a}	3.04±0.00^{a}
2.5	2.5	83.72^{a}	12.06^{b}	14.41±0.26^{b}	2.86±0.11^{a}
2.5	3.0	70.00^{a}	7.90^{b}	11.29±0.28^{bc}	2.40±0.10^{b}

SE=Standard error. Means followed by the same letters within columns are not significantly different at the 5% level according to Tukeys test. Data recorded after 8 weeks of culture

Fig. 3.1. Multiple shoot formation from nodal explants of *N. khasiana*. (a) Initiation of shoot buds in half-strength MS, (b) Multiple shoot induction in half-strength MS + 2.5 mg/l KN, (c) Multiple shoot induction in half-strength MS + 2.0 mg/l BAP, and (d) Multiple shoot induction in half-strength MS + 2.5 mg/l KN + 2.0 mg/l BAP. (Bar 5 mm)

used singly, the maximum number of shoots was initiated in the medium supplemented with 2.5 mg/l of KN with an average of only 4.04±0.72 shoots per explant with the BFC index of 2.02 (Table 3.1; Fig. 3.1b). However, with 2.0 mg/l BAP in the medium, the number of shoots was increased to 5.04±0.62 shoots per explant with the BFC index of 3.75 (Fig. 3.1c). 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) resulted in significant enhanced 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 maximum number of shoots per explant (19.16±0.23) with the BFC index of 17.48 and shoot length of 3.04 cm (Fig. 3.1d).

3.3.3. Rooting and acclimatization

In the present study, NAA was found to be more effective for root induction than IBA. A significant response in terms of root induction was observed within 4 weeks of culture in the medium supplemented with NAA. The regenerated shoots cultured in half-strength MS supplemented with 2.0 mg/l NAA resulted in maximum response of 95.54% with an average of 9.04±0.46 roots/shoot (Table 3.2; Fig. 3.2a, b). However, addition of IBA in the medium did not have any significant effect on root induction.

Table 3.2. Rooting of in *vitro* regenerated shoots of *N. khasiana* in half-strength MS medium supplemented with NAA and IBA

NAA (mg/l)	IBA (mg/l)	% of response	Average root number	Mean root length (cm)
Control	-	18.23^{f}	1.40 ± 0.45^{e}	1.32 ± 0.12^{ef}
0.5	-	54.16^{bc}	2.66 ± 0.52^{de}	1.07 ± 0.20^{cef}
1.0	-	62.50^{ac}	3.41 ± 0.57^{cd}	2.00 ± 0.32^{bce}
1.5	-	79.18^{ad}	4.41 ± 0.51^{bc}	3.39 ± 0.36^{ad}
2.0	-	95.54^{a}	9.04 ± 0.46^{a}	3.58 ± 0.16^{a}
2.5	-	70.86^{ade}	5.12 ± 0.70^{b}	2.10 ± 0.40^{cde}
-	0.5	33.35^{c}	1.50 ± 0.49^{e}	0.37 ± 0.11^{f}
-	1.0	41.12^{ce}	1.70 ± 0.43^{e}	0.91 ± 0.22^{ef}
-	1.5	50.00^{cd}	3.25 ± 0.68^{f}	2.04 ± 0.50^{e}
-	2.0	62.64^{ac}	$2.66\pm0.44d^{ef}$	1.21 ± 0.19^{ef}
-	2.5	45.74^{cd}	2.04 ± 0.47^{f}	1.39 ± 0.31^{e}

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

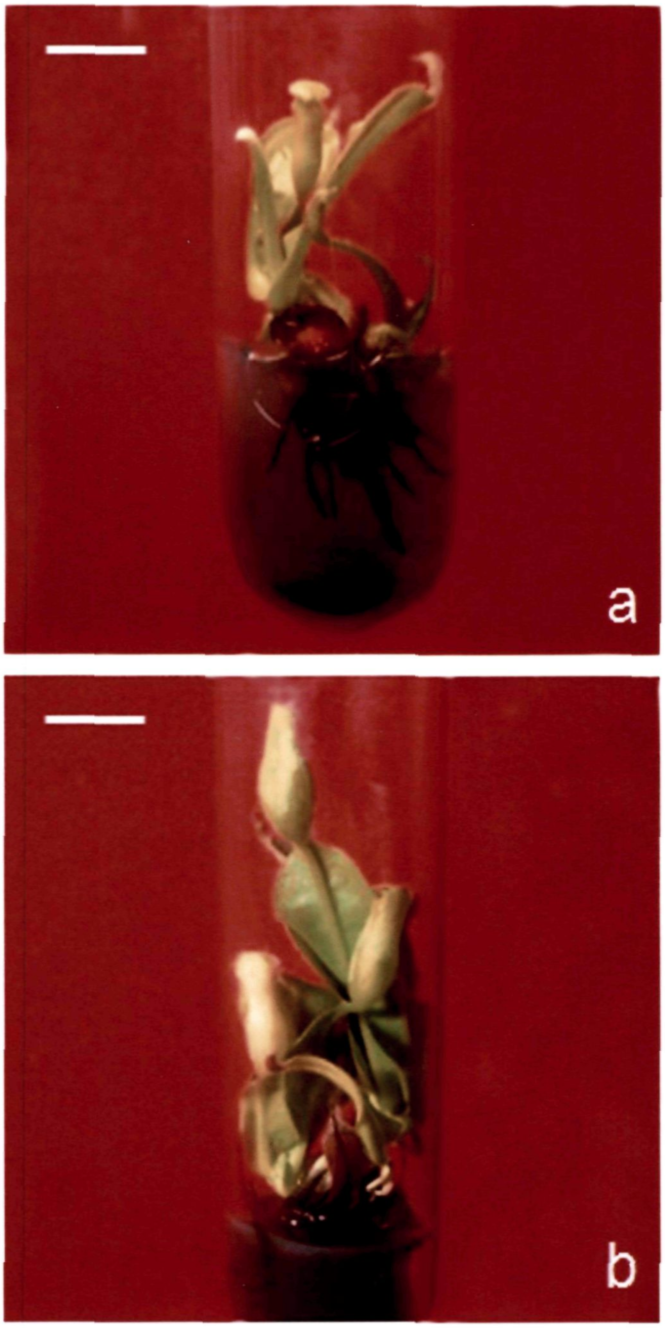


Fig. 3.2. Rooting of *in vitro* regenerated shoots of *N. khasiana*. (a) plantlet with roots in half-strength MS + 2.0 mg/l NAA, and (b) complete plantlet with well developed pitchers. (Bar 5mm)

Of the different potting mixtures tested, garden soil with sand in the ratio of 1:1(v/v) was found to be the best suited for the hardening of the complete plantlets with 91.66% of survival rate after 8 weeks (Table 3.3; Fig. 3.3). The plantlets in this compost were healthy with well-developed pitchers. In the compost containing only garden soil, 80.55% survival was observed, however, the number of pitchers per plant was only 2.00±0.23. Also, the other composts used did not support the growth of the plantlets. The plantlets were established in the glass house in about 3-4 months time. The plantlets which were observed to be morphologically similar to the parental plant were subsequently transferred to earthen pots for further growth and development.

The growth of the micropropagated plantlets in different regenerations varied. The average number of multiple shoots induced from the mother plant was 19.16±0.23 shoots per explant with the BFC index of 17.48 which decreased to 15.75±0.34 shoots per explant from the plantlets of the first regeneration with the BFC index of 13.12 and 13.58±0.54 shoots per explant from the plantlets of second regeneration with the BFC index of 12.46 (Table 3.4; Fig. 3.4a-f). The parameters *viz.*, number of leaves per plant, leaf size, number of pitchers per plant, pitcher size, plantlet height and root length were considered for comparing the morphology of the hardened plantlets of the three consecutive regenerations and it was observed that there was no significant difference in the growth parameters of the plantlets of the different regenerations (Table 3.5).

Table 3.3. *Ex vitro* establishment of *N. khasiana* plantlets eight weeks after transfer to different potting mixture

Sl. No.	Potting mixture	Survival (%)	No. of pitcher/plant (Mean±SE)	Pitcher size (cm) (Mean±SE)	Plantlet height (cm) (Mean±SE)
1.	Garden soil	80.55^{a}	2.00 ± 0.23^{b}	3.17 ± 0.12^{b}	4.34 ± 0.17^{b}
2.	Garden soil and fine stone particles (1:1)	55.92^{bc}	2.47 ± 0.28^{ab}	2.31 ± 0.13^{c}	4.09 ± 0.13^{b}
3.	Garden soil, sand and charcoal powder (1:1:1)	69.44^{ac}	3.00 ± 0.31^{a}	3.39 ± 0.13^{b}	3.56 ± 0.08^{c}
4.	Garden soil and sand (1:1)	91.66^{a}	3.21 ± 0.15^{a}	4.04 ± 0.12^{a}	5.10 ± 0.09^{a}
5.	Garden soil, sand and decaying litter (1:1:1)	52.77^{c}	3.26 ± 0.15^{a}	2.10 ± 0.14^{c}	3.72 ± 0.12^{bc}

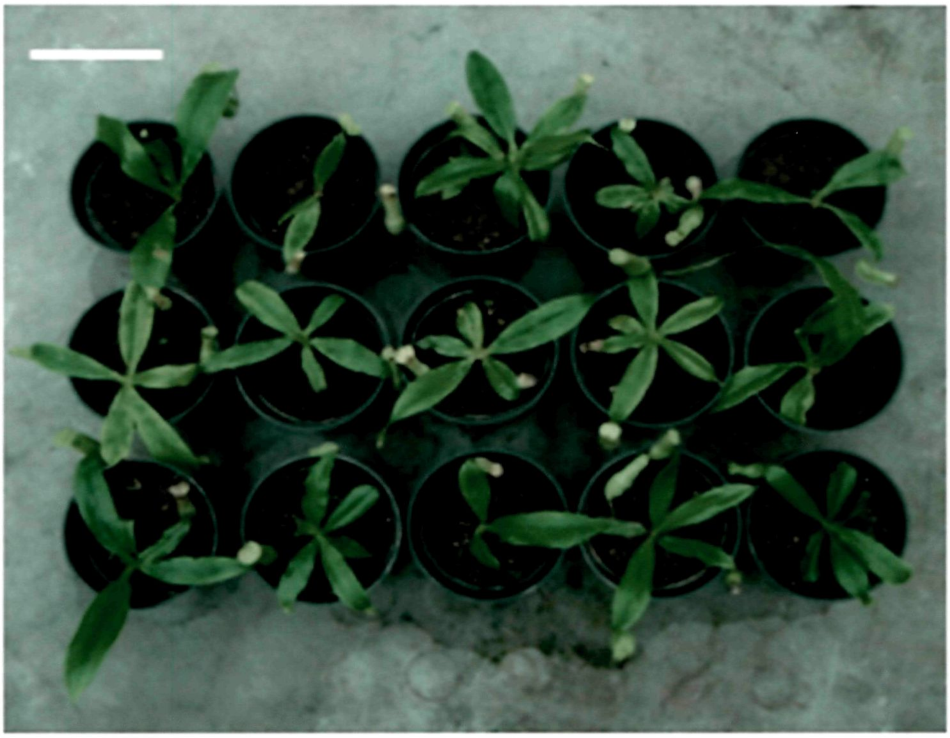


Fig. 3.3. Hardened plantlets of *N. khasiana*. (Bar 1 cm)

Table 3.4. Comparision of growth in the micropropagated plantlets of three consecutive regenerations of *N. khasiana*

Sl. No.	Regenerations	% of response (shoot)	Average shoot number (Mean±SE)	BFC	Mean shoot length (cm) (Mean±SE)	% of response (root)	Average root number (Mean±SE)	Mean root length (cm) (Mean±SE)
1.^{s}	1st	91.24^{a}	19.16±0.23^{b}	17.48^{b}	1.02±0.02^{a}	95.54^{a}	9.04±0.46^{a}	3.58±0.16^{a}
2.st	2nd	83.33^{a}	15.75±0.34^{a}	13.12^{b}	1.69±.07^{a}	84.32^{b}	8.19±0.12^{a}	3.73±0.12^{a}
3.^{s}	3rd	79.16^{a}	13.58±0.54^{a}	12.46^{b}	1.96±0.08^{a}	83.56^{b}	8.04±0.13^{a}	3.34±0.10^{a}

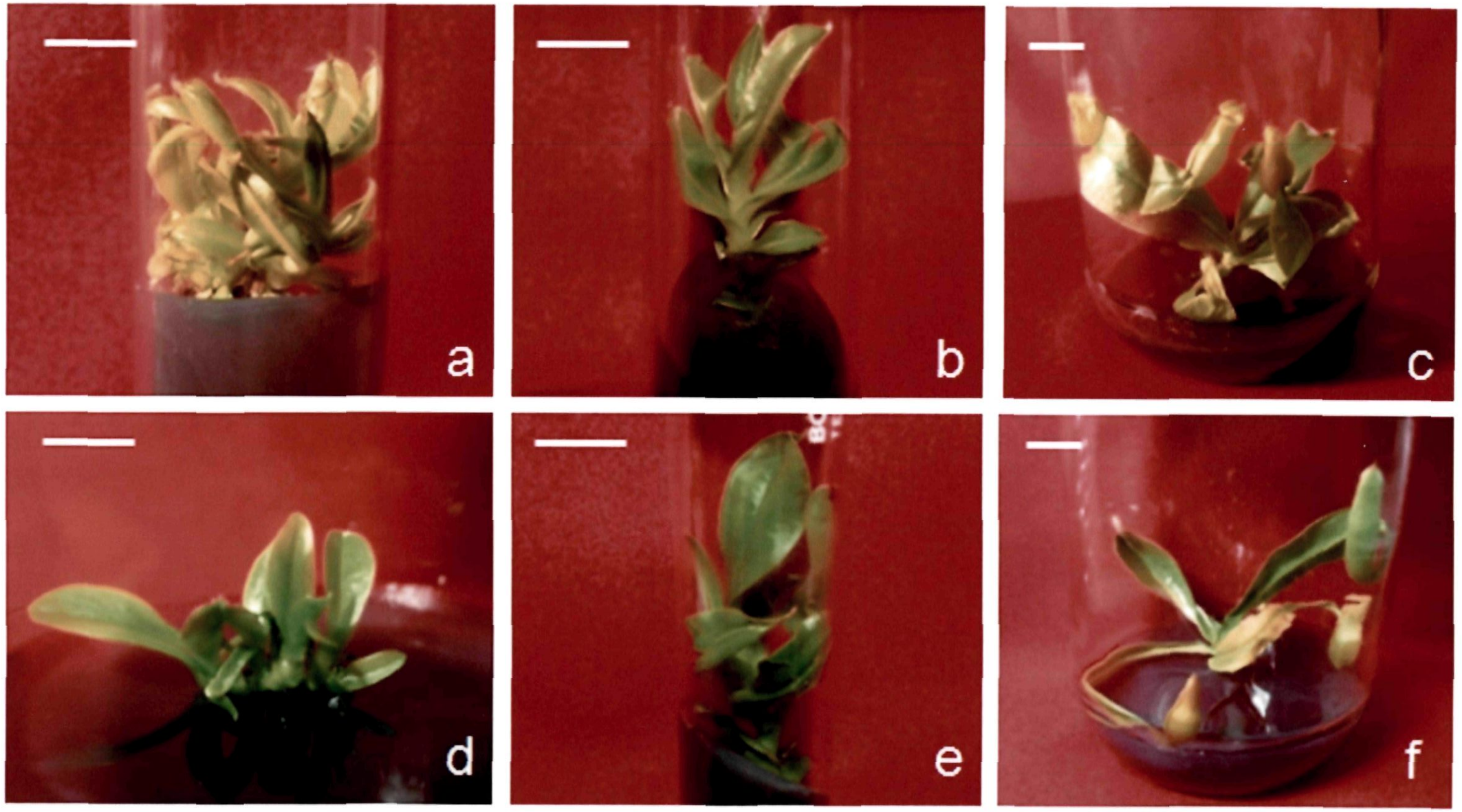


Fig. 3.4. Micropropagation of the second regeneration (a-c), and the third regeneration (d-f) of *N. khasiana*. (Bar 5mm)

Table 3.5. Comparisons of morphological characteristics in the hardened plantlets of the three consecutive regenerations of *N. khasiana*

Sl. No.	Regenerations	No. of leaves/plant	Leaf size (cm) (Mean±SE)	No. of pitchers/plant (Mean±SE)	Pitcher size (cm) (Mean±SE)	Plantlet height (cm) (Mean±SE)	Root length (cm) (Mean±SE)
1.	1st	5.95±0.12 ^{a}	4.65±010 ^{a}	4.12±0.12 ^{a}	3.42±0.05 ^{a}	4.97±0.096 ^{b}	5.32±0.11 ^{a}
2.	2nd	6.29±0.17 ^{a}	4.72±0.08 ^{a}	4.20±0.12 ^{a}	3.52±0.08 ^{a}	5.12±0.08 ^{ab}	5.62±0.09 ^{a}
3.	3rd	6.33±0.20 ^{a}	4.60±0.06 ^{a}	3.95±0.15 ^{a}	3.48±0.07 ^{a}	5.40±0.07 ^{a}	5.39±0.18 ^{a}

3.4 Discussion

Organogenic potentiality of a species depends upon physiological status of the explants, culture medium and growth regulators incorporated in the nutrient medium for triggering the inherent regenerative capacity of the explants. Although different explants are capable of producing adventitious shoots, it is usually found that the explants excised from different organs, vary in morphogenic capacity. Therefore, the propagation rate and morphogenic response significantly vary to a greater extent according to the explant type. Thus, successful micropropagation depends not only on the selection of the most suitable explant, but also on the correct combination of growth regulators used in the medium for the explants response (Basu and Chand 1996; Martin 2004). Evaluation of medium for the metabolic needs of the cultured cells and tissues is a prerequisite step for developing efficient protocol for plant regeneration. Composition of the medium plays a major role in enhancing shoot proliferation. The nutritional requirement varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture (Balakrishnan *et al.* 2009).

In the present study, the major difficulty encountered during the initial establishment of culture was the problem of fungal contamination which could be overcome through standardization of surface-sterilization procedure. For a successful micropropagation protocols, identification and maintenance of completely contamination-free culture is an absolute prerequisite (Rani and Dantu 2012). The browning of culture medium and cultured explants of *N. khasiana* could be checked by incorporating ascorbic acid (50 mg/l) and activated charcoal (500 mg/l) in the medium. This might be attributed to the ability of ascorbic acid and activated charcoal to adsorb phenolic compounds produced by the explants. Earlier report suggested the use of ascorbic acid (50 mg/l), citric acid (10 mg/l) and polyvinylpyrrolidone (PVP) (500 mg/l) to reduce the browning of the culture (Rathore *et al.* 1991). Incorporation of activated charcoal was also found to be effective to reduce browning of culture medium (Madhusudhanan and Rahiman 2000; Bahadur et al. 2008)). Addition of ascorbic acid in the medium has proven to be beneficial in overcoming the browning of cultures of *Musa* and *Carizzo* as well (Dantu *et al.* 2009; Ko *et al.* 2009; Germana *et al.* 2011).

Concentration of salts in the basal medium often influences regeneration capacity of the explants. In the present study, the reduced strength of MS medium was used for shoot multiplication from nodal explants. Earlier reports have suggested that a low concentration of nutrients is required for growth of *N. khasiana* (Kitching and Schofield 1986). In *Centaurea ultreiae* also, half-strength of MS salts was found to be optimum for its *in vitro* regeneration (Mallon *et al.* 2010).

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between growth regulators supplied in the medium and the growth substances produced endogenously. The incorporation of growth regulators in the culture medium at various concentrations resulted in differential response with regard to number of shoots produced, growth and development of plantlets in the present study. Shoot bud induction and shoot proliferation could be achieved in the presence of cytokinins, BAP or KN incorporated singly and in combination in the medium. KN, when used alone in the culture medium, resulted in an optimum of 4.04 ± 0.72 shoots per explant with 50.23% of response, whereas the explants inoculated in the medium supplemented with BAP produced an optimum of 5.04 ± 0.62 shoots per explant with 75.13% of response. Similar results were reported in shoot proliferation from nodal segments of *Piper barberi* in presence of BAP and KN incorporated singly in the culture medium (Anand and Rao 2000). Superiority of BAP to KN in inducing multiple shoot formation has been reported in several plants such as *Medicago truncatula*, *Cypripedium flavum*, and *Justicia gendarussa* (Neves *et al.* 2001; Yan *et al.* 2006; Thomas and Yoichiro 2010; Magyar-Tabori *et al.* 2010). Similarly, the incorporation of BAP singly in the medium has been reported to promote shoot proliferation in *Tuberaria, Maesa, Passiflora,* and *Metabriggsia* (Goncalves *et al.* 2010; Faizal *et al.* 2011; Garcia *et al.* 2011; Ma *et al.* 2011). However, in several other cases, KN was found to be more suitable for shoot proliferation as in *Houttuynia* and *Castilleja* (Chakraborti *et al.* 2006; Martinez-Bonfil *et al.* 2011). The promotory effect of BAP over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the enzyme cytokinin oxidase, or receptor abundance in its perception apparatus which interacts with the coupling elements in the signal transduction chain (Burch and Stuchbury 1987). Shoot proliferation, in the present study, was tremendously increased when the medium was supplemented with BAP and KN in combination. Similar results of high frequency of shoot multiplication using combination of two cytokinins has been reported in other plants (Rajeswari and Paliwal 2006; Frabetti *et al.* 2009; Swarna and Ravindhran 2012; Singh *et al.* 2012). The stimulatory effect of BAP and KN in combination on efficient shoot multiplication has been also well documented in many other plant species such as *Cinnamomum camphora*, *Stevia rebaudiana, Artemisia vulgaris, Pinus kesiya*, *Vitex agnuscastus*, *Portulaca grandiflora, Jatropha curcas, Ilex khasiana* (Babu *et al.* 2003; Ahmed *et al.* 2007; Sujatha and Kumari 2007; Tandon *et al.* 2007; Balaraju *et al.* 2008; Srivastava and Joshi 2009; Kumar *et al.* 2010; Dang *et al.* 2011). In the present study, the number of shoots per explant and percentage of response were decreased with an increase in the levels of KN and BAP in the medium suggesting the inhibitory effect of cytokinins at higher concentrations; confirming the results of Indhra and Dhar (2000). This might 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). The BFC index is an efficient indicator of bud induction as it takes into consideration both the number of explants showing bud induction as well as the number of buds formed per explant (Tandon *et al.* 2007). In case of *N. khasiana*, the BFC was higher in the explants inoculated in the medium supplemented with BAP as compared to KN. This index was maximum (17.48) in case of explants inoculated in the medium incorporated with BAP (2.0 mg/l) and KN (2.5 mg/l) in combination. Elevation of BAP level beyond this concentration drastically reduced BFC index which could be due to the toxic effect of BAP at higher concentrations.

Synthetic auxins NAA and IBA are commonly used in the nutrient medium to induce root meristem differentiation (Supriyanto and Rohr 1994; Cheng *et al.* 1995; Zhang *et al.* 2006). Earlier reports have shown the effectiveness of NAA on root initiation in *N. khasiana* (Rathore *et al.* 1991; Tandon and Rathore 1994; Latha and Seeni, 1994; Nongrum *et al.* 2008). Similarly, the efficiency of NAA on rooting has been reported in several other plant species (Kalia *et al.* 2007; Viehmannova *et al.* 2007; Bhuyan *et al.* 2011). Maximum rooting was induced when the culture medium was supplemented with NAA in case of *Portulaca grandiflora*, *Pinus massoniana*, *Chrysanthemum morifolium* (Jain and Bashir 2010; Zhu *et al.* 2010; Verma 2012).

Hardening and acclimatization of plantlets is essential for survival of plantlets under *ex vitro* conditions. The transfer of plantlets from the culture vessels to the glass house is a crucial step as the environmental conditions required for *ex vitro* growth of plants are different from those under controlled conditions (Hazarika 2003). *In vitro* plants are exposed to invariably controlled growth conditions such as high amount of organic and inorganic nutrients, plant growth regulators, carbon source, high humidity, low light and poor gaseous exchange. These may support rapid growth and multiplication, however, the controlled conditions induce structural and physiological changes in plants rendering them unfit to survive when transferred directly to the field. Thus, a gradual acclimatization of plantlets from laboratory to field condition is necessary. Plants often die during the transfer from *in vitro* to *ex vitro* conditions (Pospisilova *et al.* 1999). The overall success of the *in vitro* propagation depends on the successful hardening and transplantation of the plantlets in the field. Under controlled culture conditions, the anatomical and morphological conditions of *in vitro* plantlets such as development of cuticle, hairs, photosynthetic ability and conducting tissues, etc., required for the growth and development of plantlets remains non-functional. The stomata in *in vitro* cultured plants remain open. Generally, *in vitro* plantlets are very delicate and therefore wilt rapidly on direct transfer to normal green house conditions. In the acclimatization process, excessive water loss from plantlets is a major problem (Hazarika 2006). Therefore, during acclimatization, the first and foremost requirement for successful transplantation is the maintenance of plantlets under very high humidity conditions (90-100%) for the first 10-15 d followed by gradual decrease in humidity (60-70%). The plantlets are then gradually shifted from high moisture environment to regular environment so as to gradually develop the protective system (Bhojwani and Razdan 1983). Temperature is also very crucial for higher survival rate and growth of transplanted plants. During summer, plants are exposed to high irradiance and temperature (30-40°C), and low humidity. Carbon dioxide enrichment in the greenhouse for the cultivation of ornamental plants has a positive impact on production. Increased carbon dioxide concentration also lessens water stress of microcuttings by closing the stomata as reported by Matysiak and Nowak (1995). The plantlets hardened under such conditions showed better survival when transferred to the field. Successful transplantation also depends on suitable size of the plantlets and their state of growth *in vitro*. The *in vitro*-raised plantlets need proper care before they are transferred to the outside environment. Thus, careful step-wise procedure is to be followed when *in vitro* plants are transferred to pots or in field conditions. In the present study, healthy plantlets with vigorously growing roots were transferred for higher growth and easy establishment. Compost containing garden soil with sand in the ratio of 1:1(v/v) gave the best results for the hardening of the complete plantlets. The compost used was suitable for growth of plantlets as it might have facilitated proper drainage and aeration for root respiration. Covering the plantlets with polythene bags was beneficial as it could retain moisture for the growth of the plants. The surviving plantlets had a healthy root system and leaves with well-developed pitchers ensuring proper growth. Decrease in BFC index was observed in the subsequent regenerations. This might be due to reduced regenerative capacity of the *in vitro*-raised plantlets in culture conditions. However, the plantlets of the three successive regenerations were observed to be morphologically very similar.

Chapter 4

Assessment of genetic fidelity through cytological analysis in regenerants of *Nepenthes khasiana*

4.1 Introduction

Nepenthes khasiana belonging to the order Caryophyllales is a challenging taxon from taxonomical and phylogenetic point of view. Therefore, it is not surprising to note that there is still lack of basic information about somatic chromosome number of several species belonging to the genus *Nepenthes.* Although certain remarks about the chromosome counts were made by few researchers (Heubl and Wistuba 1997), authentic details of chromosome biology could not be traced till date. A critical perusal of published literature on cytogenetic of the order Caryophyllales reveals that while the genus *Drosera* has received the attention of chromosome workers, the genus *Nepenthes* belonging to the same order has failed to generate any enthusiasm among cytologists. Heubl and Wistuba (1997) probably were the first to report somatic chromosome number of $2n=80$ in about 15 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. veeitchii*, *N. albomarginata*, *N. reinwardtiana*, *N. tentaculata,* and *N. clipeata.* An enormous spectrum of chromosome numbers has been reported in Droseraceae which is closely allied to Nepenthaceae and $x=5$ or $x=10$ was found to be the most frequent basic number. It has been reported that diploid and lower polyploids have already gone extinct in *Nepenthes* and restricted to high ploidy level thereby challenging the cytotaxonomic position of the genus (Heubl and Wistuba 1997). Due to several ambiguous assumptions and diverse spectrum of chromosome number, accurate cytotaxonomic studies of the single species of *Nepenthes* from Indian subcontinent *viz.*, *N. khasiana* are by and large lacking. However, the chromosome complements of *N. khasiana* were investigated for ascertaining the somatic chromosome number in various plant collections and the somatic chromosome number of 2n=80 has been recorded in all the cells studied. The investigations revealed the chromosomes to be small sized making it impractical to determine the position of the centromere(s) convincingly. Therefore, the approach of focusing on chromosome counts alone in micropropagated plants of *N. khasiana* could be helpful in the assessment of genetic fidelity at three successive regeneration stages.

Nuclear instability of *in vitro*-raised plant is very common and has been demonstrated in several plant species (D'Amato 1975; Sunderland 1977; Orton 1980). This instability is often manifested in the form of numerical and structural alterations in chromosomes of cultures as well plants regenerated from them (Kao *et al.* 1970; Sree Ramulu *et al.* 1983; McCoy and Bingham 1987). The frequency of such aberrations is reported to be very high, especially in the callus-mediated regenerants (Sunderland 1977; Yeoman and Street 1977; Roy 1980) as compared to direct regeneration (Sheridan 1974; Mathur *et al.* 1987; Sen and Sharma 1991). In addition to biochemical, histological and molecular approaches, cytogenetical analysis is also one of the most reliable techniques to assess any change in the genetic make-up during the process of regeneration. Chromosomal abnormalities have been observed in several tissue culture-derived plants and their progenies (Ahloowalia 1976, 1983, 1986; Duncan 1997; Roth *et al.* 1997; Kaeppler *et al.* 1998; Gupta 1998). Changes in chromosome number and structure disfunction of mitotic spindle are the cytological characteristics which have been commonly reported as somaclonal variations (Bayliss 1980; Lee and Phillips 1988; D'Amato 1990). Although alterations of chromosome numbers have been reported to be more common, structural chromosome changes have been observed with greater frequency in some species (Joachimiak *et al.* 1993). Chromosomal abnormalities associated with translocations, inversions and duplications or deletions are some of the frequently reported chromosomal aberrations (Kaeppler *et al.* 2000). Such aberrations which could have resulted due to chromosomal breaks, chromatin abnormalities such as anaphase bridges and chromosome stickiness and spindle failures, lagging chromosomes/chromatids, aneuploidy or polyploidy were considered as mitotic abnormalities. The chromosomal aberration types and frequencies have been reported to be dependent on the genotype of the explants, composition of nutrient medium especially the kind and concentrations of plant growth regulators, overall culture conditions as well as duration of cultures (Edallo *et al.* 1981; McCoy *et al.* 1982; Lee and Phillips 1987).

4.2 Materials and methods

4.2.1. Plant material and regeneration

Axillary cultures of *N. khasiana* were established *in vitro* through nodal explants as described earlier in Chapter 3.

4.2.2. Cytological preparations for mitotic complements

The root tips of about 0.5-1.0 cm long were excised from both *in vivo* (mother plant) and *in vitro*-raised plantlets of the three successive regenerations. The root tips were pretreated with 0.002 M 8-hydroxyquinoline solution for 3 h at room temperature before being fixed in Carnoy's fluid consisting of propanol and propanoic acid in the ratio of 3:1 for 24 h at room temperature. The root tips were transferred to 70% (v/v) ethanol and stored in a refrigerator till utilized for squash preparations. For making squash preparation of chromosomes, the fixed root tips were thoroughly washed with distilled water 3-4 times and hydrolyzed in 5 N HCl for 1 h at room temperature. The hydrolyzed root tips, after washing thoroughly, were subsequently transferred to leuco-basic fuschin solution and were kept for 45 min at room temperature 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.

4.2.4. Microphotography

The micro-photographs were taken using *Jenoptik* CCD camera (Germany) attached to *Labomed* LX 400 fluorescent microscope. A minimum of five regenerants of *in vitro* cultures were selected from each regeneration. At least five slides were prepared from the root tips of each micropropagated plantlet and on average six cells with clearly countable chromosomes from each slide were scored for the determination of chromosome counts.

4.2.5. Magnification

The illustrations in the present investigation were magnified at x1000 to the original dimensions of the image, with no further increase in the magnification during processing stage.

4.3 Results

Chromosome complements of *N. khasiana* were studied for ascertaining the somatic chromosome number in various plant collections. Somatic chromosome number as 2n=80 has been unambiguously recorded in all the cells studied (Fig. 4.1.a, b) with no evidence of any numerical variations, whatsoever. As the chromosomes are observed to be of small size, it was inconvenient to study the karyomorphology of the chromosomes. Therefore, the study was focused on chromosome counts alone.

A total of 30 cells were analyzed from the mother plant and all the cells analyzed showed normal somatic chromosome number of 2n=80 with no evidence of numerical variations (Table 4.1; Fig. 4.2.a-d).

In the plantlets raised in the first regeneration, a total of 30 cells were analyzed out of which 76.66% cells showed somatic chromosome number as 2n=80 while the remaining 23.33% cells showed chromosome number of 2n=76, 78 (Fig.4.3).

Fig. 4.1. (a-b) Chromosome complements in *N khasiana*, 2n=80 (Bar 10 μm)

Table 4.1. Analyses of root tip cells observed at metaphase in the mother plant (control) and the regenerated plantlets of *N. khasiana*

Regen-erations	No. of cells analyzed	Cells with normal chromosome		Cells with deviant chromosome		Deviant chromosome no.
		No.	%	No.	%	
Control	30	30	100	0	0	-
1st	30	23	76.66	7	23.33	2n=76 (2) 2n=78 (5)
2nd	30	20	66.66	10	33.33	2n=70 (6) 2n=76 (1) 2n=78 (1) 2n=84 (1) 2n=86 (1)
3rd	30	18	60.00	12	40.00	2n=70 (7) 2n=76 (3) 2n=84 (1) 2n=86 (1)

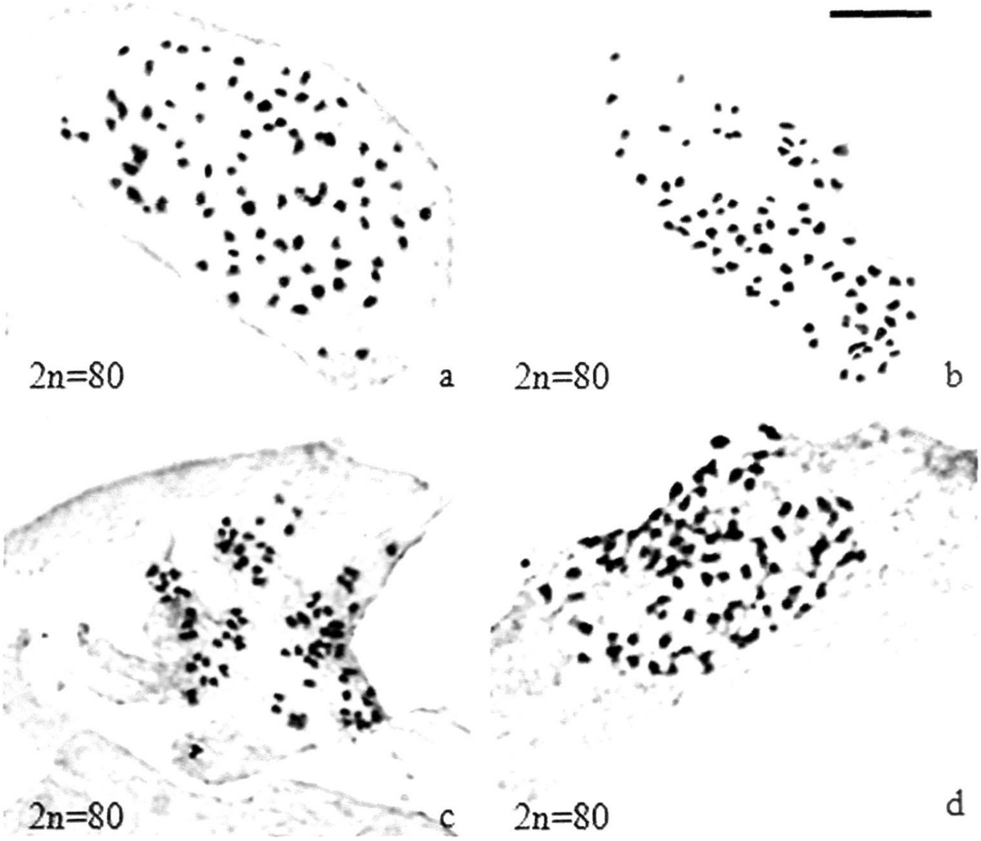


Fig. 4.2. (a-d) Chromosome complements in the mother plant of *N. khasiana*, 2n= 80 (Bar 10 μm)

Out of 30 cells analyzed in the plantlets of the second regeneration, 66.66% cell showed normal somatic chromosome number of 2n=80 while the remaining 33.33% cells were observed to possess deviant chromosome numbers of 2n=70, 76, 78, 84, 86 (Fig. 4.4).

In the plantlets of the third regeneration, 60% cells showed normal somatic chromosome number of 2n=80 and deviant chromosome complements of 2n=70, 76, 84, 86 were recorded in 40% cells (Fig. 4.5).

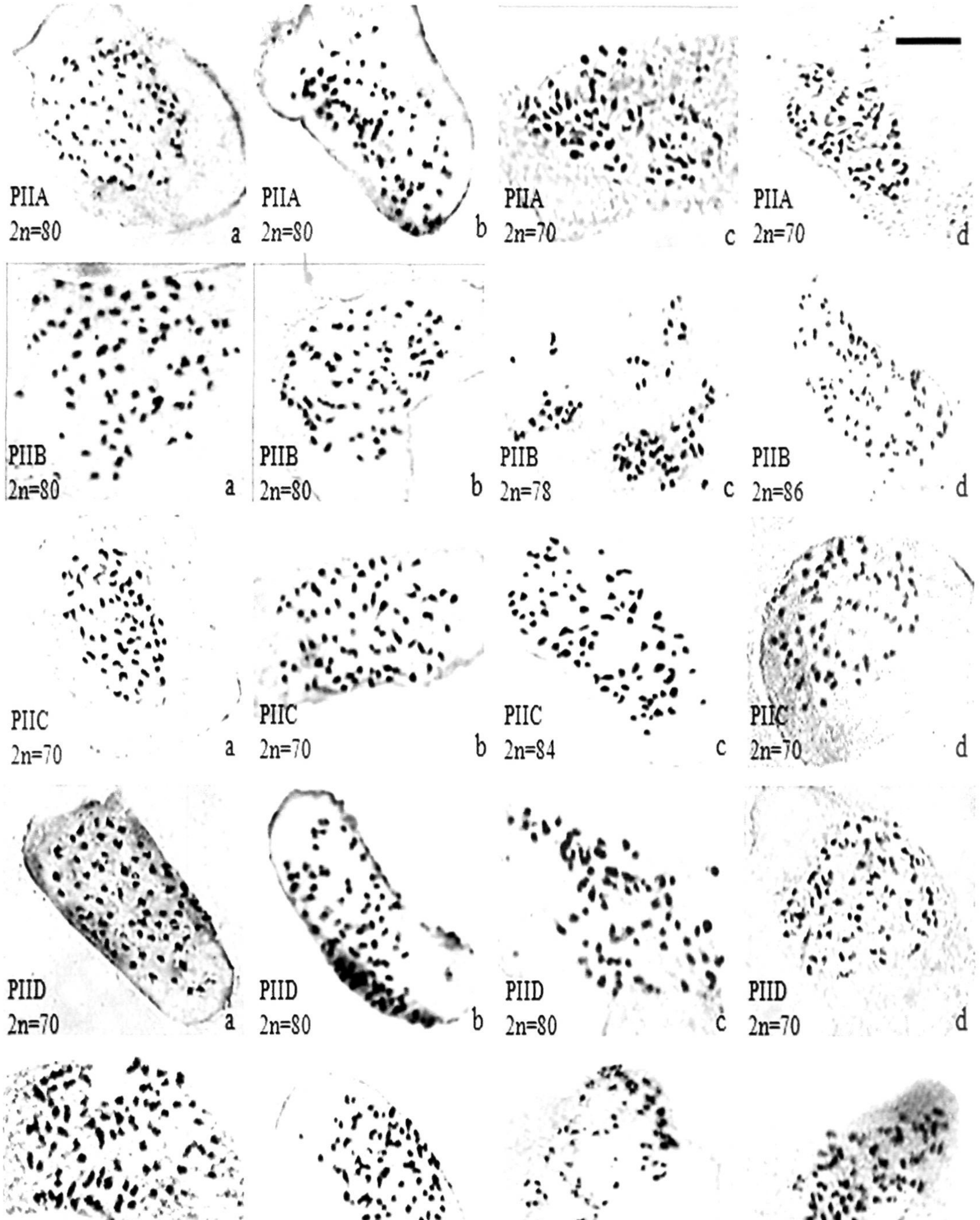


Fig. 4.4. (a-d) Chromosome complements in cells of PIIA, PIIB, PIIC, PIID and PIIE of *N. khasiana*. (Bar 10 μm). PIIA, PIIB, PIIC, PIID and PIIE represent plantlets A, B, C, D and E of the second regeneration.

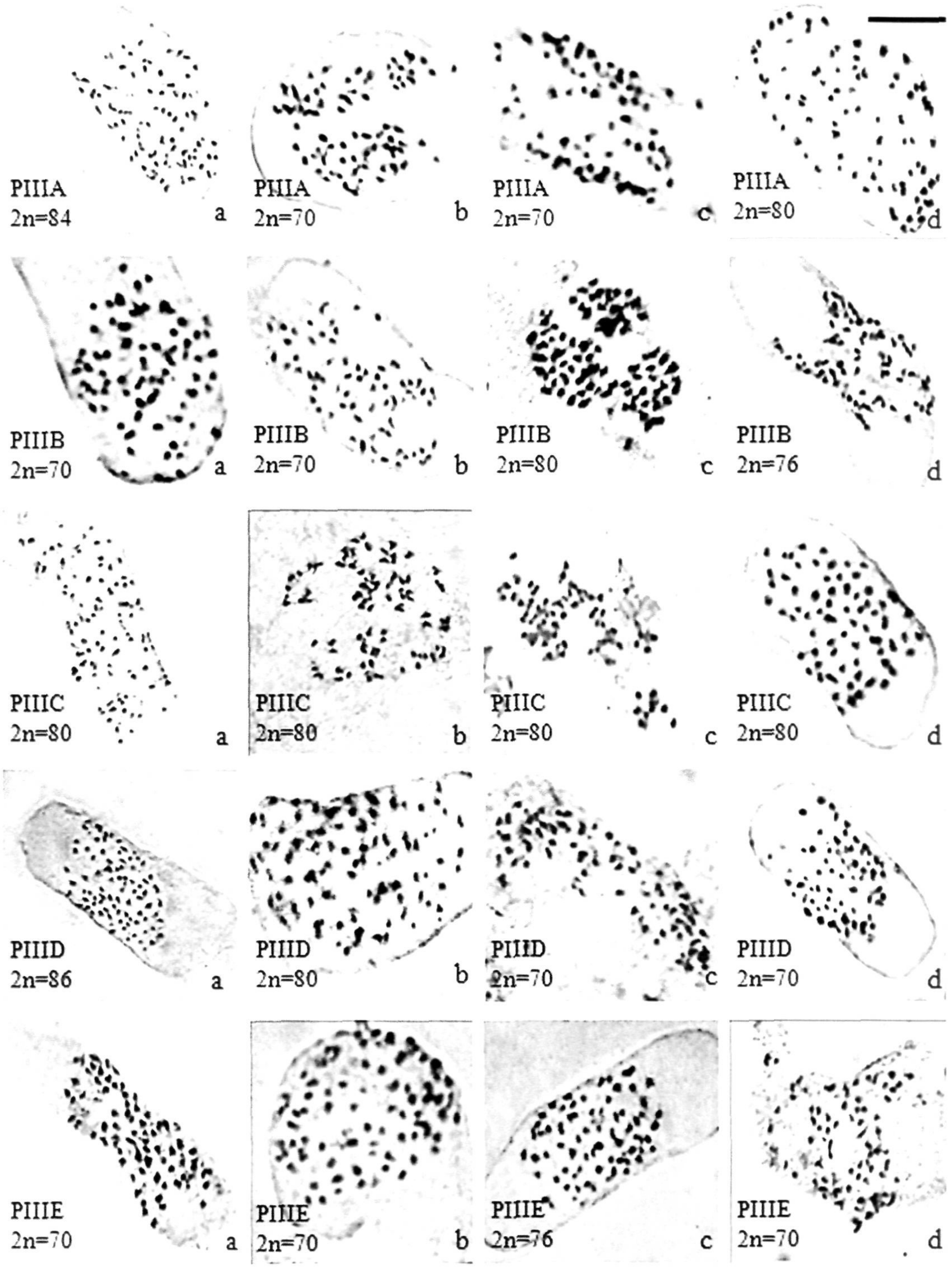


Fig. 4.5. (a-d) Chromosome complements in cells of PIIA, PIIB, PIIC, PIID and PIIE of *N. khasiana*. (Bar 10 μm). PIIA, PIIB, PIIC, PIID and PIIE represent plantlets A, B, C, D and E of the second regeneration.

4.4 Discussion

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 15 species of *Nepenthes* as mentioned earlier in the present chapter. Members of the family Nepenthaceae are known for remarkable uniformity in chromosome numbers of 2n=80 without any indication of numerical variations. The present studies are in agreement with these reports. However, such observations need to be substantiated with informations on more number of species representing the entire genetic spectrum of the genus. It was also observed that chromosomes lack a single localized centromere in *N. khasiana*. The chromosomes of most eukaryotes have 'localized centromere' which presents as a primary constriction. However, 'non-localized centromere' or 'diffuse centromere', which does not show any constriction or localized centromere position on chromosome, are known in some plants such as *Luzula* (Castro *et al.* 1949), and members of the family Cyperaceae (Hakansson 1958). In *Drosera* which is very closely related taxa with the genus *Nepenthes*, distinct primary constrictions or localized centromere has not been observed supporting the diffuse centromere hypothesis (Kondo *et al.* 1976; Kondo and Segawa 1988; Sheik *et al.* 1995). Similarly, in *N. khasiana*, the somatic chromosomes were observed to be very small in size and clear chromosomal gap between sister chromatids was not seen. In other words, the centromeres might have been probably diffused along the entire length of chromosome suggesting the holocentric nature of the chromosomes. Since the diffused type possesses centromere function dispersed along the whole chromosome length, in theory all fragments of this type of chromosomes are stably transmitted after cell division. On the contrary, Kondo and Lavarack (1984) observed distinct primary constrictions in the larger chromosome of *Nepenthes*. Also, Junichi *et al.* (2011) reported that two larger chromosomes of *Drosera arcturi* showed primary constrictions and *D. regia* had localized-centromeric position or well-differentiated primary constrictions in most metaphase chromosomes.

Chromosome size, condensing behavior of chromatin and interphase nuclei of the genus *Nepenthes* 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 support the assumption that in *Nepenthes*, diploid and lower polyploids have already gone extinct (Heubl and Wistuba 1997). Based on this study, *N. khasiana,* could be regarded as a polyploidy taxa (8x or 16x) 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 the 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 their hybrid derivatives are essential to ellucidate taxonomic and phylogenetic relationships among the species of the genus *Nepenthes*.

It is a common belief that seedlings produced in nature through vegetative propagation are genetically more stable as compared to those produced by seeds since the desirable phenotypic characteristics of the parent remain undisturbed in the former. Vegetative propagation can take place through rhizome, corm, offsets, bulbs, tubers, suckers, etc. Stem cuttings, air layering, budding, grafting are some of the conventional methods employed for such propagation. However, these methods of propagation impose several limitations for large-scale production. Plant tissue culture holds great promise for rapid mass propagation of valuable genotypes in a limited space, especially in case of rare, threatened or critically endangered plants (Prance 1997; Feijoo and Iglesias 1998; Tandon and Kumaria, 1998; Lynch 1999). However, *in vitro* culture techniques are known to be associated with genetic instability in plants due to influencing factors like somaclonal variations (Karp 1989; Cullis 1992; Phillips *et al.* 1994). Tissue culture-induced variations could be a very serious problem resulting in the production of undesirable plant off-types (Karp 1993; Cassells *et al.* 1999).

N. khasiana, naturally propagate mostly through seedlings produced from the rhizomatous basal portion of the stem (Bordoloi 1977). In the present study, it is clearly established that the plantlets were regenerated directly from nodal explants of *in vivo N. khasiana* plant. The regenerated plantlets were maintained for the three successive regenerations and genetic variability, if any, was assessed in tissue culture raised plants. From the present observation, it was seen that the plantlets of the first regeneration showed less quantum of variation with 76.66% normal cells as compared to the plantlets of the second and the third regenerations recording 66.66% and 60% normal cells, respectively. Plant cells are subjected to stress at different stages of culture in artificial medium which leads to somaclonal variations in varying percentage of cells (Bairu *et al.* 2006). In the first regeneration, only 23.33% cells showed deviant chromosome numbers which was increased to 33.33% and 40% in the second and the third regenerations respectively. The causes for origin of somaclonal variations have been reported to be manifold related to both external and internal aspects of plant tissue culture (Karp 1991). The level of genetic instability may be attributed to naturally occurring variation or accumulation of mutations during the culture period. Studies have shown that the presence or absence of variations during tissue culture depends upon the source of explants and the mode of regeneration including levels of growth regulators (Goto *et al.* 1998; Martin *et al.* 2006).

The explant tissue can affect the frequency and nature of somaclonal variations (Kawiak and Lojkowska 2004; Chuang *et al.* 2009). It has been reported that somaclonal variations can arise from pre-existing mutations already present in the tissues of the donor plant or may be induced during the culture phase (Bairu *et al.* 2011). Cellular organization is a critical factor for plant growth and loss of cellular control gives rise to disorganized growth under *in vitro* conditions ultimately leading to genetic variations. Although direct organogenesis from meristem cultures, minimises the possibility of instability, the stabilising influence of the meristem is sometimes lost when the cells are grown in culture (Vasil 1994). In contrast, highly differentiated tissues generally produce more variants, probably due to redifferentiation phase, than explants with pre-existing meristems illustrating the importance of the explants source from the donor plant with respect to its inherent genetic composition and genome uniformity, in any of its components (Sharma *et al.* 2007). The greater the cellular disorganization and the longer the duration of the disorganized phase, greater are the chances of somaclonal variations. In the present study, the nodal explants with the pre-existing buds were used for multiple shoot induction through direct organogenesis. However, 23.33% cells showed deviant chromosome numbers which might be due to the loss of the stabilizing ability of the meristems present in the pre-existing buds as reported by Vasil *et al.* (1994).

The primary events, controlled by exogenously applied plant growth regulators (PGRs) that trigger morphogenesis via cell-cycle disturbance might induce variabilities (Peschke and Phillips 1992). In addition to natural growth hormones found in plants, incorporation of PGRs in tissue culture for promoting cell division and growth, subject the explants to *in vitro* stress ultimately leading to genetic instability. It is believed that growth regulators preferentially increase the rate of division of genetically abnormal cells (Bayliss 1980). There are evidences which indicate that growth regulators enhance somaclonal variations during the culture phase through their effect on cell division, degree of disorganized growth and selective proliferation of specific cell types (Roels *et al.* 2005; Siragusa *et al.* 2007; Radhakrishnan and Kumari 2008). PGRs such as auxins and cytokinins preferentially increase the rate of division in cells (Bayliss 1980). The genetic composition of a cell population can therefore, be influenced by the relative levels of growth regulators, especially synthetic compounds which have been reported to be associated with somaclonal variations (D'amato 1975; LoSchiavo 1989; Vidal and De Garcià 2000). The possibility of unbalanced concentrations of auxins and cytokinins inducing polyploidy was also highlighted (Swartz 1991). High levels of BAP also greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with low level of BAP (Oono 1985). Munthali *et al.* (1996) detected genetic variations in tissue cultured-raised plants of sugarbeet regenerated by adventitious shoot budding in which BAP was supplemented in the medium in combination with auxin. The presence of a relatively high concentration of BAP was implicated in inducing the chromosomal abnormalities in a somaclonal variant CIEN BTA-03 derived from the banana cultivar 'Williams' (Gimenez *et al.* 2001). However, the exposure of the banana cultivar 'Nanjanagudu Rasabale' to relatively high concentrations of BAP and KN was reported to cause no somaclonal variations (Venkatachalam *et al.* 2007). Feyissa *et al.* (2007) detected the occurrence of genetic variations in tissue culture-derived plantlets of *Hagenia abyssinica* regenerated using axillary buds in presence of BAP in combination with IBA. In another study, all somaclones regenerated from different parts of *Solanum tuberosum* investigated showed chromosomal instability detecting aneuploid and polyploid cells at high frequency (57-89%) when BAP was used in high concentrations (Jelenic *et al.* 2001). However, PGRs did not influence the genetic stability of the micropropagated plants of *Foeniculum vulgare* regenerated through somatic embryogenesis (Bennici *et al.* 2004). In the present study, the culture medium was supplemented with 2.0 mg/l KN and 2.5 mg/l of BAP in combination for successful multiple shoot induction from the nodal segments. Both of these cytokinins are well known for inducing cell growth and development through enhanced cell division.

In the present investigation, it was observed that the micropropagated plantlets of *N. khasiana* which is characterized by higher ploidy (probably at 8x level), produced cells with deviant chromosome numbers *in vitro*. Genetic variations among plants regenerated are always higher among polyploids and plants with high chromosome numbers than those with low ploidy and low chromosome number species (Watson *et al.* 1992; Skirvin *et al.* 1994). Plants regenerated from mesophyll protoplast of tetraploid British cultivar 'Maris Bard' and 'Fortyfold' and Dutch cultivar 'Bintje' of potato revealed extensive variations in chromosome number reflecting the polyploid nature of potato ($2n=2x=48$), and its concomitant higher tolerance to chromosomal changes. Numerical and structural variations in chromosome of tissue culture-raised plants are strong evidences for possible change in genetic composition of an organism (Kunitake *et al.* 1995; Al-Zahim *et al.* 1999). Karp and Maddock (1984) detected substantial changes in both number and structure of chromosomes in plants of four different wheat (*Triticum aestivum* $2n = 6x = 42$) cultivars regenerated via somatic embryogenesis.

The frequency of somaclonal variations increases as the number of subcultures and their duration increases, especially in cell suspensions and callus cultures (Reuveni and Israeli 1990; Rodrigues *et al.* 1998; Bairu *et al.* 2006). Moreover, the rapid multiplication of a tissue or long-term cultures may also affect genetic stability and thus lead to somaclonal variations (Israeli *et al.* 1995). Hartmann *et al.* (1989) showed that the long period in culture increased the number of somaclonal variants in wheat regenerants.

Rodrigues *et al.* (1998) observed 1.3% somaclonal variants in the fifth subculture during micropropagation of *Musa* which was found to be increased to 3.8% in the twelveth subculture. Similarly, Bairu *et al.* (2006) observed an increase in the rate of occurrence of variants with progressive sub-culturing of micropropagated plants of *Musa* spp. Zhenxun and Hongxian (1997) also reported that aneuploidy and mixoploidy were predominantly observed in banana regenerants cultured for long durations. A statistical model has been proposed for predicting the theoretical mutation rate with the number of multiplication cycles as the primary parameter and two main conclusions were derived from the model that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and, variable off-type percentages can be expected after a given number of multiplication cycles (Cote *et al.* 2001). However, the model had limited applications because of the complexity of biological systems. Etienne and Bertrand (2003) described the effects of age of embryogenic cell suspensions on frequency and phenotype of variants of *Coffea arabica*, where somaclonal variation was increased to 25% in plants produced from 12-month-old cell suspensions in plants as compared to1.3% in plants produced from 3-month-old cell suspensions. Therefore, it is not surprising that subsequent increase of genetic variations in the plantlets of the second and the third regenerations of *N. khasiana* was observed in the present study. However, the plantlets of the three consecutive generations were morphologically very similar. This may be attributed to the ability of the normal cells to overcome the incompetent abnormal cells with decreased potential resulting in the normal phenotypic characters. Similarly, Browers and Orton (1982) observed that aberrant chromosome numbers occurred at an appreciably high frequency in morphologically indistinguishable plants of celery derived through somatic embryogenesis. Although abnormal cells with deviant chromosome numbers are ubiquitous in tissue culture, regeneration acts as a sieve mostly permitting the growth of stable normal cells (D'Amato 1977). Moreover, chromosomal abnormalities such as loss or addition of few chromosomes arising from tissues of polyploid or hybrid origin can be tolerated (Heinz and Mee 1971; Sree Ramulu 1987; Jelenic *et al.* 2001).

The present study reveals that the regenerated plantlets of the first regeneration of *N. khasiana* showed low percentage of cells with deviant chromosome numbers which was subsequently increased in the plantlets of the second and the third regenerations indicating that genetic stability could not be maintained in the regenerated plants of *N. khasiana* kept for longer duration of time in culture. However, no morphological variation was observed in the plantlets of the three consecutive regenerations.

Chapter 5

Assessment of genetic fidelity in regenerants of *Nepenthes khasiana* based on differential flourochrome binding pattern

5.1 Introduction

Heterochromatin regions are the most intensively studied and best known chromosome markers in both plants and animals. The distribution pattern(s) of heterochromatin have been employed as a reliable marker for identification of individuals and/or intra- or inter-specific chromosomal variations (Marks and Schweizer 1974; Schweizer and Ehrendorfer 1976; Greilhuber and Speta 1989). Heterochromatic regions are generally analyzed by either C-banding techniques or direct detection with base-specific fluorochromes, such as chromomycin A_3 (CMA), 4-6-diamidino-2-phenylindole (DAPI), Hoechst 33258, mythramycin, quinacrin, etc. (Schmid and Guttenbach 1988; Sumner 1990; Guerra 2000). The origin of the differential staining of heterochromatin for detection of certain defined target loci is certainly related to one of the most universal characteristics of heterochromatin, i.e. the presence of tandem repetitive DNA sequences.

Fluorochrome-based detection has the advantage of being a simpler, more reproducible and less destructive technique, as compared to the other known chromosome approaches (Guerra 1993). It is important to evaluate the distribution of each heterochromatin type separately by cyto-molecular techniques as the bands of a single karyotype may be composed of different families of repetitive DNA with independent distribution patterns (Flavell 1982). This technique has been applied successfully for studying the distribution of heterochromatin in plant species such as *Secale*, *Scilla*, *Hordeum*, *Aegelops* (Appels *et al.* 1978; Deumling and Greilhuber 1982; Brandes *et al.* 1995; Badaeva *et al.* 1996). Heterochromatin associated with the nucleolar organizing region (NOR) can be specifically identified by fluorescence *in situ* hybridization (FISH) using a probe which can localize 45S rDNA sites in plants (Hizume *et al.* 1992; Galasso *et al.* 1996). An alternative way of discriminating heterochromatin types is to stain the chromosome with flourochromes that have a preferential affinity for AT- or GC- rich DNA which can differentiate the distribution of heterochromatin in the chromosome (Guerra 2000). Conventional staining techniques may show heteropycnotic regions that form the so-called prophase condensation pattern (Ikeda 1988; Fukai and Mukai 1988; Benko-Issepon and Morawetz 1993). Such regions have often been confused with heterochromatin patterns which may be very different from each other (Guerra 1988; Marawetz 1991). However, staining with base-specific fluorochrome has been recognized as a reliable method of discriminating different types of heterochromatin in plants (Vosa 1970, 1976; Schweizer 1976).

The specific binding of the fluorochromes with the chromosomes depends mainly on the nitrogenous base composition of the DNA molecule(s), in such a way so that each region of the chromosome may show a positive (+) or negative (-)

reaction with a given fluorochrome (Schweizer 1981). The fluorochromes CMA and DAPI are the most intensively used fluorochromes in plants, as these exhibit preferential staining for GC- and AT-rich DNA sequences, respectively. This allows the identification of different types of heterochromatin in a more convincing note. Morphological differences in the karyotypes can be effectively revealed using fluorescent based detection staining method. It is also possible to measure the differences in the amount and site of GC- and AT-rich base pairs on the chromosome complements, thereby, distinguishing different genomes. The base-specific fluorochrome reaction of heterochromatin for 58 species of plants showed the heterochromatin to be preferentially located in similar regions of chromosome, regardless of the distance from the centromere (Guerra 2000). Proximal bands were found to be more common in small sized chromosomes suggesting the possible role of the chromosome size in heterochromatin localization. In *Allium subvillosum*, heterochromatin has been reported to be localized in the telomeric regions of all the short arms, and in the interstitial regions of all the long arms (Guerra 2000).

Insectivorous plants have always been the source of attention due to their extraordinary abilities for trapping a wide range of insects. In spite of the difficulties in establishing a clear relationship among the *Drosera* species using conventional karyomorphological and the molecular data, some researchers have attempted to differentiate the species based on the base-specific fluorochrome staining pattern (Sheik and Kondo 1995; Junichi *et al.* 2011). Sheik and Kondo (1995) observed the fluorophore binding sites at the terminal region revealing that GC- and AT-rich segments were mostly aggregated at the ends of *Drosera* chromosomes (telomeric regions). They suggested that *D. helodes* and *D. sewelliae* are closely related on the basis of chromosome number and fluorescent binding AT/GC rich loci. They also observed that distinct primary constriction or centromere was conspicuously absent in any of the chromosomes of *Drosera* species. Their results confirmed that *Drosera* chromosome(s) might have diffused centromeres along their entire length. However, Junichi *et al.* (2011) observed primary constriction in *D. regia* contradicting the earlier hypothesis. In addition, based on the differential fluorescent staining they found AT and GC base pairs to be equally dispersed in the heterochromatin of all the chromosomes in *D. arcturi* and *D. regia*.

From the forgoing reports, it is amply clear that the repetitive sequences found in the heterochromatin of the nuclear genome are vulnerable to major changes especially under culture conditions. Therefore, in the present chapter, an attempt has been made to observe such changes in the nuclear genome repetitive sequences of *in vitro*-raised plantlets of *N. khasiana*.

5.2 Materials and methods

5.2.1. Cytological preparation for CMA and DAPI binding sites

Root tips of the mother plant and the plantlets of the three consecutive regenerations were pretreated with 0.002 M 8-hydroxyquinoline solution for 3 h at room temperature before being fixed in Carnoy's fluid consisting propanol and propanoic acid (3:1) for 24 h at room temperature. The root tips were subsequently preserved in 70%

(v/v) ethanol and stored in a refrigerator till utilized. For fluorescent staining with CMA and DAPI, the root tips were subsequently washed with distilled water (2-3 times) and then macerated in an enzymatic mixture containing 2% cellulase and 20% pectinase at 37°C for 1-2 h. Root tips were rewashed with distilled water and squashed in a drop of 45% acetic acid under the cover glass which was subsequently removed by freezing in liquid nitrogen. Later the slides were stained with DAPI (2 µg/ml) : glycerol (1:1, v/v) solution to permit selection of the best cells. The slides were destained in ethanol/glacial acetic acid (3:1, v/v) for 30 min at room temperature and dehydrated in absolute ethanol for two hours at room temperature. The slides were subsequently air dried and aged at -20°C for 3-4 d for CMA/DAPI staining. The aged slides were stained with 0.1 mg/ml CMA for 1 h and counterstained with 1 µg/ml DAPI for 30 min, before being mounted in 1:1 (v/v) McIlvaine's buffer supplemented with 2.5 mM $MgCl_2$ (pH 7.0) and glycerol. The slides were kept in the dark at room temperature for 3 d before observation. The cytogenetic preparations were observed and photographed under a fluorescence microscope with a BV (blueviolet) and UV (ultraviolet) filter cassette for CMA and DAPI staining respectively.

5.2.2. Microphotography

The micro-photographs were taken using Leica DFC310 FX camera (Germany) attached to Leica Microsystems CMS GmbH fluorescent microscope. A minimum of five plantlets of *in vitro* cultures were selected from each regeneration. At least five slides were prepared from the root tips of each regenerant and on average six cells from each slide were scored for the determination of specific fluorescent sites.

5.2.3. Magnification

The illustrations in the present investigation were magnified at x 1000 to the original dimensions of the image, with no further increase in the magnification during processing stage.

5.3 Results

A total of 30 cells were analyzed from the mother plant and the plantlets of the three consecutive regenerations. Distribution of CMA and DAPI sites in the interphase nuclei of the mother and the regenerated plantlets of the three regenerations are summarized in Table 5.1. In case of the mother plant, the number of $DAPI^+$ sites was 5.33±0.73, on an average, with the range of 2-8 (Fig. 5.1). The number of $DAPI^+$ sites was increased to 5.74±0.47 with the range of 2-8 in the plantlets of the first regeneration which was further increased to 6.61±0.39 with the range of 5-12 and 6.74±0.57 with the range of 3-12 in the plantlets of the second and the third regenerations respectively (Fig. 5.2-5.4).

The number of CMA^+ sites observed, in case of the mother plant, was 5.11±0.47, on an average, with the range of 2-6 (Fig. 5.1). In the plantlets of the first regeneration, the number of CMA^+ sites was 5.00±0.30 with the range of 4-9 which was further decreased to 4.63±0.45 with the range of 1-8 and 4.16±0.47 with the range of 0-8 in the plantlets of the second and the third regenerations respectively (Fig. 5.2- 5.4).

Table 5.1. Distribution of CMA and DAPI sites in the interphase nuclei of the mother plant (control) and the regenerated plantlets of *N. khasiana*

Regenerations	Total no. of cells analyzed	DAPI$^+$		CMA$^+$		DAPI$^+$/CMA$^+$	
		Mean±SE	Range	Mean±SE	Range	Mean±SE	Range
Control	30	5.33±0.73	2-8	5.11±0.47	2-6	0.16±0.16	0-1
1st	30	5.74±0.47	2-8	5.00±0.30	4-9	0.61±0.23	0-3
2nd	30	6.61±0.39	5-12	4.63±0.45	1-8	0.77±0.20	0-3
3rd	30	6.74±0.57	3-12	4.16±0.47	0-8	0.77±0.35	0-5

The number of CMA$^+$/DAPI$^+$ sites, on an average, was 0.16±0.16 with the range of 0-1 in the mother plant (Fig.5.1). In the plantlets of the first regeneration, the number of CMA$^+$/DAPI$^+$ sites was 0.61±0.23 with the range of 0-3 (Fig. 5.2). In case of plantlets of the second regeneration, the number of CMA$^+$/DAPI$^+$ sites was 0.77±0.20 with the range of 0-3 (Fig. 5.3). After the second regeneration, there was not much change in the number of CMA$^+$/DAPI$^+$ sites. The number of CMA$^+$/DAPI$^+$ sites observed was 0.77±0.35 with the range of 0-5 in the plantlets of the third regeneration (Fig. 5.4).

The number of CMA$^+$ sites (GC-rich regions) was fewer than DAPI$^+$ (AT-rich regions) sites in the mother plant and the same trend was observed in the regenerated plantlets studied in the three subsequent regenerations, with little deviation found in the first regeneration. This indicates that the AT base pairs are more than GC base pairs in the heterochromatin regions of *N. khasiana*.

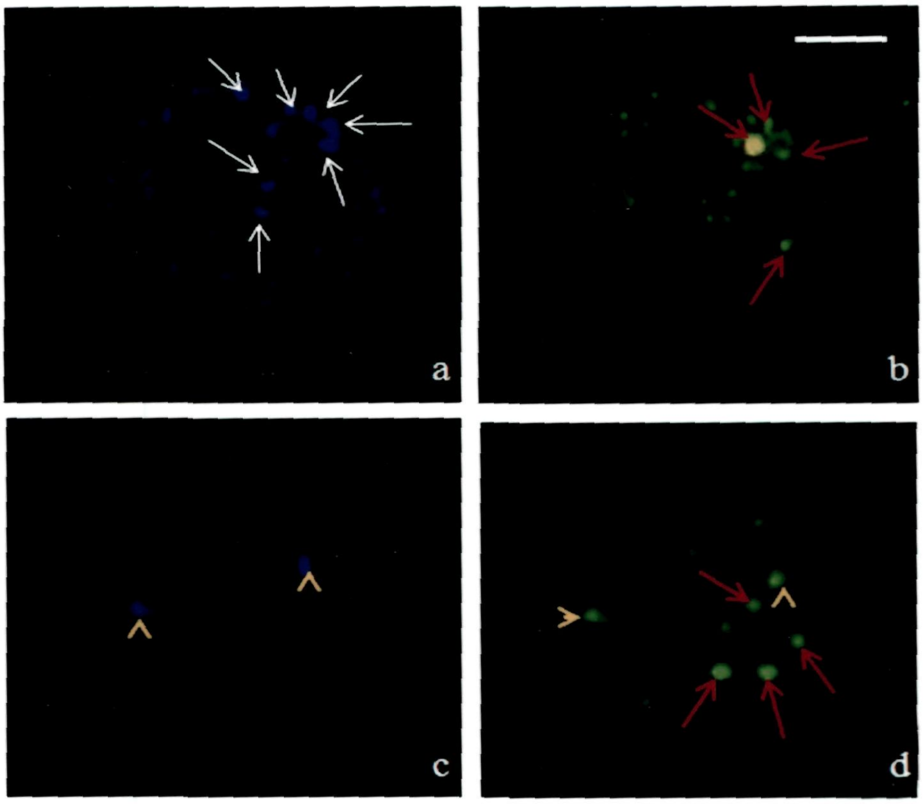


Fig. 5.1. Interphase nuclei of the mother plant of *N. khasiana* stained with DAPI (a, c) and CMA (b, d). (Bar 10 µm). Arrows indicate DAPI+ and CMA+ sites and arrowheads indicate CMA+/DAPI+ sites.

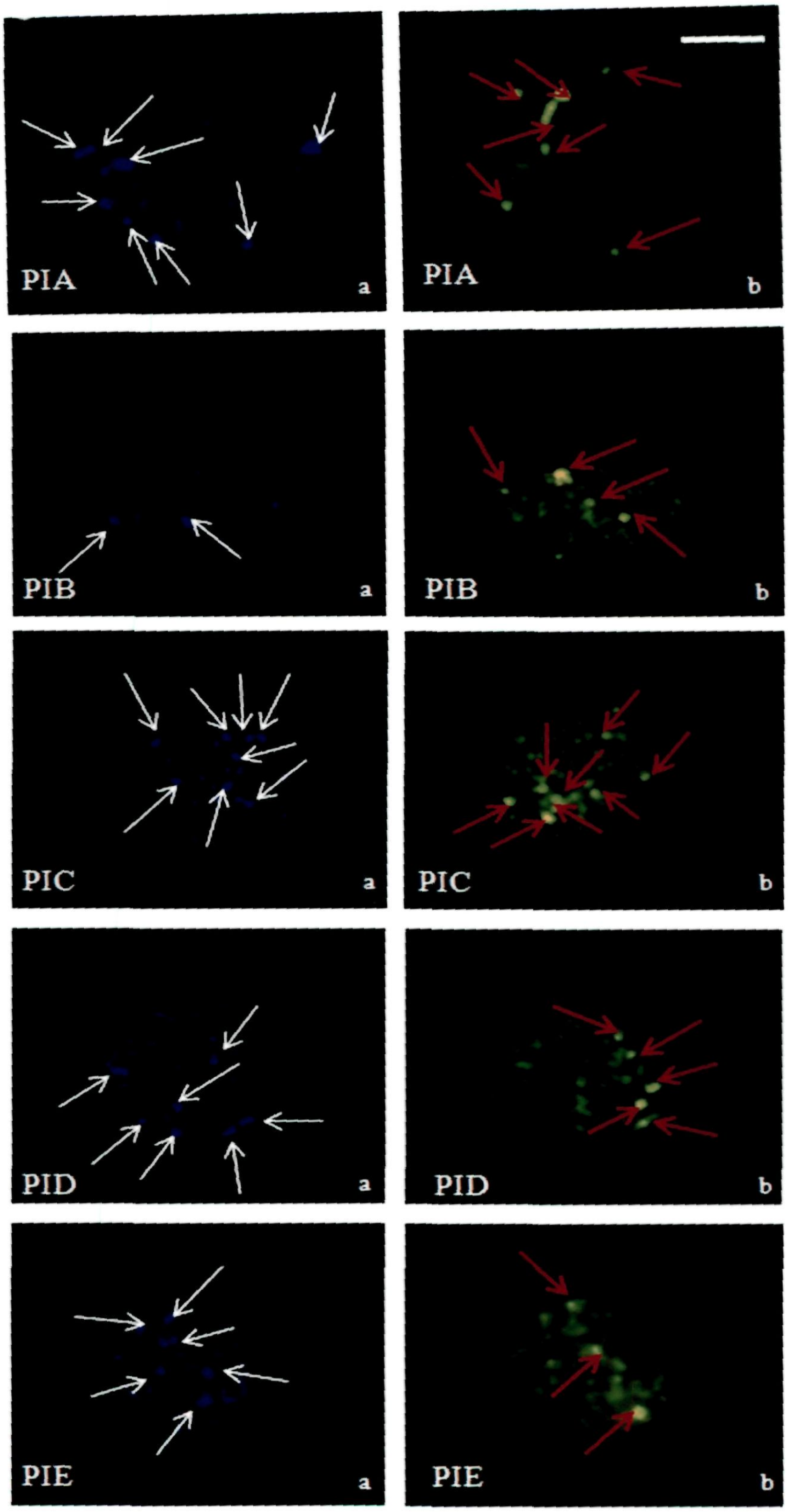


Fig. 5.2. Interphase nuclei of the micropropagated plantletlets of the first regeneration of *N. khasiana* tained with DAPI (a), and CMA (b). (Bar 10 µm). Arrows indicate DAPI+ and CMA+ sites.

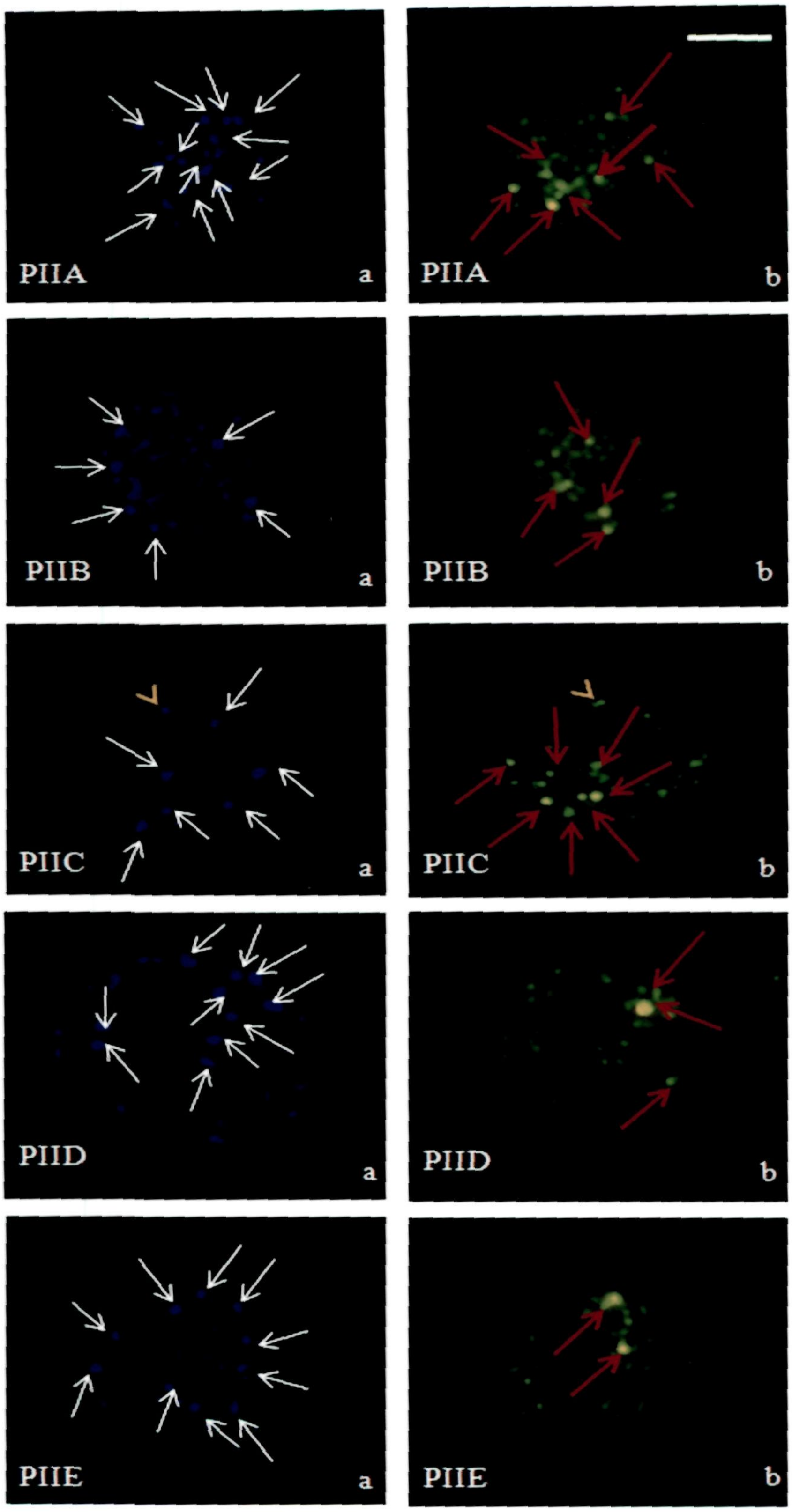


Fig. 5.3. Interphase nuclei of the micropropagated plantlets of the second regeneration of *N. khasiana* stained with DAPI (a), and CMA (b). (Bar 10 μm). Arrows indicate DAPI+ and CMA+ sites and

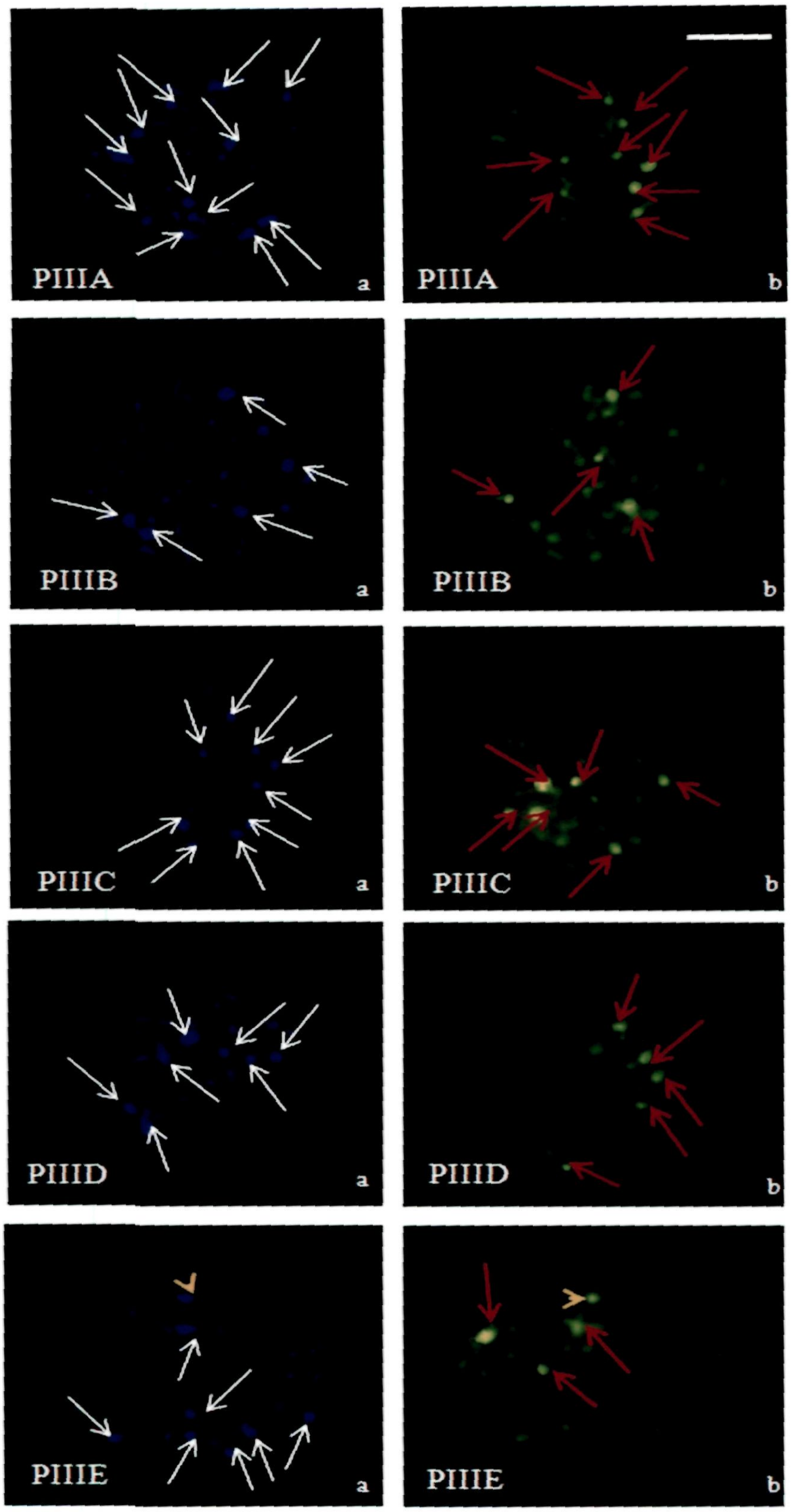


Fig. 5.4. Interphase nuclei of the micropropagated plantletofs of the third regeneration of *N. khasiana* stained with DAPI (a), and CMA (b). (Bar 10 µm). Arrows indicate DAPI+ and CMA+ sites and arrowheads

5.4 Discussion

Specific flourochrome-based detection has been employed as a reliable method for assessing the genetic stability in the tissue culture-raised plants. Tandem repetitive sequences found in the heterochromatin regions are reportedly associated with the nuclear genomic instability among the plants regenerated *in vitro* (Sutherland and Richards 1994; Gupta 1998). Changes in the copy number of repetitive sequences are reflected in the number of fluorescent based detection sites. Therefore, fluorochrome-based detection method, being more reliable and more consistent, was employed in the present investigation for determination of genetic stability in the micropropagated plantlets of *N. khasiana*.

In the present study, the number of DAPI$^+$ sites was increased in the first regeneration as compared to the mother plant. The number of DAPI$^+$ sites was increased subsequently in the plantlets of the second and the third regenerations. This indicates that the heterochromatin regions of these plantlets showed an overall increase of AT base pairs. However, it was observed that there was a corresponding decrease in the number of CMA$^+$ sites in the three consecutive regenerations indicating that the amount of GC base pairs was decreased in the micropropagated plantlets. The number of DAPI$^+$ sites, in the present study, was found to be approximately the same with the number of the CMA$^+$ sites in the mother plant. This result suggests that heterochromatin regions of the mother plant are composed of equal amount of AT and GC base pairs. Thus, the amount of AT and GC bases are expected to be the same in the regenerated plantlets. However, the number of DAPI$^+$ sites was found to be little higher than the number of CMA$^+$ sites in the plantlets of the first regeneration suggesting higher amount of AT base pairs in these plantlets. Similarly, in case of the plantlets of the second and the third regenerations, the number of $DAPI^+$ sites was found to be more as compared to the number of CMA^+ sites. This indicates that the amount of AT base pairs is more as compared to the amount of GC base pairs in the heterochromatin regions of the plantlets of the second and the third regenerations.

Thus, the amount of AT and GC base pairs in the plantlets of all the three regenerations were different as compared to the mother plant signifying the changes in the copy number of tandem repetitive sequences in the heterochromatin regions of the *in vitro*-raised plants. Nevertheless, the pattern of distribution of AT and GC base pairs was observed to be the same in both the micropropagated plantlets and the mother plant implying that the heterochromatin regions of most of the micropropagated plantlets are mainly composed of AT base pairs as in the case of the mother plant. The changes in the amount of the repetitive sequences, in the present investigation, correspond to the changes in the chromosome number in the micropropagated plantlets in which cells with deviant chromosome numbers were found to be subsequently increased in the plantlets of the second and the third regenerations. The number of AT base pairs was higher than the number of GC base pairs in all the three subsequent regenerations indicating the instability of the chromosomes which might have ultimately led to the changes in the number of chromosome. Therefore, the tandem repeat sequences located in the heterochromatin regions must have involved in distressing the normal cell cycle leading to chromosomal abnormalities as reported in earlier studies (Lapitan *et al.* 1984; Johnson *et al.* 1987; Peschke and Phillips 1992; Phillips *et al.* 1994).

The primary cytological changes observed among the regenerated plants and their progeny include chromosome rearrangements, numerical and structural changes in chromosome (Fluminhan *et al.* 1996). Cytological analysis of regenerated plants has shown that chromosome breakage and its consequences (deficiencies, duplications, translocations and inversions) are events quite frequently observed in plant tissue culture, and that breakpoints are often associated with late-replicating chromosome regions which are heterochromatic in nature. These results were observed by Sacristan (1971) in *Crepis capillaris*, where 82% of rearrangements induced *in vitro* involved chromosome breaks at the heterochromatic region of the long arm of the Sat-chromosome. McCoy *et al.* (1982) reported a high frequency of chromosome breakage in the late-replicating heterochromatic regions of the centromere in oat chromosomes. Similarly, break-points involved in translocations and deletions were also observed in heterochromatic regions in regenerated wheat and rye hybrids (Lapitan *et al.* 1984). However, in maize, it was reported that chromosome breakages occurred between the centromere and the distal heterochromatic blocks called knobs (Lee and Phillips 1987). The involvement of heterochromatin in the breakage events led to the hypothesis that normally late-replicating heterochromatic regions may replicate even later under the culture environment leading to the formation of anaphase bridges due to delayed separation of sister chromatids at heterochromatic regions and subsequent chromosome breakage (Lee and Phillips 1987, 1988). This model is based on the observation that heterochromatic knobs are involved in chromosome breakage events in high-loss stocks of maize, presumably due to incomplete replication before mitosis (Rhoades and Dempsey 1972, 1973). In addition, quantitative changes in repetitive DNA sequences have been observed in regenerated plants of several species, for eg. amplification of repeated sequences observed in tissue culture-regenerated plants derived from wheat and rye (Lapitan *et al.* 1988; Karp *et al.* 1992).

In the present study, it is clearly indicated that DAPI$^+$ sites and CMA$^+$ sites found in the late replicating heterochromatin regions are variable in number in the micropropagated plantlets and the instability in the amount of AT and GC base pairs reveals genetic variations in the regenerated plantlets of *N. khasiana*. However, the pattern of the distribution of AT and GC base pairs in the nuclear genomes of *N. khasiana* was undisturbed in the micropropagated plantlets. In Chapter 4, it has been shown that the regenerated plantlets of the second and the third regenerations show significant variations in the chromosome number. The corroboratory results of molecular fluorescent studies reveal heterochromatin related abnormalities due to the changes in the AT and GC base pairs.

Chapter 6

Assessment of genetic fidelity in regenerants of *Nepenthes khasiana* using DNA-based markers

6.1 Introduction

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant achievement in the field of molecular genetics. Molecular markers offer numerous advantages over conventional assessment of variations on the basis of phenotypic characters as they are stable, detectable in all kinds of tissues regardless of growth, differentiation, development, and are not affected by the environment, pleiotropic and epistatic effects. DNA-based molecular markers are versatile tools in the fields of taxonomy, physiology, embryology, genetic engineering, population genetics (Schlotterer 2004). In the recent years, molecular techniques are used as the most desirable and valuable tools in the analysis of genetic variations induced by tissue culture in regenerated plants (Sabir *et al.* 1992; Isabel *et al.* 1993; Rani *et al.* 1995; Hashmi *et al.* 1997; Goto *et al.* 1998; Rout and Das 2002; Rady and Nazif 2005; Feyissa *et al.* 2007; Gao *et al.* 2010; Khan *et al.* 2011; Singh *et al.* 2012; Khateeb *et al.* 2013). At present, a number of DNA-based markers such as RFLP, AFLP, RAPD, and SSRs are available to detect sequence variations between closely related species. More recently, PCR-based SPAR marker which includes RAPD, ISSR and DAMD methods has come up as an effective tool for studying genetic diversity in plants and it collectively provides a comprehensive description of the extent of the existing diversity (Bhattacharya *et al.* 2005; Ranade *et al.* 2009; Sharma *et al.* 2010; Kumar *et al.* 2011). RAPD is advantageous being rapid, cost-efficient in terms of operational aspects, very small amount of DNA is required and information on template DNA sequence is not needed. Similarly, ISSR is simple and does not require previous knowledge of the sequence of the genome being tested. The technique, involves amplification of the region between two identical microsatellite repeats within the genome and its main advantage is the capability of analyzing multiple loci in a single reaction. DAMD profiles are generated from minisatellite rich regions and reveal similarities among the repetitive sequence families. Thus, these three methods involve regions which have substantially different evolutionary histories and genome coverage (Powell *et al.* 1996; Parsons *et al.* 1997). These markers have been used widely, either alone or in tandem with morphological markers, to obtain more consistent information on the genetic variations in micropropagated plants. The combined data analysis of these methods considered together is expected to reveal a comprehensive pattern of genetic information amongst the regenerated plants. SPAR technique, therefore, would be more precise for the establishment of genetic fidelity in the micropropagated plants before being transferred to the field. However, very little information on study of genetic fidelity of *in vitro*-raised plants using SPAR approach can be traced in the published literature. Mishra *et al.* (2008) have successfully employed three single primer amplification reaction techniques *viz.* RAPD, ISSR and DAMD for the assessment of genetic stability in micropropagated plants of *Aegle marmelos*. In the present study, it is attempted to assess the genetic stability of the *in vitro*-raised plants of *N. khasiana* using three different SPAR methods. Data generated by these three markers were analyzed for the three successive regenerations.

6.2 Materials and Methods

6.2.1. Genomic DNA extraction

Total genomic DNA was extracted from fresh leaves of the mother plant and the *in vitro*-raised plantlets of all the three successive regenerations using modified CTAB method (Porebski *et al.* 1997). The extraction buffer was supplemented with 3% CTAB, 3 M NaCl and 1% PVP followed by purification of extracted DNA. 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.

6.2.2. Quality check of genomic DNA

Agarose gel electrophoresis was performed to check the quality of the isolated DNA of each plant sample. The tray and comb were washed thoroughly with distilled water and wiped with ethanol. Agarose gel (0.8%) was prepared by dissolving agarose in 1X TBE (Tris-Borate-EDTA) to which 2 μl of ethidium bromide (10 mg/ml stock) was added. The gel solution was allowed to cool to about 60°C and poured slowly into the well leveled tray fitted with comb. The gel was allowed to polymerize for about 1h at room temperature and subsequently the comb was removed. The gel tray was placed into the submarine tank already filled with 1X TBE. Samples for mupid electrophoresis check were prepared by mixing 3 µl of isolated genomic DNA and 1µl of 10X loading buffer (0.21% bromophenol blue, 0.21% xylene cyanol FF, 0.2 M EDTA at pH 8.0, and 50% glycerol) and finally the volume was raised to 10 µl with 1X TBE. Samples were then spinned down in a centrifuge and loaded in agarose gel submerged in 1X TBE buffer and electrophoretically separated with appropriate voltage of current (50-100 V). The gels were then observed in the gel documentation system and photographed under UV with Gel Logic 100 Imaging System (Biosteps, Germany). Fragment size and nature of DNA were determined by comparing with 1 kb ladder which was loaded in the first well of the gel.

6.2.3. Quantification of DNA

The quantity and purity of the isolated DNA was checked using UV spectrophotometer. Five microlitre of the DNA sample was mixed with 295 µl of TE buffer and the absorbance was recorded at 260 nm and 280 nm in a spectrophotometer with TE buffer as blank. The ratio of absorbance at two wavelengths (A_{260}: A_{280}) was compared with the standard ratio of pure DNA. The ratio i.e., A_{260}: A_{280} of a preparation of pure sample of DNA equal to 1.8 was taken as standard.

If the ratio of A_{260}: A_{280} is < 1.8, the DNA sample is considered to be contaminated by protein and phenol, and

If the ratio of A_{260}: A_{280} is > 1.8, the sample is considered to be contaminated with RNA.

The concentration of DNA was calculated as follows:

$$\text{Concentration of DNA } (\mu g/\mu l) = \frac{\text{A260 X 50 X Dilution Factor}}{1000}$$

(For double stranded DNA, 1 O.D. at 260 nm = 50 µg/µl DNA)

A 100 µl (10 ng/µl) working DNA stock solution was prepared by diluting in ultra pure water and stored at 4°C until use.

6.2.4. Single primer amplification reaction methods

Four RAPD kits (OPA, OPC, OPK and OPH) comprising 20 decamer random primers per kit (total 80 primers) were procured from Operon Technologies, Alameda, CA, USA. A total of 36 ISSR primers and 20 DAMD primers were custom synthesized from Metabion Inc. Ltd., Germany.

6.2.5. PCR optimization, primer survey and final amplification

Varying concentrations of (i) template DNA (20, 30, 40, 50 and 60 ng), (ii) Taq DNA polymerase (0.5 – 3 U), and (iii) Mg^{++} salt (1- 5 mM) were used in various combinations to optimize the reaction conditions for PCR. The optimal amplification conditions for all the three SPAR methods, namely, RAPD, ISSR and DAMD were established. Eighty RAPD, 36 ISSR and 20 DAMD primers were screened to identify the most reproducible primers. After PCR optimization and primer screening, final amplification reactions with selected primers were carried out as per the optimized conditions with the plantlets of all the three successive regenerations and the mother plant.

6.2.6. SPAR and gel electrophoresis

On the basis of PCR optimization and primer survey, all further reactions were carried out in 25 µl volumes containing 30 ng of template DNA, 200µM of each of the four dNTPs, 1X PCR buffer (10 mM Tris pH 9.0; 50 mM KCl), 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase (Bangalore Genei, India), and 5 pmol of primers (in case of RAPD) and 10 pmol of primers (in case of ISSR and DAMD).

The reactions for RAPD were performed according to protocol developed by Williams *et al.* (1990). PCR was performed with a programme consisting of pre-PCR cycle at 95°C for 4 min and 30 sec; 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. In case of ISSR, the reaction programmes were set at 94°C for 3 min for initial denaturation followed by 40 cycles of 1 min at 92°C, 1 min at annealing temperature (42-58°C depending on the primer's Tm and/or according to GC contents), 2 min elongation at 72°C and a cycle of final extension at 72°C for 10 min. For DAMD, amplification reactions were carried out at 94°C for 3 min for denaturation followed by 40 cycles of 92°C for 1 min, 2 min at 55°C, 2 min elongation at 72°C and a cycle of final extension at 72°C for 10 min. All the reactions were performed in a thermal cycler 2720 (Applied Biosystems Gene Amp PCR System, USA). After the genomic DNA had been amplified, the PCR products were mixed with 2.5 µl 10X blue dye and subsequently separated by electrophoresis using 1.2% agarose gel for RAPD, and 1.5% agarose gel for ISSR and DAMD in 1X TBE buffer stained with ethidium bromide under 70V constant power supply for 3 h.

Photography was done under UV with Gel logic 100 imaging system (Biosteps, Germany). Molecular ladders 500bp (Bangalore Genei, India) for RAPD and 100bp in case of ISSR and DAMD (Bangalore Genei, India) were used as markers for determining the size of the amplicons.

6.2.7. Scoring and data analyses

Each amplification product was scored across all the samples and considered as a marker. Bands were recorded as present (1) or absent (0). Faint bands with low intensity were not considered for final scoring. All amplifications were repeated at least twice and only reproducible bands were considered for analyses. A data set of amplified bands was scored manually from the gel profiles and this included only the well-separated and distinct bands. The data were scored individually, first for all the primers in a SPAR method and subsequently the data sets for all the three methods collectively, which were combined together for the final neighbor-joining (NJ) analysis. A pair-wise matrix of similarity between the micropropagated plantlets and the mother plant was determined cumulatively for all the three methods using Jaccard's coefficient (Jaccard 1902) by the NTsys-pc, version 2.02k (c).

6.3 Results

6.3.1. Quality check and quantification of isolated DNA

Purity of the isolated DNA was found to be close to the standard ratio of pure DNA. The ratio at A_{260}: A_{280} for the isolated DNA samples ranged within 1.5 to 2.0. The yield was high with less shearing.

6.3.2. Standardization of RAPD, ISSR and DAMD protocols and primer screening

Of the different concentrations of template DNA (20, 30, 50, 60 ng), 30 ng was found to be the most suitable as it yielded good and scorable products. In addition, 1.5 mM of $MgCl_2$, and 0.6 U Taq DNA polymerase were the most suitable among various concentrations tested. Of 136 primers screened, 41 primers (Table 6.1) produced analyzable amplification products for RAPD, ISSR, DAMD and were finally selected for further profiling.

6.3.3. SPAR analysis and profile polymorphism

6.3.3.1. SPAR analysis across the micropropagated plantlets of the three regenerations collectively

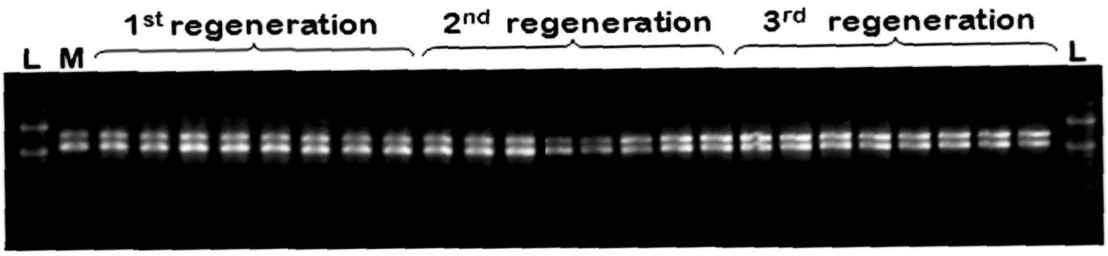
A total of 80 RAPD primers were used for initial screening out of which only 14 primers resulted in 74 clear, well-separated and reproducible bands of which 10 bands were polymorphic (13.51%) with an average of 0.71 polymorphic bands per primer across the three regenerations (Table 6.2; Fig. 6.1-6.3). A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.94 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6.4).

Table 6.1. Details of RAPD, ISSR and DAMD used in the present study

Sl. No.	Primer name	Primer sequence (5'-3')
RAPD		
1	OPH-1	GGTCGGAGAA
2	OPH-5	AGTCGTCCCC
3	OPH-6	ACGCATCGCA
4	OPH-11	CTTCCGCAGT
5	OPK-10	GTGCAACGTG
6	OPK-11	AATGCCCCAG
7	OPK-12	TGGCCCTCAC
8	OPK-13	GGTTGTACCC
9	OPK-14	CCCGCTACAC
10	OPK-17	CCCAGCTGTG
11	OPK-18	CCTAGTCGAG
12	OPK-19	CACAGGCGGA
13	OPC-7	GTCCCGACGA
14	OPA-11	CAATCGCCGT
ISSR		
1	N1	ACACACACACACACACT
2	N2	TGTGTGTGTGTGTGTGA
3	N3	GAGAGAGAGAGAGAGAYT
4	N4	CACACACACACAGG
5	N5	CACACACACACAAC
6	N6	CACCACCACGC
7	N7	GAGGAGGAGGC
8	N8	CACACACACACAGT
9	N9	ACACACACACACACACAG
10	N10	ACACACACACACACACAA
11	N11	ACGACGACGACGACGACG
12	N12	ACACACACACACACACGA
DAMD		
1	URPIF	ATCCAAGGTCCGAGACAACC
2	URP2F	GTGTGCGATCAGTTGCTGGG
3	URP2R	CCCAGCAACTGATCGCACAC
4	URP6R	GGCAAGCTGGTGGGAGGTAC
5	URP9F	ATGTGTGCGATCAGTTGCTG
6	URP13R	TACATCGCAAGTGACACAGG
7	URP17R	AATGTGGGCAAGCTGGTGGT
8	URP25F	GGACAAGAAGAGGATGTGGA
9	URP30F	GGACAAGAAGAGGATGTGGA
10	URP32F	TACACGTCTCGATCTACAGG
11	URP38F	AAGAGGCATTCTACCACCAC
12	HBV5	GGTGTAGAGAGGGGT
13	HVR	CCTCCTCCCTCCT
14	INS	ACAGGGGTGGGG
15	YN73	CCCGTGGGGCCGCCG

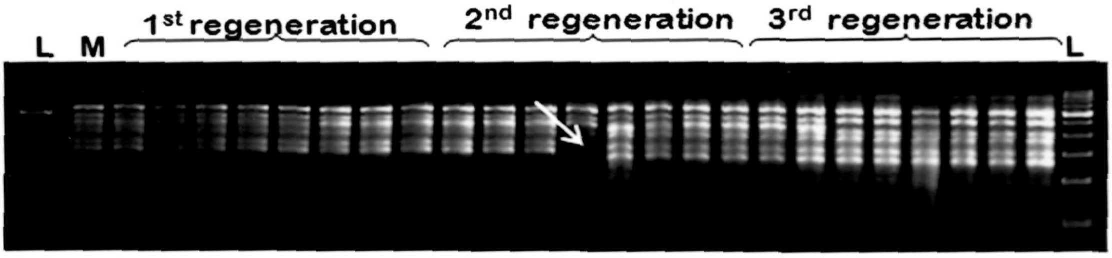
Table 6.2. Collective SPAR analysis for the plantlets of the three regenerations of *N. khasiana* together

Sl. No.	SPAR approach	No. of primer used	Total bands amplified	Average bands/ primer	Total no. of polymorphic bands	Average no. of polymorphic bands/ primer	% of polymorphism	Distance range (Jaccard's Coefficient)
1.	RAPD	14	74	5.28	10	0.71	13.51	0.94-1.00
2.	ISSR	12	50	4.16	7	0.58	14.00	0.94-1.00
3.	DAMD	15	60	4.00	10	0.66	16.66	0.91-1.00
4.	RAPD+ISSR +DAMD	41	184	4.48	27	0.65	14.67	0.95-1.00

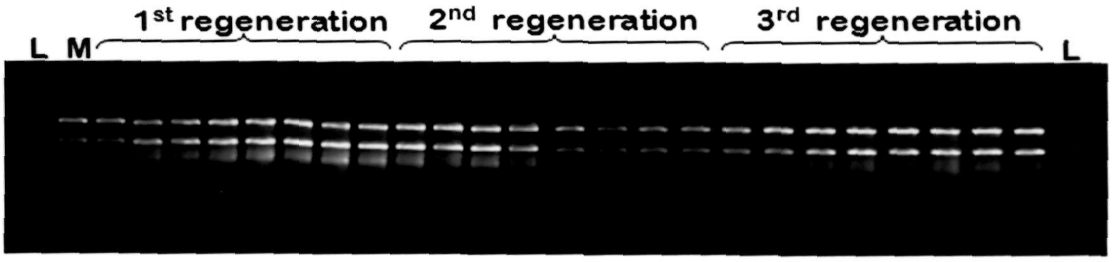


A OPH1

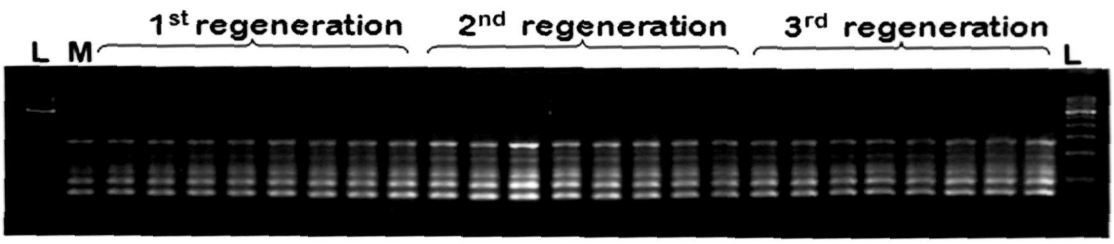
B OPH5



C OPH6

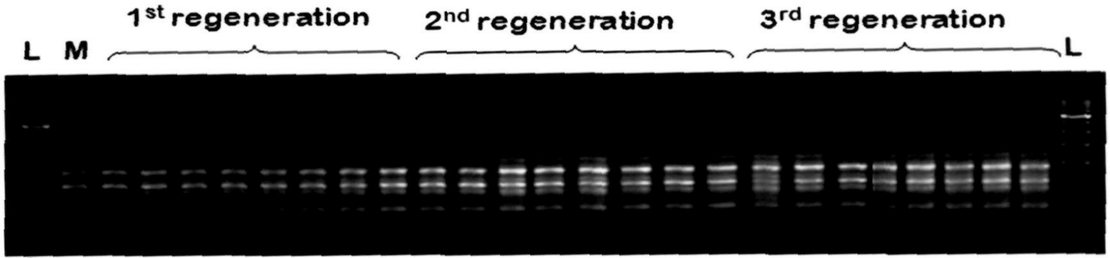


D OPH11



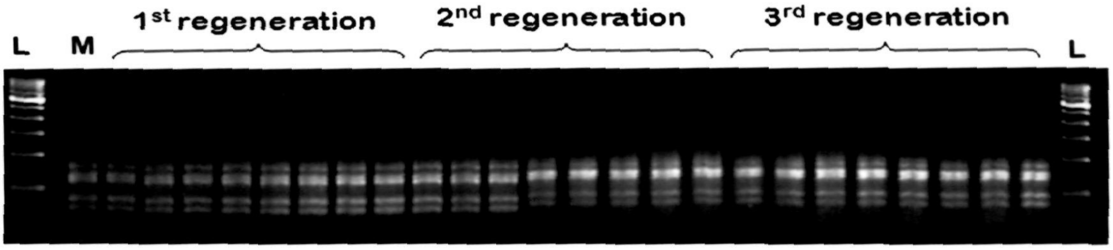
E OPK10

Fig. 6.1. RAPD profiles of *N. khasiana*. (A) OPH1, (B) OPH5, (C) OPH6, (D) OPH11, and (E) OPK 10; Lane L: 500 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.



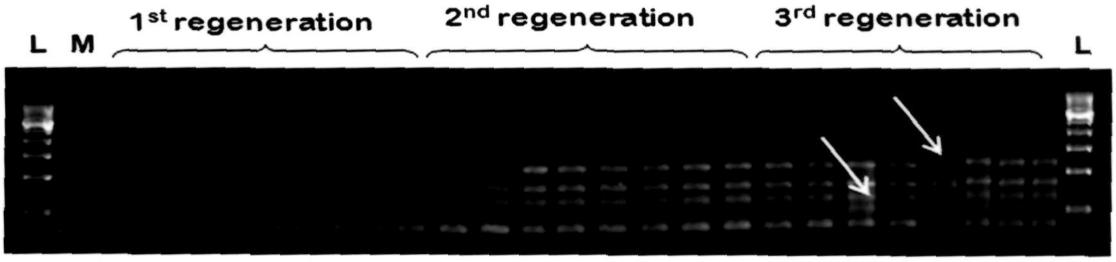
A

OPK11



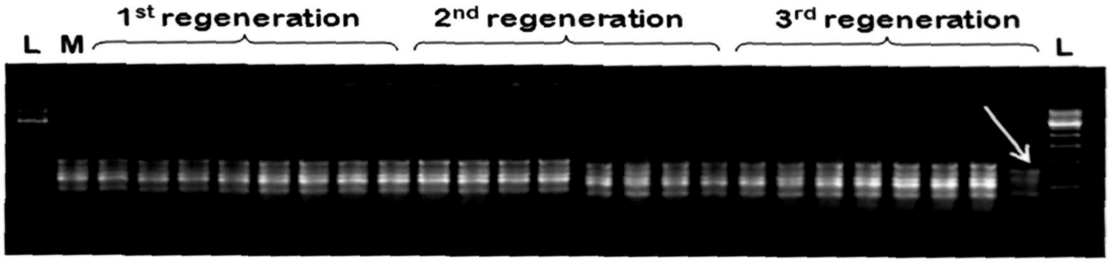
B

OPK12



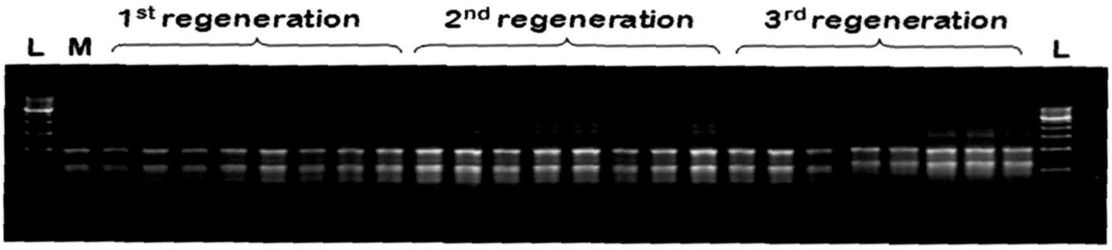
C

OPK13



D

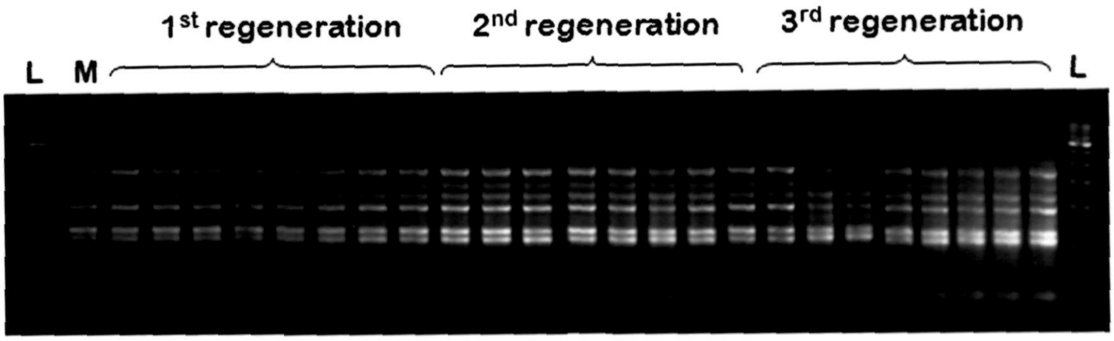
OPK14



E

OPK17

Fig. 6.2. RAPD profiles of *N. khasiana*. (A) OPK11, (B) OPK12, (C) OPK13, (D) OPK14, and (E) OPK 17; Lane L: 500 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.

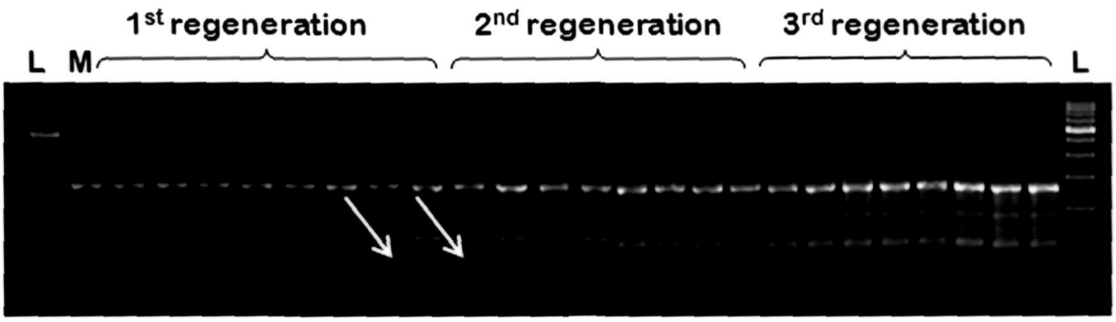


A

OPK18

B

OPK19



C

OPC7

D

OPA11

Fig. 6.3. RAPD profiles of *N. khasiana*. (A) OPK18, (B) OPK19, (C) OPC7, and (D) OPA11 Lane L: 500 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.

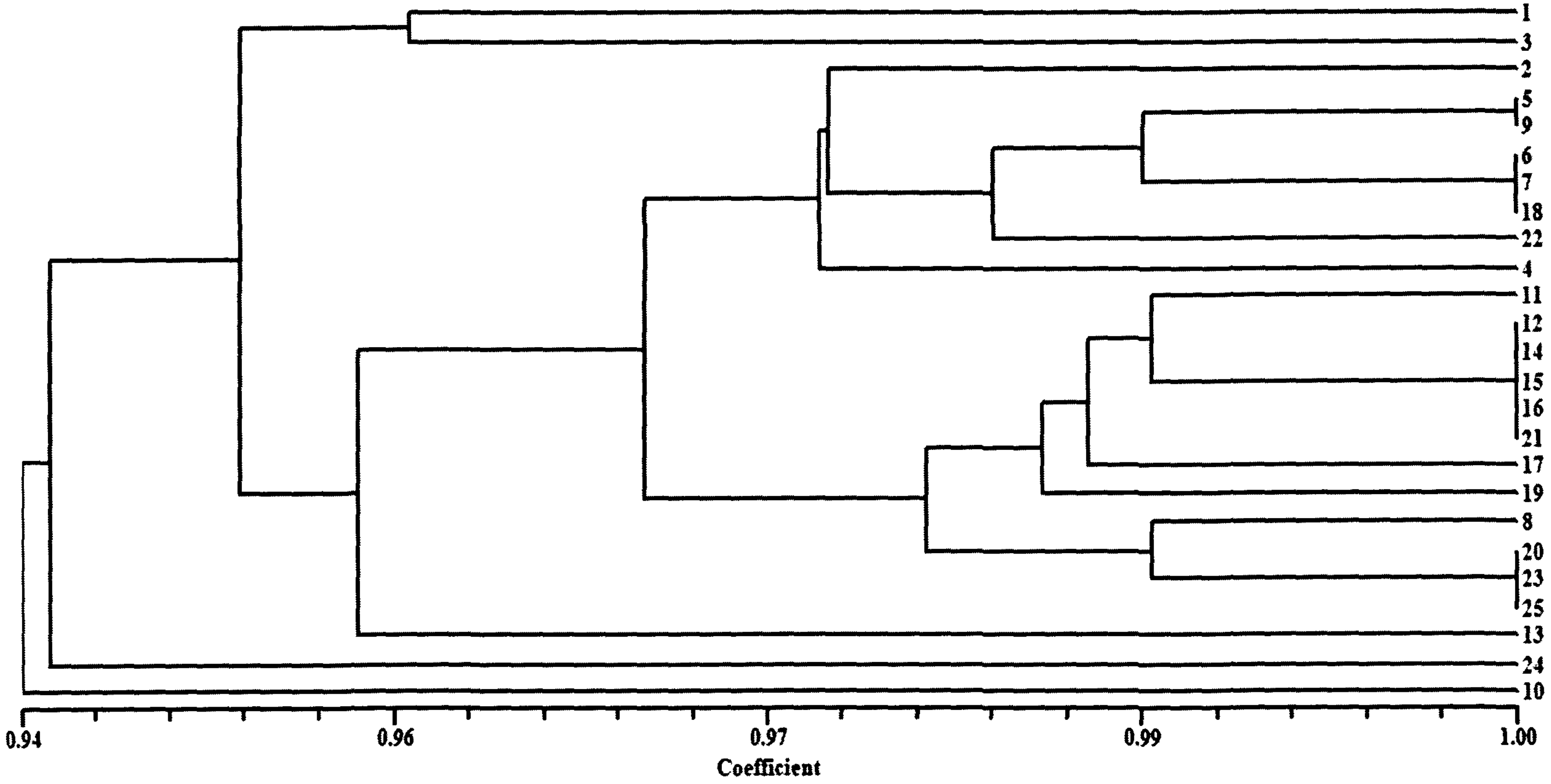


Fig. 6.4. UPGMA dendrogram generated from RAPD illustrating coefficient similarities among the micropropagated plantlets of the three consecutive regenerations and the mother plant of *N. khasiana*.

In case of ISSR, out of 36 primers, 12 primers generated a total of 50 distinct and scorable bands of which 7 bands were polymorphic (14%) with an average of 0.58 polymorphic bands per primer (Table 6.2; Fig.6.5-6.7). A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.94 to 1.00 among the micropropagated plants and the mother plant (Fig. 6.8).

In DAMD profiling, 15 primers resulted in 60 uniform and scorable bands out of which 10 fragments (16.66%) with an average of 0.66 polymorphic bands per primer (Table 6.2; Fig.6.9- 6.11). Of the three molecular markers used, DAMD revealed higher polymorphism (16.66%) in comparison to RAPD (13.51%) and ISSR (14%) respectively, across the three successive regenerations. A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.91 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6. 12).

Collectively a total of 136 primers (80 RAPD, 20 DAMD and 36 ISSR) were screened collectively and 41 primers (14 RAPD, 15 DAMD and 12 ISSR) were finally selected for further profiling (Table 6.2). The cumulative analysis was also carried out for the three molecular markers commonly regarded as SPAR in which a total of 184 fragments were produced collectively, of which 27 fragments were polymorphic (14.67%) with an average polymorphic bands of 0.65 fragments per primer. The cumulative data were also used to compute pairwise distances by Jaccard's coefficient

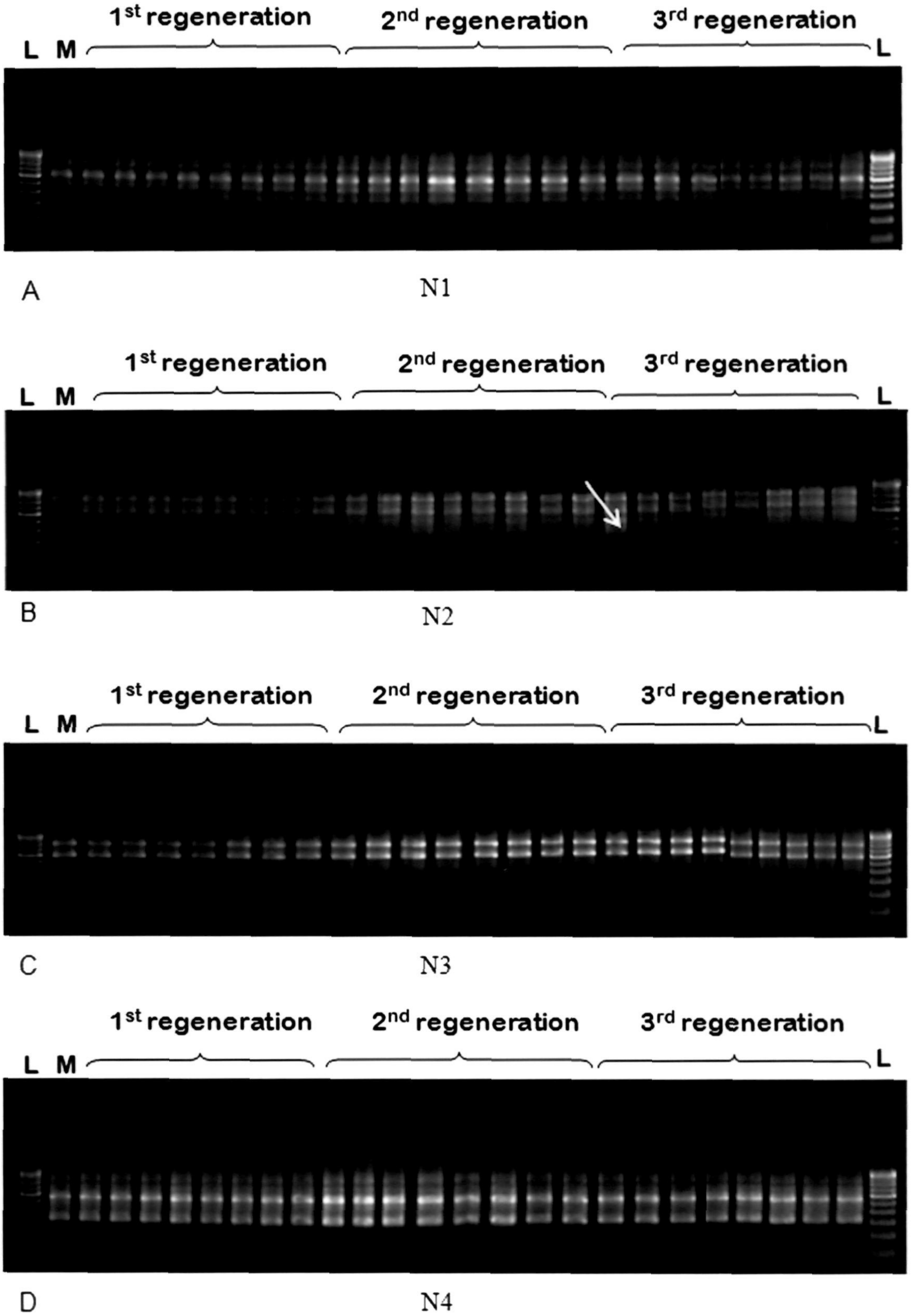


Fig. 6.5. ISSR profiles of *N. khasiana*. (A) N1, (B) N2, (C) N3, and (D) N4; Lane L: 100 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.

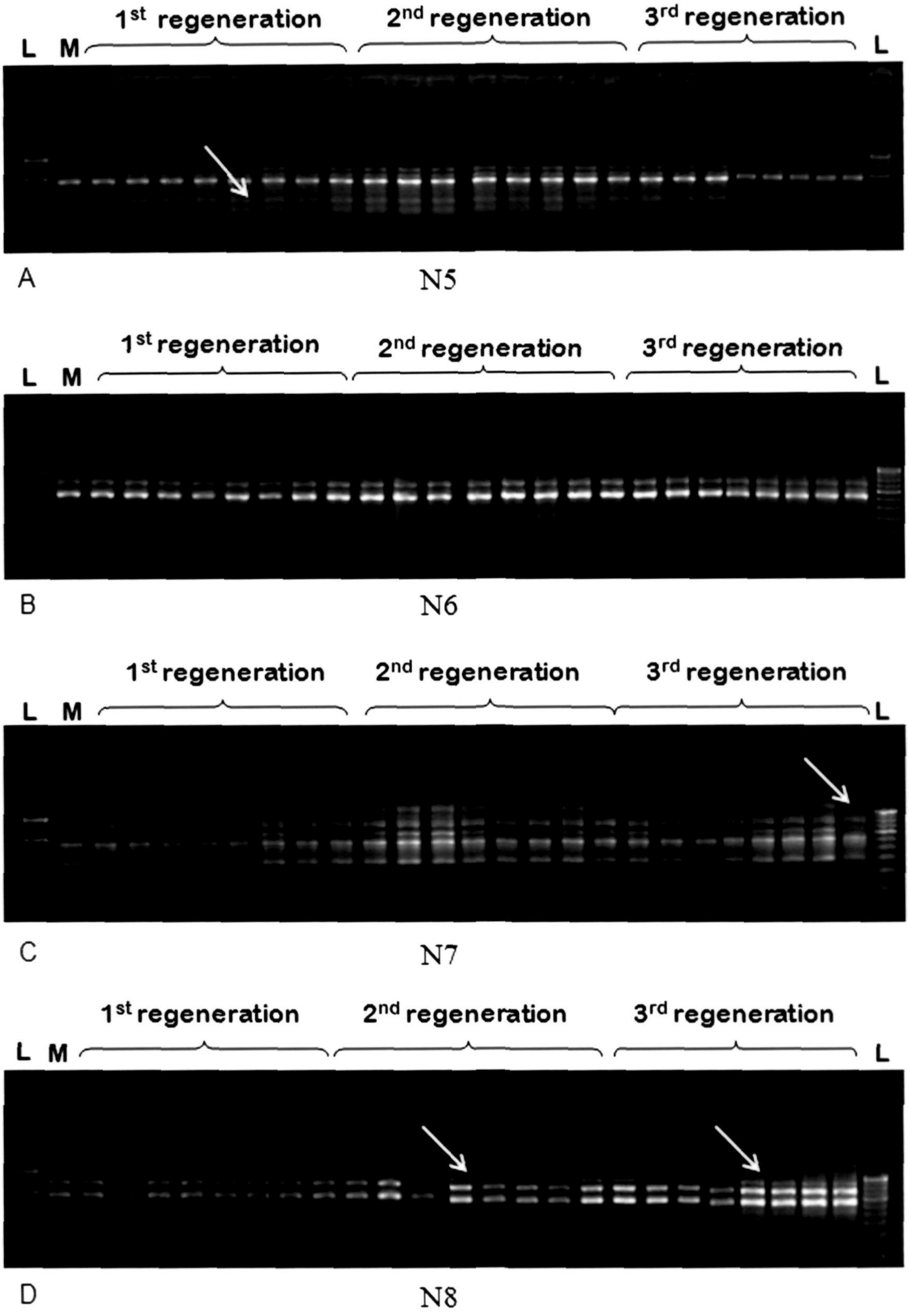


Fig. 6.6. ISSR profiles of *N. khasiana*. (A) N5, (B) N6, (C) N7, and (D) N8; Lane L: 100 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.

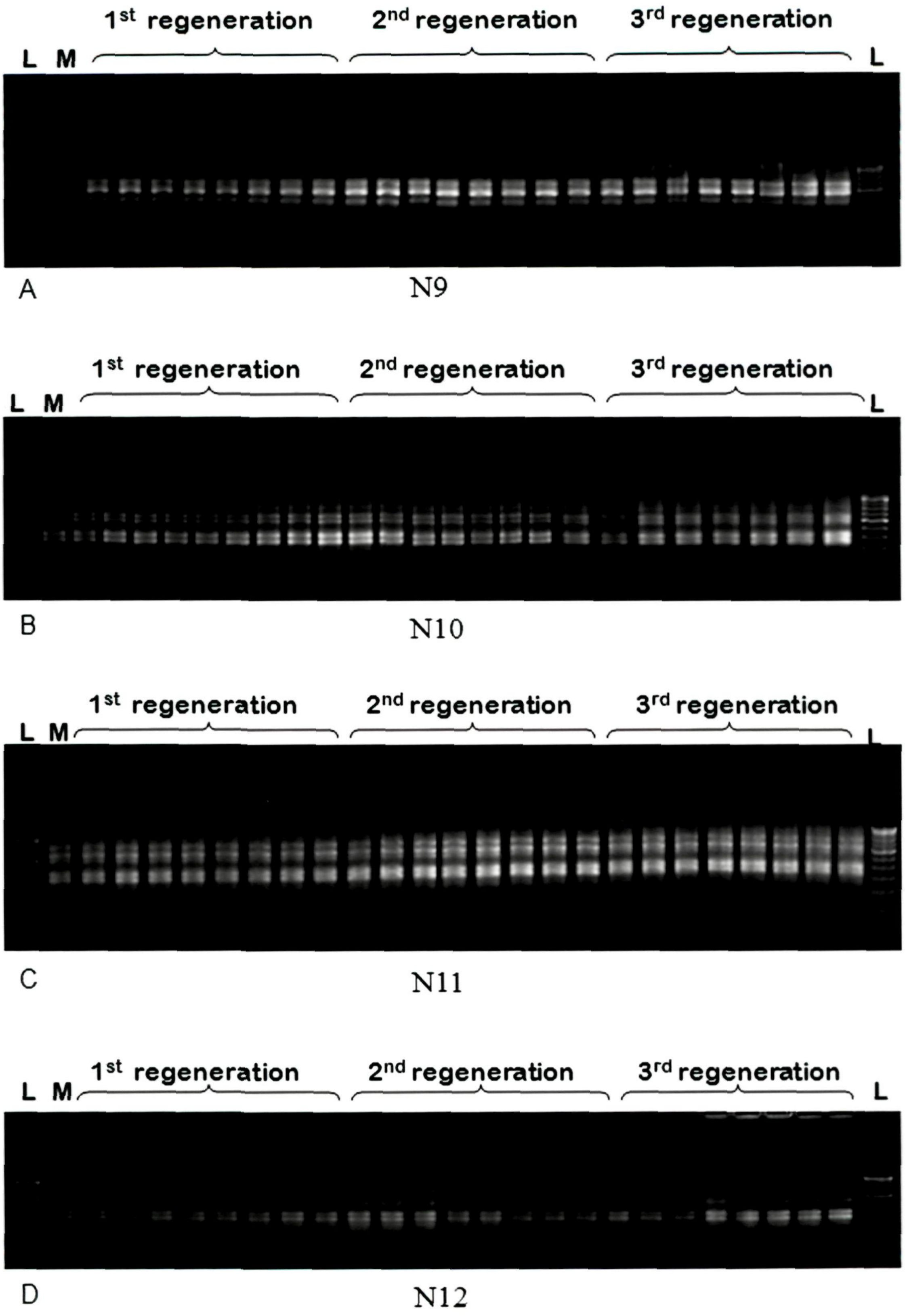


Fig. 6.7. ISSR profiles of *N. khasiana*. (A) N9, (B) N10, (C) N11, and (D) N12; Lane L: 100 bp ladder; Lane M: mother plant.

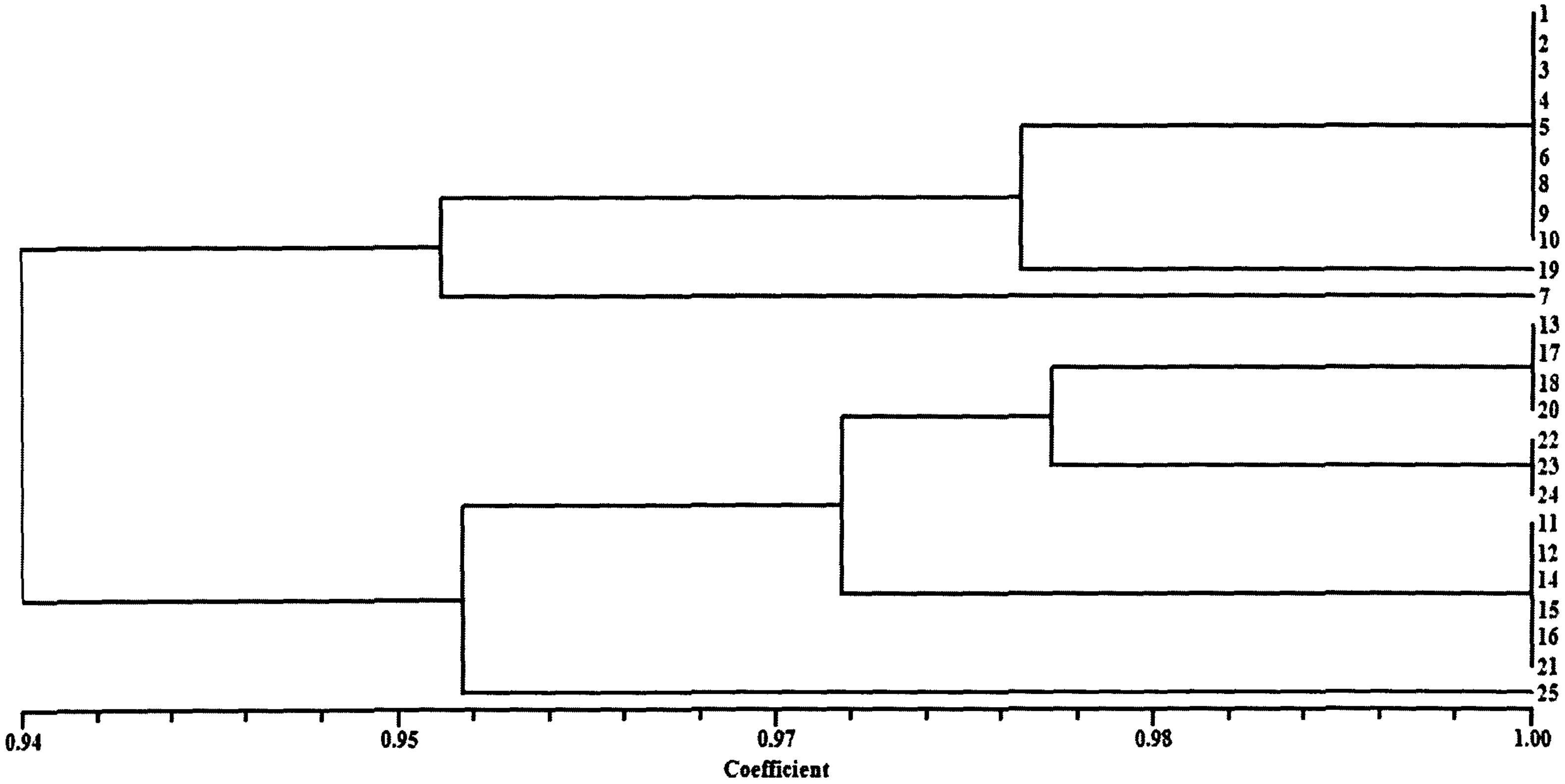
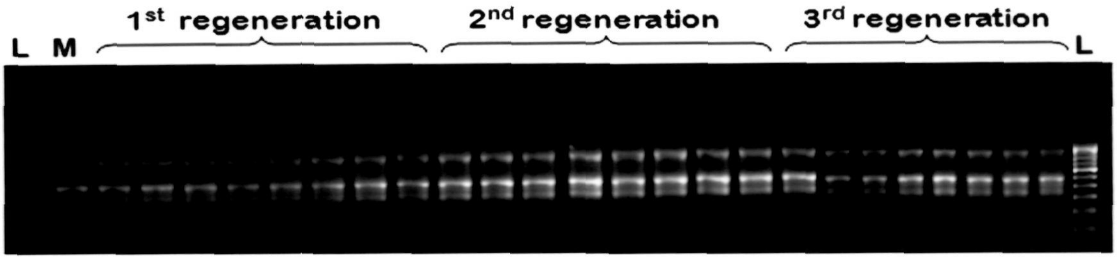
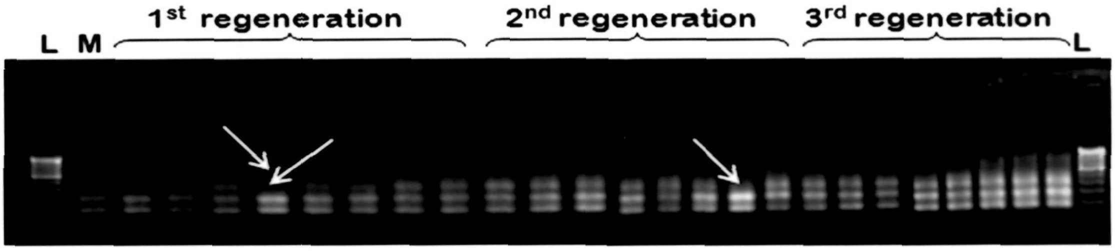


Fig. 6.8. UPGMA dendrogram generated from ISSR illustrating coefficient similarities among the micropropagated plantlets of the three consecutive regenerations and the mother plant of *N. khasiana*.



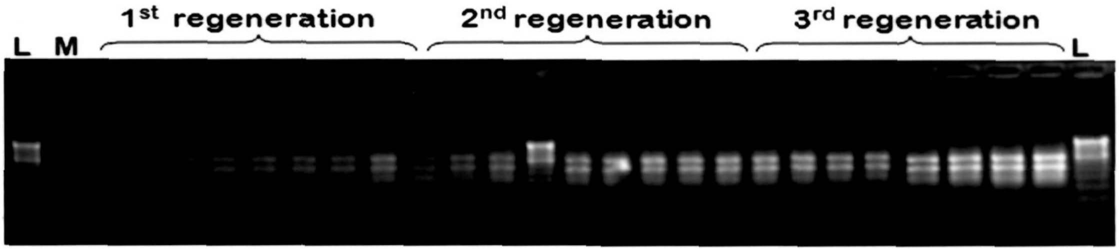
A URP1F



B URP2F

C URP2R

D URP6R



E URP9F

Fig. 6.9. DAMD profiles of *N. khasiana*. (A) URP1F, (B) URP2F, (C) URP2R, (D) URP6R, and (E) URP9F; Lane L: 100 bp ladder; Lane M: mother plant. Arrows indicate differences in the banding profiles.

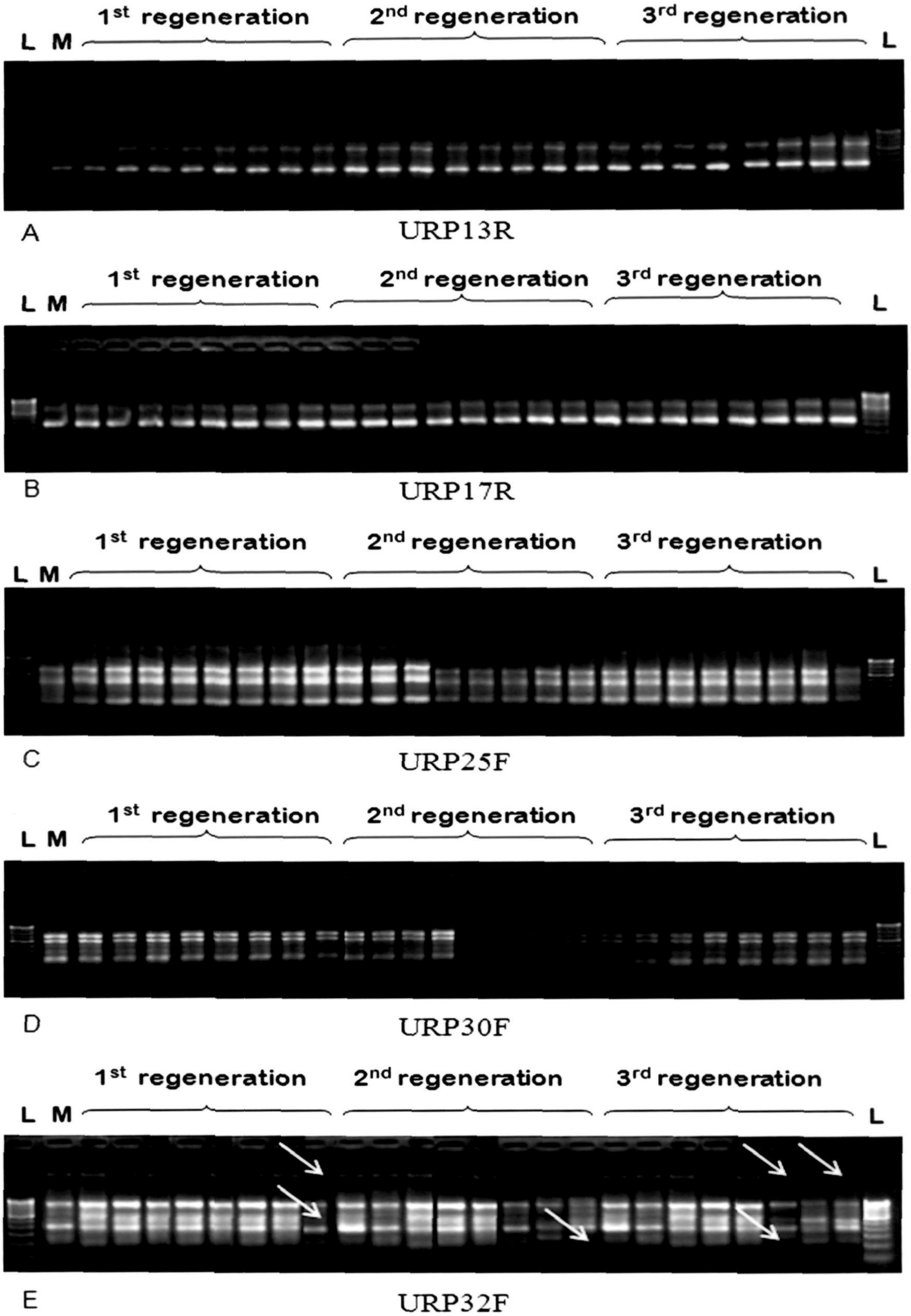
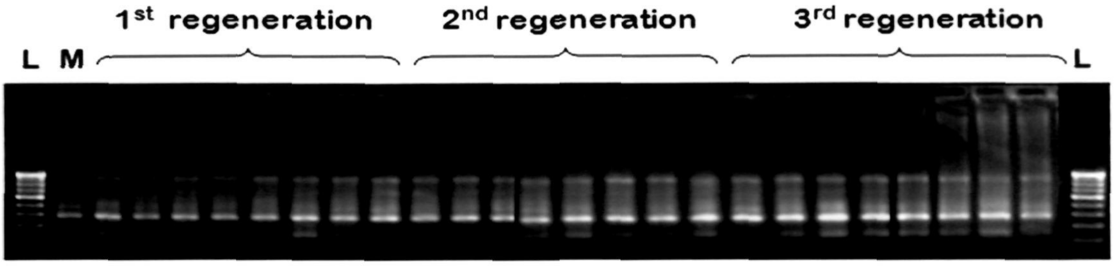
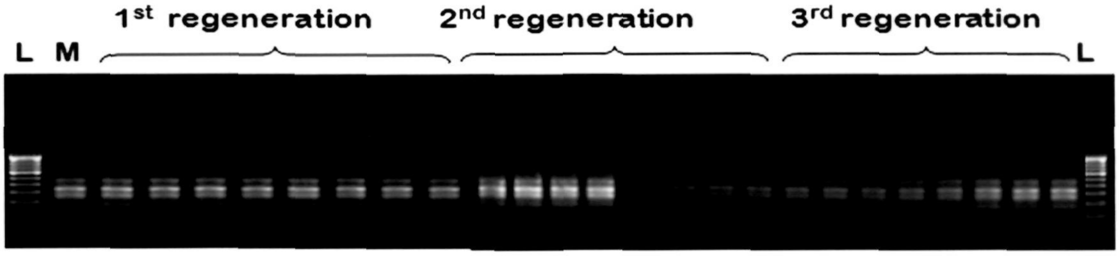


Fig. 6.10. DAMD profiles of *N. khasiana*. (A) URP13R, (B) URP17R, (C) URP25F, (D) URP30F, and (E) URP32F; Lane L: 100 bp ladder; Lane M: mother plant. Arrows indicate the differences in the banding profiles.



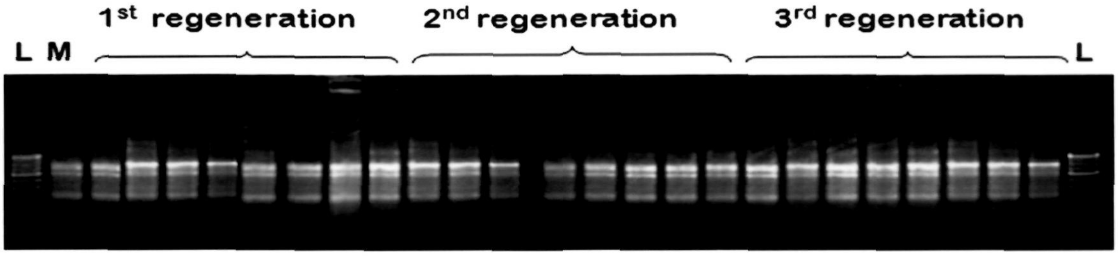
A

URP38F



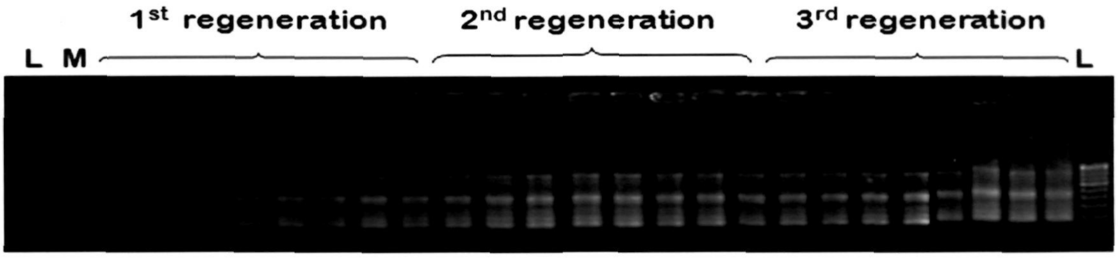
B

HBV (71845)



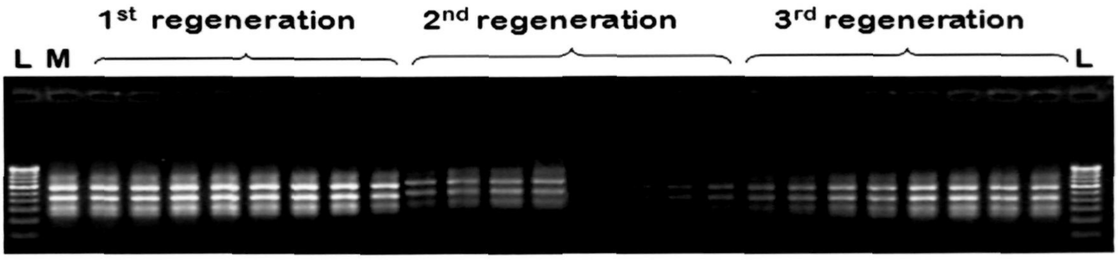
C

HVR (71846)



D

INS (71850)



E

YN73 (71851)

Fig. 6.11. DAMD profiles of *N. khasiana*. (A) URP38F, (B) HBV (71845), (C) (71846), (D) INS (71850), and (E) YN73 (71851); Lane L: 100 bp ladder; Lane M: mother plant.

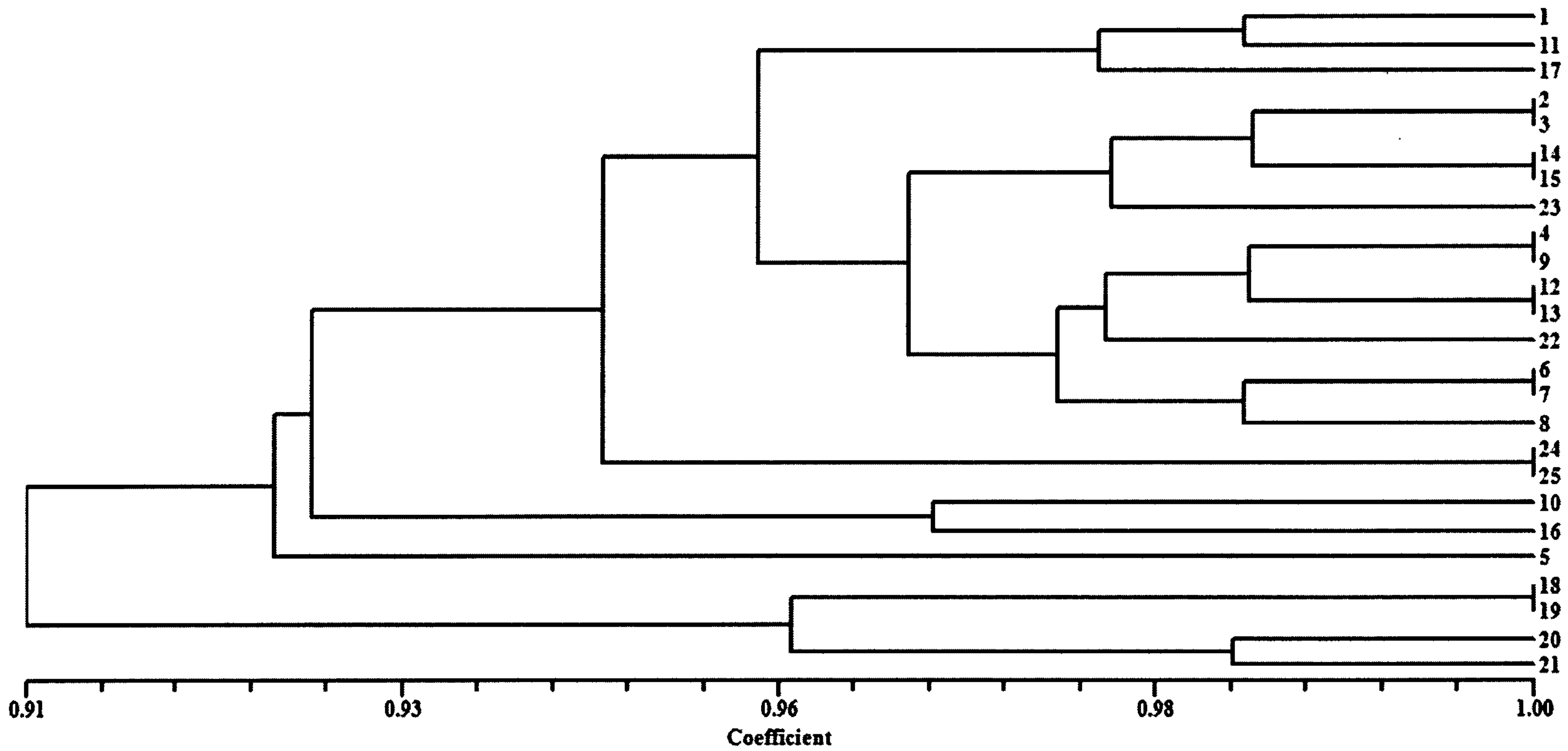


Fig.6.12. UPGMA dendrogram generated from DAMD illustrating coefficient similarities among the micropropagated plantlets of the three consecutive regenerations and the mother plant of *N. khasiana*.

which showed a distance range of 0.95-1.00 among the micropropagated plantlets and the mother plant (Fig.6.13).

6.3.3.2. SPAR analysis for the plantlets of the first regeneration

SPAR analysis for the plantlets of the first regeneration is shown in Table 6.3. A total of 80 RAPD primers were used for initial screening out of which 14 primers resulted in 72 clear, well-separated and reproducible fragments of which 3 were polymorphic (4.1%) with an average of 0.21 polymorphic bands per primer (Fig. 6.1-6.3). A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.98 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6.14a).

In case of ISSR profiling, out of 36 primers screened, 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. 6.5-6.7). A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.96 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6.14b).

Out of 20 DAMD primers screened, 15 primers resulted in 59 clear and scorable bands of which 5 bands were polymorphic (8.47%) with an average of 0.33 polymorphic bands per primer (Fig. 6.9-6.11). A dendrogram generated by cluster analysis using the UPGMA method based on Jaccard's coefficient indicated genetic similarity ranging from 0.94 to 1.00 among the micropropagated plantlets and the mother plant (Fig.6.14c).

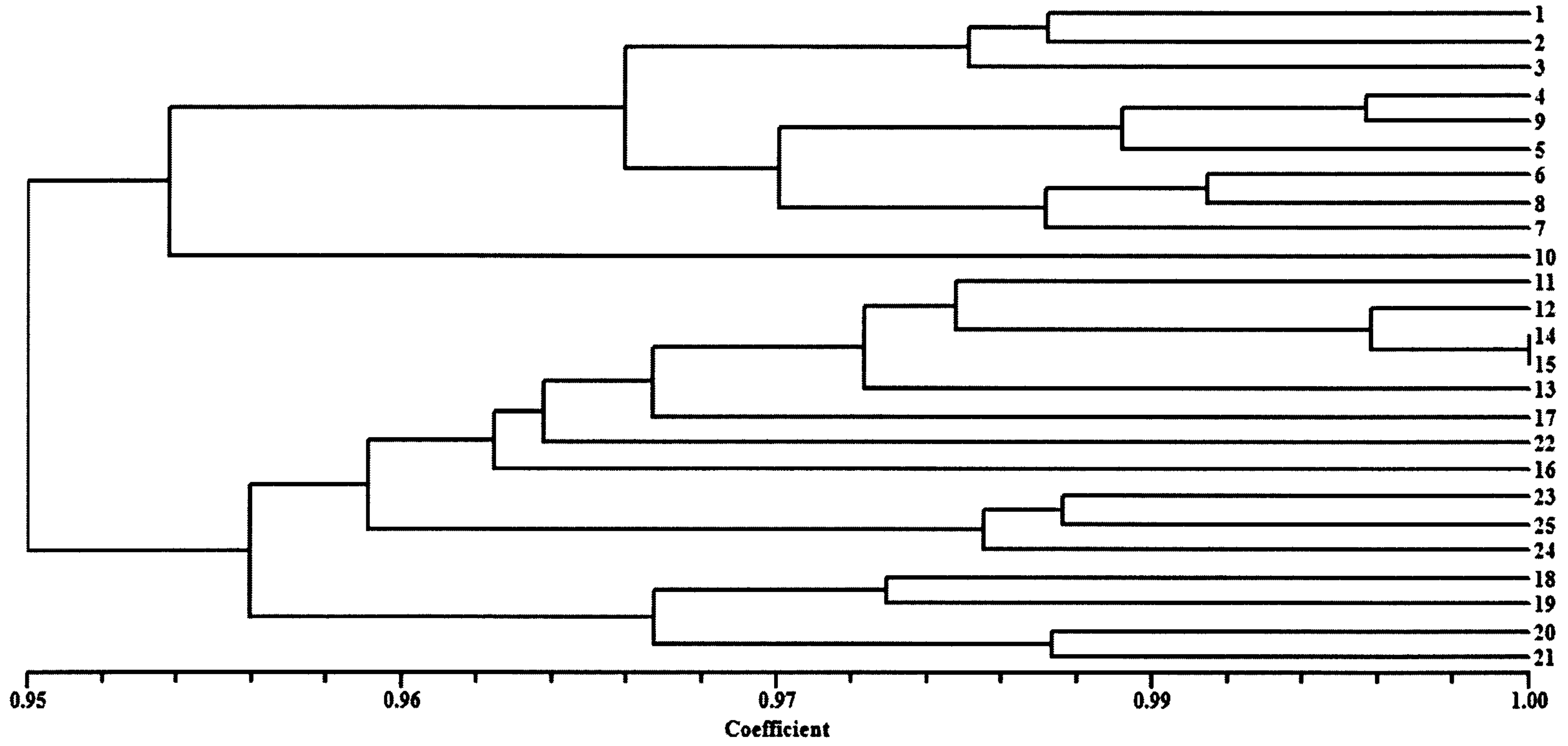


Fig. 6.13. UPGMA dendrogram generated from SPAR illustrating coefficient similarities among the micropropagated plantlets of the three consecutive regenerations and the mother plant of *N. khasiana*.

Table 6.3. SPAR analysis for the plantlets of the first regeneration of *N. khasiana*

Sl. No.	SPAR approach	No. of primer used	Total bands amplified	Average bands/ primer	Total no. of polymorphic bands	Average no. of polymorphic bands/ primer	% of polymorphism	Distance range (Jaccard's Coefficient)
1.	RAPD	14	72	5.10	3	0.21	4.10	0.98-1.00
2.	ISSR	12	46	3.80	2	0.16	4.30	0.96-1.00
3.	DAMD	15	59	3.94	5	0.33	8.47	0.94-1.00
4.	RAPD+ISSR +DAMD	41	177	4.31	10	0.24	5.65	0.98-0.99

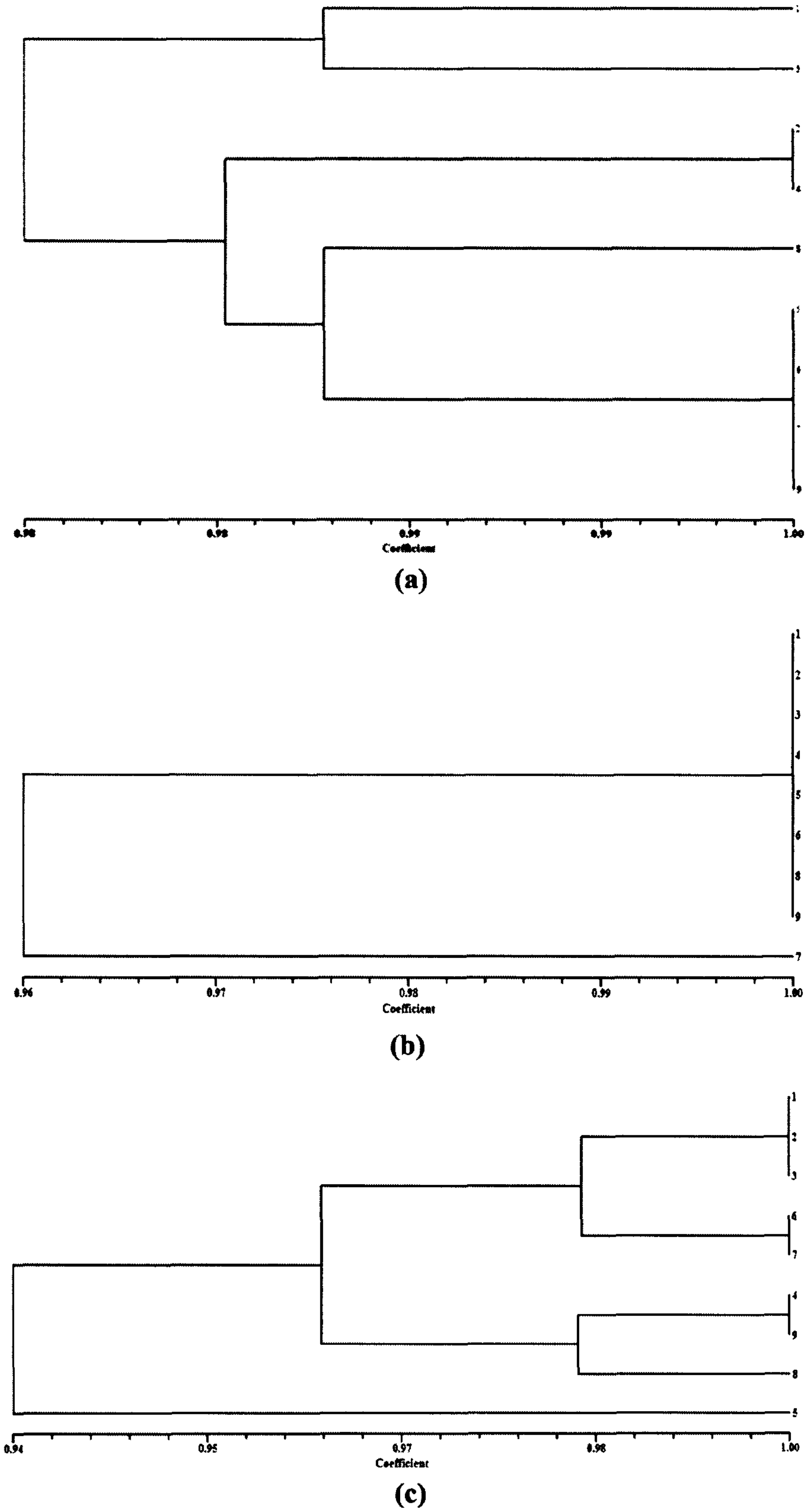


Fig. 6.14. UPGMA dendrogram illustrating coefficient similarities among the micropropagated plantlets of the first regeneration and the mother plant of *N. khasiana*: (a) RAPD; (b) ISSR; and (c) DAMD.

Cumulative dataset was used to estimate the efficiency of SPAR to determine genetic variation between the micropropagated plantlets of the first regeneration and the mother plant of *N. khasiana*. Out of 177 fragments produced collectively, 10 bands were polymorphic (5.65%) with an average polymorphic band of 0.24 polymorphic bands per primer (Table 6.3). The cumulative data were also used to compute pairwise distances by Jaccard's coefficient which showed a distance range of 0.98-0.99 among the micropropagated plantlets and the mother plant (Fig. 6.15).

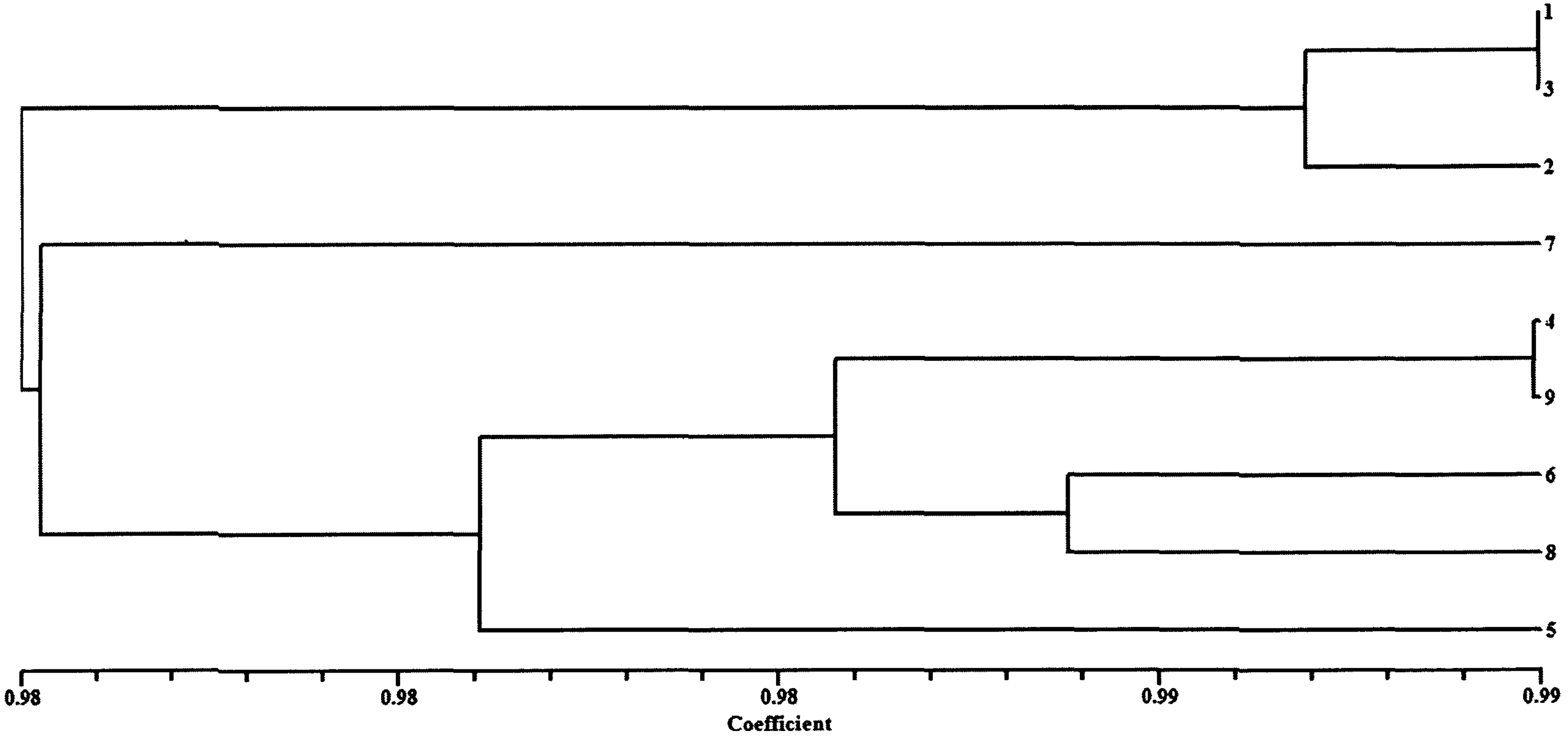


Fig.6.15. UPGMA dendrogram generated from SPAR illustrating coefficient similarities among the micropropagated plantlets of the first regeneration and the mother plant of *N. khasiana*.

6.3.3.3. SPAR analysis for the plantlets of the second regeneration

SPAR analysis for the plantlets of the second regeneration is presented in Table 6.4. Fourteen RAPD primers resulted in 72 clear, well-separated and reproducible fragments of which 5 fragments were polymorphic (6.9%) exhibiting an average polymorphic bands of 0.28 per primer (Fig. 6.1-6.3). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.96-1.00 among the micropropagated *plantlets and the mother plant (Fig. 6.16a).*

In case of ISSR profiling, 12 primers generated a total of 49 fragments of which 3 bands were polymorphic (6.1%) with an average polymorphic band of 0.25 per primer (Fig. 6.5-6.7) and showed a genetic distance of 0.95-1.00 among the micropropagated plantlets and the mother plant (Fig. 6.16b).

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. 6.9- 6.11). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.92 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6.16c).

Out of 180 fragments produced collectively in second regeneration, 14 fragments were polymorphic (7.77%) with an average polymorphic band of 0.34 fragments per primer (Table 6.4). The cumulative data were also used to compute pairwise distances by Jaccard's coefficient which showed a distance range of 0.96-1.00 among the micropropagated plantlets and the mother plant (Fig. 6.17).

Table 6.4. SPAR analysis for the plantlets of the second regeneration of *N. khasiana*

Sl. No.	SPAR approach	No. of primer used	Total bands amplified	Average bands/ primer	Total no. of polymorphic bands	Average no. of polymorphic bands/ primer	% of polymorphism	Distance range (Jaccard's Coefficient)
1.	RAPD	14	72	5.10	3	0.21	4.10	0.98-1.00
2.	ISSR	12	46	3.80	2	0.16	4.30	0.96-1.00
3.	DAMD	15	59	3.94	5	0.33	8.47	0.94-1.00
4.	RAPD+ISSR +DAMD	41	177	4.31	10	0.24	5.65	0.98-0.99

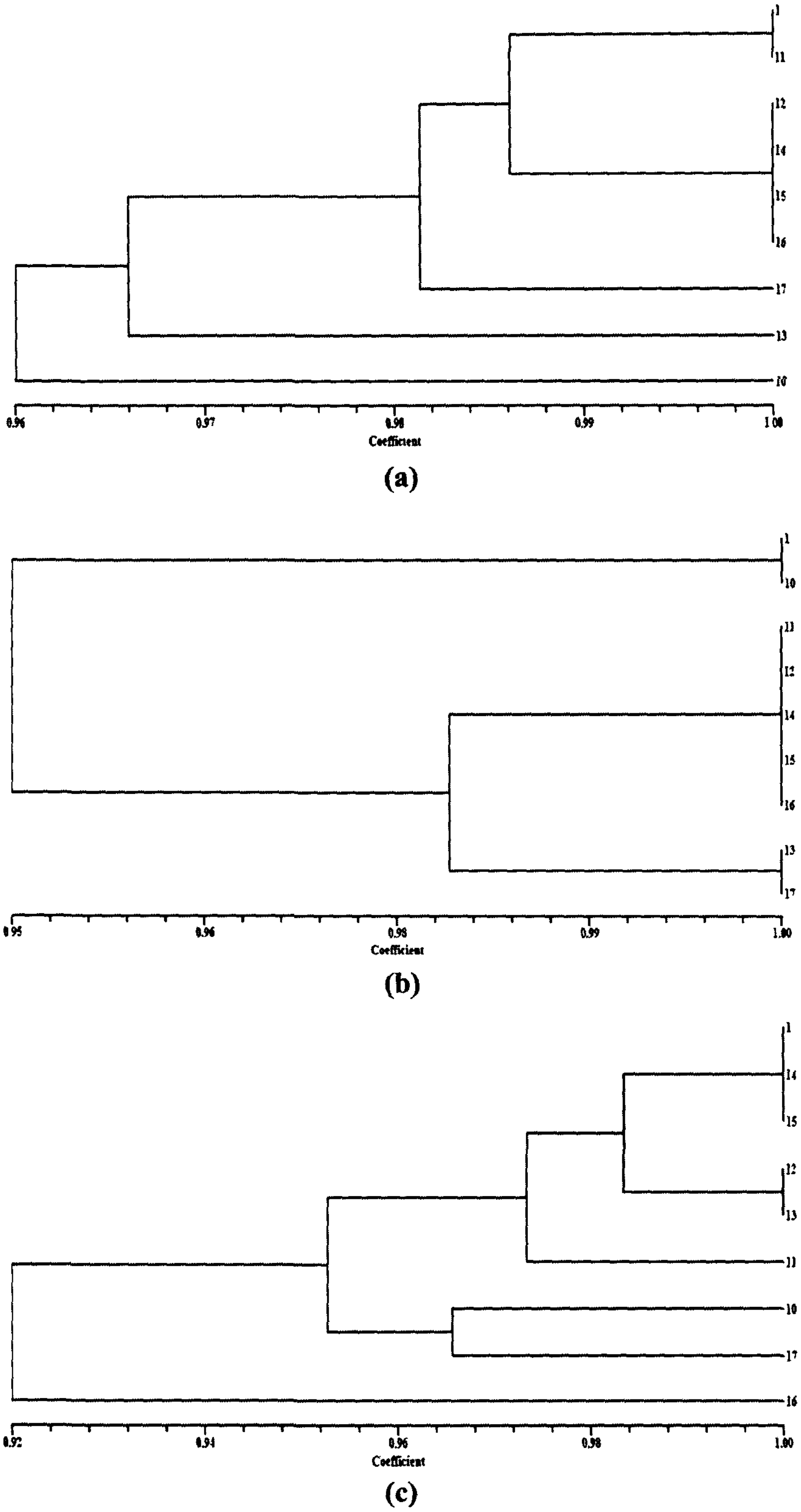


Fig. 6.16. UPGMA dendrograms illustrating coefficient similarities among the micropropagated plantlets of the second regeneration and the mother plant of *N. khasiana*: (a) RAPD; (b) ISSR; and (c) DAMD.

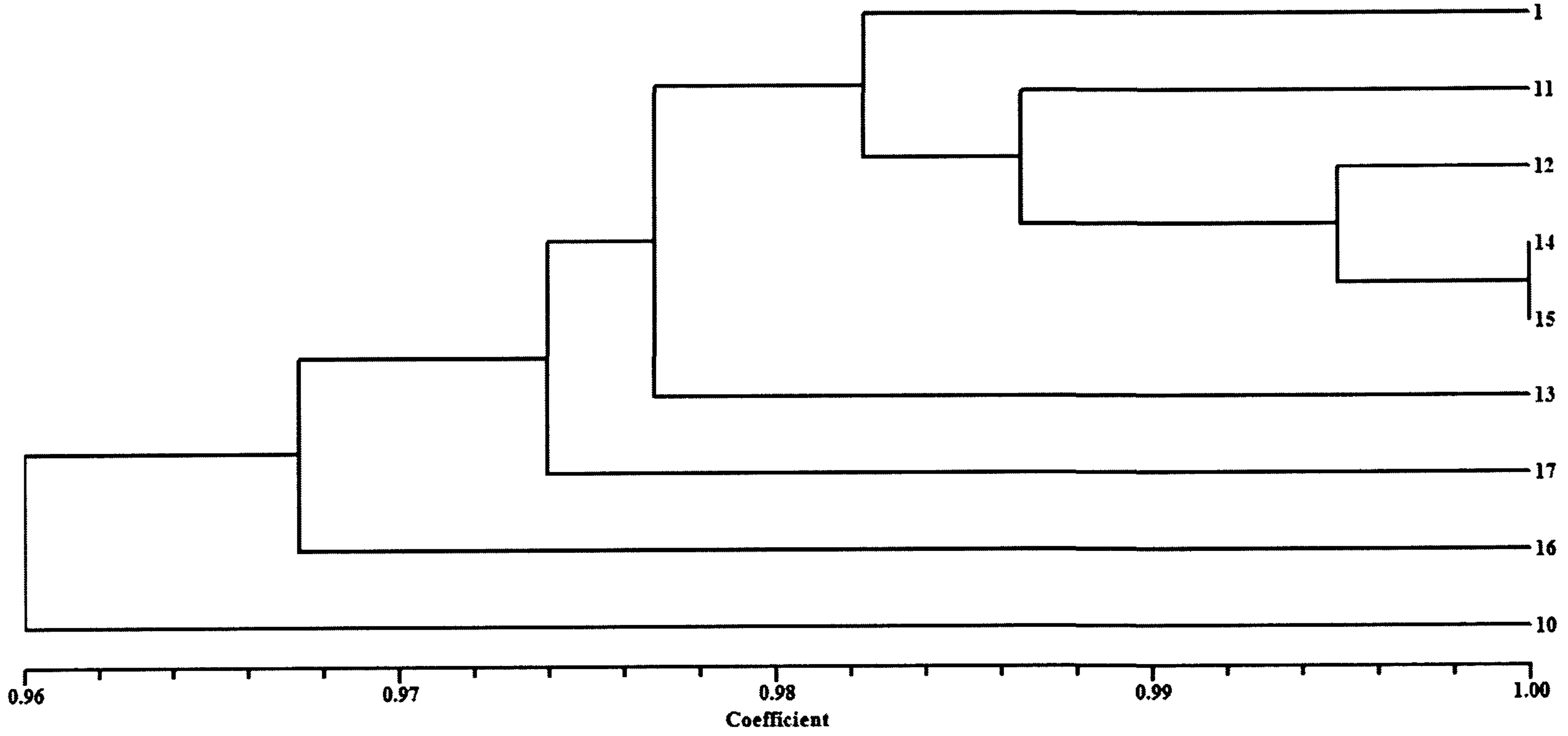


Fig. 6.17. UPGMA dendrogram generated from SPAR illustrating coefficient similarities among the micropropagated plantlets of the second regeneration and the mother plant of *N. khasiana.*

6.3.3.4. SPAR analysis for the plantlets of the third regeneration

SPAR analysis for the plantlets of the third regeneration is summarized in Table 6.5. Out of 74 scorable bands produced in case of RAPD, 7 bands were polymorphic (9.4%) with an average of 0.5 polymorphic bands per primer (Fig. 6.1-6.3). The genetic distance among the micropropagated plantlets and the mother plant recorded was 0.95-1.00 (Fig. 6.18a).

Twelve ISSR primers generated a total of 50 fragments of which 5 fragments were polymorphic (10%) with an average of 0.41 polymorphic bands per primer (Fig. 6.5-6.7). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.94 to 1.00 among the micropropagated plantlets and the mother plant (Fig. 6.18b).

In 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. 6.9-6.11). The genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.91 to 1.00 (Fig. 6.18c).

A total of 184 bands were generated collectively of which 20 fragments were polymorphic (10.87%) with an average of 0.48 polymorphic bands per primer (Table 6.5), and genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.95 to 1.00 (Fig. 6.19).

Table 6.5. SPAR analysis for the plantlets of the third regeneration of *N. khasiana*

Sl. No.	SPAR approach	No. of primer used	Total bands amplified	Average bands/ primer	Total no. of polymorphic bands	Average no. of polymorphic bands/ primer	% of polymorphism	Distance range (Jaccard's Coefficient)
1.	RAPD	14	74	5.28	7	0.50	9.40	0.95-1.00
2.	ISSR	12	50	4.16	5	0.41	10.00	0.94-1.00
3.	DAMD	15	60	4.00	8	0.53	13.33	0.91-1.00
4.	RAPD+ISSR +DAMD	41	184	4.48	20	0.48	10.87	0.95-1.00

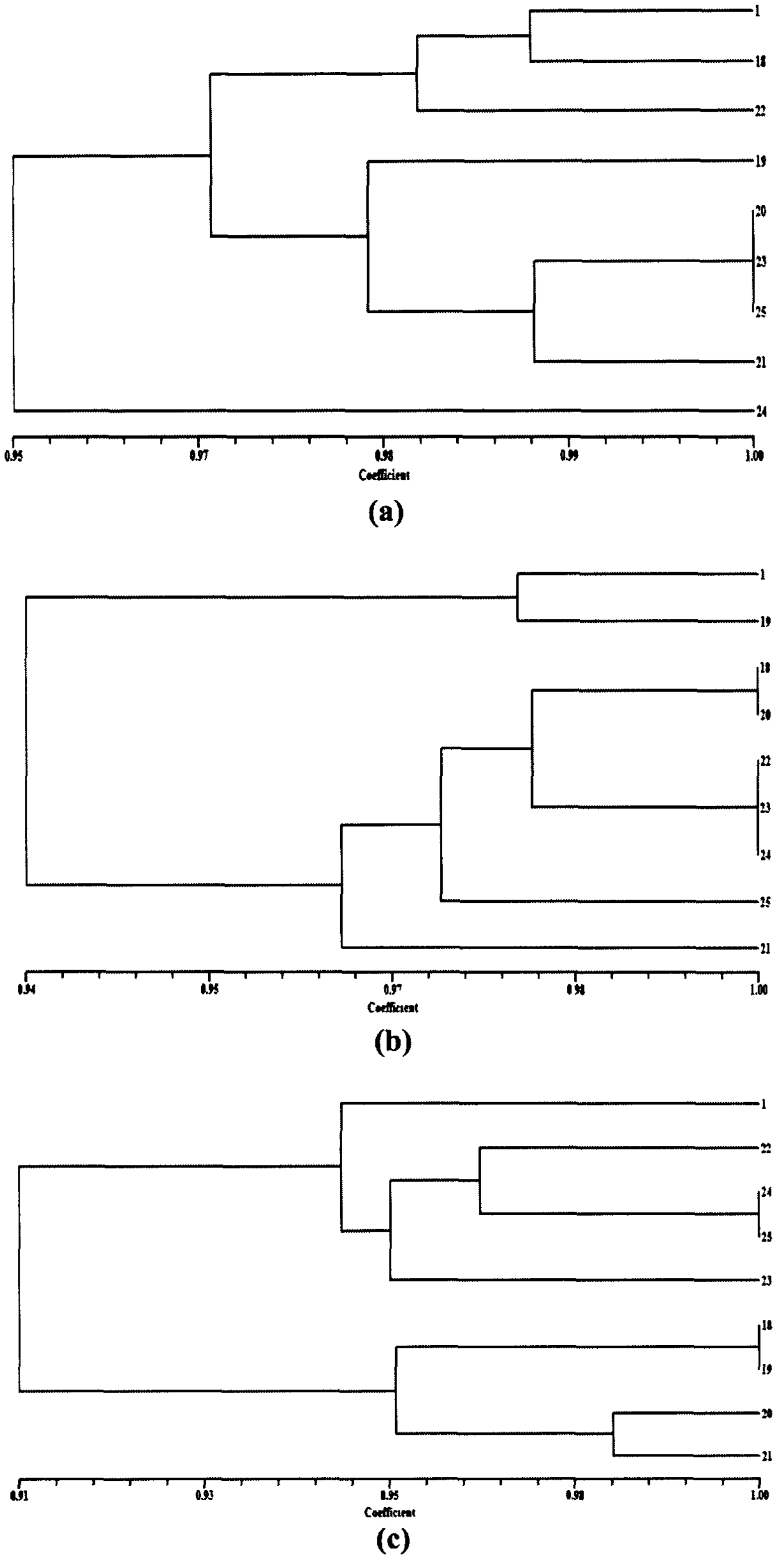


Fig. 6.18. UPGMA dendrograms illustrating coefficient similarities among the micropropagated plantlets of the third regeneration and the mother plant of *N. khasiana*: (a) RAPD; (b) ISSR; and (c) DAMD.

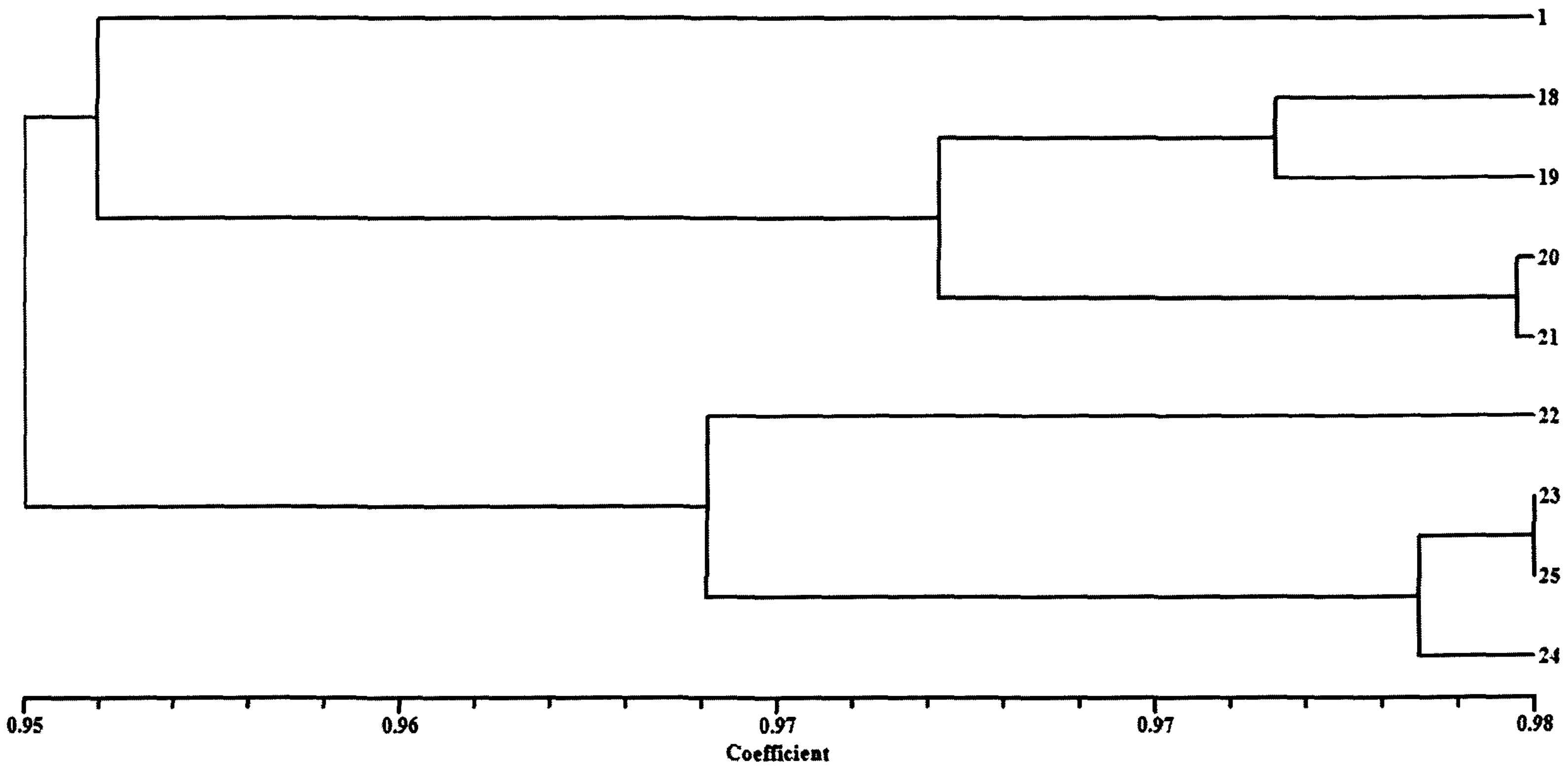


Fig. 6.19. UPGMA dendrogram generated from SPAR illustrating coefficient similarities among the micropropagated plantlets of the third regeneration and the mother plant of *N. khasiana*.

The efficiency of the molecular markers was also compared for assessing the genetic fidelity in the micropropagated plantlets of *N. khasiana* in the three successive regenerations. It was observed that DAMD markers detected higher polymorphism of 8.47% in the plantlets of the first regeneration which was subsequently increased to10.10% and 13.33% in the plantlets of the second and the third regenerations respectively, as compared to RAPD (4.1%, 69% and 9.4% in the plantlets of the first, the second and the third regenerations respectively) and ISSR (4.3%, 6.1% and 10% in the plantlets of the first, the second and the third regenerations respectively) (Table 6.6). However, collective data regarded as SPAR approach detected polymorphism of 4.65% in the plantlets of the first regeneration which was subsequently increased to 7.77% and 10.87% in the plantlets of the second and the third regenerations respectively.

Table 6.6. Comparision of molecular methods for assessing genetic fidelity in the micropropagated plantlets of the three successive regenerations of *N. khasiana*

Sl. No.	Regenera-tions	RAPD		ISSR		DAMD		SPAR	
		% of poly-morphism	Jaccard's Co-efficient range	% of poly-morphism	Jaccard's Co-efficient range	% of poly-morphism	Jaccard's Co-efficient range	% of poly-morphism	Jaccard's Co-efficient range
1.	1st	4.1	0.98-1.00	4.3	0.96-1.00	8.47	0.94-1.00	4.65	0.98-1.00
2.	2nd	6.9	0.96-1.00	6.1	0.95-1.00	10.10	0.92-1.00	7.77	0.96-1.00
3.	3rd	9.4	0.95-1.00	10.0	0.94-1.00	13.33	0.91-1.00	10.87	0.95-1.00

6.4 Discussion

Molecular variation in tissue culture-derived plants has been characterized both at DNA and protein level. Variation at the DNA level has been most extensively studied using restriction enzyme analysis. The work by Botstein *et al.* (1980) on the construction of genetic maps using RFLP was the first reported molecular marker technique in the detection of DNA polymorphism. Somaclonal variations are 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 (Kaeppler *et al.* 2000). The variations generated during tissue cultures are reported to be generally the consequences of chromosomal rearrangements and single gene mutations (Phillips *et al.* 1994). These may also be caused by the activation of transposable DNA hypomethylation elements, genome adaptation to different regulatory microelements and the presence of hot spots (Hirochika *et al.* 1996; Jaligot *et al.* 2000; Linacero *et al.* 2000; Lukens and Zhan 2007). As mentioned earlier, the sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variations (Martin *et al.* 2004).

For DNA isolation, the modified CTAB method described by Porebski *et al.* (1997) is generally used for plants rich in polysaccharides and polyphenols as it ensures uniformity and purity of the isolated DNA samples. This method has been successfully employed for extraction of DNA in case of plants such as *Helianthus*, *Gossypium*, *Triticum, Rheum*, *Rosa* (Horne *et al.* 2004; Hameed *et al.* 2004; Hu *et al.* 2009;

Jabbarzadeh *et al.* 2009). Standardization of optimum conditions for PCR is necessary to generate consistent and analyzable amplification products. The temperature profile, concentrations of DNA polymerase, $MgCl_2$, primer and template DNA can affect the reproducibility of SPAR analysis (Macpherson *et al.* 1993; Meunier and Grimont 1993). The concentration of genomic DNA is considered to be crucial for any DNA-based molecular marker analysis; different plants require different concentrations of DNA depending on the quality as well as quantity of the isolated DNA samples. Several workers have reported DNA concentrations ranging from 1-50 ng per 25 µl reaction volume to be optimal for generating reproducible bands. In the present investigation, DNA concentration of 30 ng per 25 µl reaction volume was found to be optimum in most of the plants. Virk *et al.* (1995) used as little as 1 ng DNA for RAPD analysis of rice germplasm whereas Karihaloo *et al.* (1995) used 25 ng DNA for the same analysis in *Solanum melongena.* On the other hand, Lescuyer *et al.* (1997) had to use as high as 100 ng DNA per 50 µl reaction volume for genetic analysis in case of *Plasmodium falciparum.*

The number of primers required is dependent on the extent of polymorphism detected per primer and the degree of variation between the genotypes and accessions to be investigated (Cao *et al.* 1998). It has been reported that the primer base composition influences amplification strength (Williams *et al.* 1990) and the total G + C content has been shown to be positively correlated with ability and strength of amplification (Fritsch *et al.* 1993). The number of bands (2-9) produced in the present study was advantageous as it reduced the chances of unpredictable level of homoplasy caused by co-migration of non-homologous DNA fragments (Thorman *et al.* 1994; Smith *et al.* 1994).

In the present investigation, RAPD marker could detect polymorphism of 4.1% in the micropropagated plantlets of the first regeneration of *N. khasiana* which was subsequently increased to 6.9% and 9.4% in the plantlets of the second and the third regenerations. The use of RAPD markers for assessing the genetic fidelity in micropropagated plants regenerated from explants has been reported in several plants (Hashmi *et al.* 1997; Watanabe *et al.* 1998; Mondal and Chand 2002; Bindiya and Kanwar 2003; Rady 2006; Goel *et al.* 2009; Santos *et al.* 2008; Bhowmik *et al.* 2009). However, in several other instances, no genetic variation could be detected using RAPD as molecular marker (Rani and Raina 2000; Rout and Das 2002; Gaafar and Sakar 2006; Mallon *et al.* 2010; Swarna and Ravindhran 2012; Goswami *et al.* 2013). Also, no polymorphism in RAPD markers was observed between plants propagated *in vitro* and donor plants of *Anethum graveolens* (Jana and Shekhawat 2011). Whether this is due to the lack of variation or detection method that is sensitive enough still is a debatable issue. However, such analysis examines only a fraction of the total genome, therefore, DNA fragment polymorphic profiles might not have been able to detect genetic variability but more probably stability in particularly selected sequences (Harding 2004). However, ISSR marker has proven to be much more efficient in assessing the genetic integrity among clonally propagated plants as reported by many workers in different plant species (Zietekiewicz *et al.* 1994; Bhatia *et al.* 2009, 2011; Mohanty *et al.* 2010). Moreover, the long length of ISSR primers (15-30 mers) as against RAPDs (10 mers) permits the use of high annealing temperatures and thus leads to higher stringency. Martins *et al.* (2004) reported genetic homogeneity using ISSR marker in almond plantlets regenerated through axillary branching after 4-6 years of *in vitro* multiplication. Homogeneity in amplification profiles was also reported for all the micropropagated plantlets in *Swertia chirayita* through ISSR marker assay by Joshi and Dhawan (2007). However, in the study on *N. khasiana*, ISSR markers detected somaclonal variation of 4.3% in the plantlets of the first regeneration which was subsequently increased to 6.1% and 10% in the plantlets of the second and the third regenerations respectively. Guo *et al.* (2006) have successfully employed ISSR markers in detecting genetic variation of 10.62% in micropropagated plants of *Robinia pseudoacacia.* Genomic variations of 2.73% were observed in *in vitro*-raised plantlets of *Ochreinauclea missionis* (Chandrika and Rai 2009). The application of ISSR markers for the analysis of genetic fidelity of micropropagated plants has been well exemplified in *Brassica*, *Musa*, *Dictyospermum*, *Bambusa*, *Psidium*, and *Artemisia* (Leroy *et al.* 2000; Venkatachalam *et al.* 2007; Chandrika *et al.* 2008; Negi and Saxena 2010; Liu and Yang 2012; Khan *et al.* 2013). Using DAMD marker, 8.47% polymorphism in the plantlets of the first regeneration could be detected in *N. khasiana.* Subsequently, the polymorphism increased to 10.10% and 13.33% in the plantlets of the second and the third regenerations respectively. In DAMD PCR, minisatellite sequences used as primers are longer than RAPD and ISSR-PCR primers and, therefore, it can be effectively carried out at relatively high stringency reactions, thus yielding reproducible DNA markers (Karaca and Ince 2008). To date DAMD technique has been successfully used to establish phylogenetic relationships and genetic diversity in several plants (Bebeli *et al.* 1997; Zhou *et al.* 1997; Ince *et al.* 2009; Hu *et al.* 2011; Ince and Karaca 2012; Kumar and Nair 2013), however, information on application of DAMD marker in determination of genetic fidelity in tissue culture-raised plants is limited.

In general, the use of one type of molecular marker to assess the stability of *in vitro* propagated plants may be insufficient. Therefore, several authors have suggested the use of multiple molecular markers to study somaclonal variations in regenerants of several plant species. In *Actinidia deliciosa* cultures, a relatively low level of polymorphism was detected with RAPD markers, whereas with SSR markers the level of polymorphism detected was higher (Palombi and Dimiano 2002). Genetic stability was analyzed in plantlets of almond (*Prunus dulcis*) regenerated by axillary branching with RAPD markers and confirmed by ISSR analysis (Sarmento 2005). The genetic fidelity of plantlets obtained by indirect somatic embryogenesis from anthers and ovaries of *Vitis vinifera* cv. Grignolino and cv. Dolcetto was detected by SSR and AFLP analysis (Gribaudo 2009). Senapati *et al.* (2013) confirmed genetic stability in *in vitro*-raised plantlets of *Celastrus paniculatus* which showed 100% monomorphism using RAPD and ISSR markers together. No variability was detected among the *in vitro*-regenerated plantlets of *Dendrocalamus hamiltonii* using both RAPD and ISSR markers (Singh *et al.* 2013).

In the present study, three SPAR methods collectively revealed 4.65% polymorphism in the plantlets of the first regeneration of *N. khasiana*. The polymorphism was subsequently increased to 7.77% and 10.87 % in the plantlets of the second and the third regenerations respectively suggesting an overall increase of genetic variations amongst the morphologically similar micropropagated plantlets of the three consecutive regenerations and the mother plant. These variations could be due to several 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 variations (Devarumath *et al.* 2002). Plant tissues in culture conditions are also under high levels of oxidative stress which has been reported to cause DNA damage, including microsatellite instability (Jackson *et al.* 1998). Genomic instability in the phenotypically normal micropropagated plantlets, 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). Using PCR-based molecular markers, polymorphism in the DNA profiles while analyzing for genetic fidelity have been reported in *Codonopsis lanceolate*, *Vanilla planifolia*, *Dictyospermum ovalifolium*, *Spilanthes calva*, (Guo *et al.* 2006; Sreedhar *et al.* 2007; Chandrika *et al.* 2008; Razaq *et al.* 2012). The overall polymorphism frequency detected in micropropagated plantlets of *N. khasiana* was 14.67%. The polymorphic bands in the micropropagated plantlets included either loss of original band present in the mother plant or gain of novel band. This clearly indicated that complete genetic stability is lacking among the micropropagated plantlets. Similar findings on genomic variations in phenotypically normal plant regenerants have been made previously in other plant species (Rahman and Rajora 2001; Carvalho *et al.* 2004; Guo *et al.* 2006; Feyissa *et al.* 2007).

In conclusion, SPAR methods proved to be highly informative for the assessment of genetic variations in the micropropagatd plants of *N. khasiana*. The combination of

RAPD, ISSR and DAMD markers could be used for estimation of genetic similarity among the micropropagated plantlets. The present investigation further supports the need for testing micropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the micropropagation protocol for its large scale production.

Summary

Nepenthes khasiana Hook f. is the only representative member of the genus *Nepenthes* found in India. It is endemic to the state of Meghalaya located in Northeast India. The species is in great demand for its ornamental value on account of the fascinating beauty of the pitchers. It also has some ethno-medicinal values. The local herbalists prescribe the fluid of the pitcher for the treatment of diabetes and painful urination. The unopened pitcher with its content is made into a paste and applied for various skin diseases, leprosy, sometimes it is taken to ease urinary troubles and blockages.

The advent of biotechnological approaches has opened up newer areas for genetic improvement and micropropagation. Micropropagation methods allow rapid propagation of a large number of plants in short period of time in a limited space thereby protecting the biological, physiological and genetic specifications of the plant which are usually affected by environmental restrictions. However, the broader utility of any micropropagation system may be limited due to occurrence of cryptic genetic changes and development of somaclones. In a micropropagation programme, it is of paramount importance to produce true-to-type planting materials as somaclonal variations of any kind, if induced, may lead to loss of the chief characteristics of the parent rootstocks. Occurrence of somaclonal variations in tissue culture is a common phenomenon which makes it mandatory to check the genetic stability of *in vitro*-raised plants. In spite of several protocols for tissue culture being reported, the regeneration efficiency has been shown to be influenced by explant type, genotype and also the morphogenetic response varying within the same explant. In the present study, *in vitro* mass multiplication of *N. khasiana* was successfully attempted through enhanced axillary branching for production of quality planting material at an accelerated pace within a short period of time. The half-strength MS (Murashige and Skoog) medium supplemented with 2.5 mg/l kinetin (KN) and 2.0 mg/l 6-benzyl aminopurine (BAP) was proven to be the best for shoot induction with maximum response of explants (91.68 %) as well as the maximum number of shoots per explant (19.16) with BFC index of 17.48. The regenerated shoots were successfully rooted in half-strength MS medium supplemented with 2.0 mg/l α-naphthalene acetic acid (NAA) with maximum response of 95.54% with an average of 9.04 roots per shoot. The ultimate success of micropropagation on a commercial scale depends on the ability of the tissue culture-raised plants to acclimatize in the natural conditions. 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 showing the survival rate of 91.66%. The plantlets of the second and the third regenerations were raised using the explants collected from the 8-months-old rooted plants in culture belonging to the first and the second regenerations respectively. Assessment of genetic fidelity among the micropropagated plantlets of the three consecutive regenerations was carried out using various approaches. First and foremost, the morphology of the plantlets of the three consecutive regenerations was compared considering several growth parameters and it was observed that there was no difference in the external morphology of the micropropagated plantlets. However, lack of any phenotypic variation among regenerants does not necessarily imply a concomitant lack of genetic changes and it is, therefore, important to assay the outcomes of *in vitro*-raised plantlets at the genotypic level.

Somatic chromosome number as 2n=80 has been confirmed with no evidence of any numerical variations in *N. khasiana*. The position of the centromere(s) could not be determined due to very small sized chromosomes. Therefore, variations only in the number of chromosomes have been studied in the micropropagated plantlets. All the cells analyzed from the mother plant showed normal somatic chromosome number of 2n=80 unambiguously. However, from the plantlets of the first regeneration showed normal somatic chromosome number of 2n=80 in 76.66% of the cells while the remaining 23.33% cells showed deviant chromosome numbers of 2n=76, 78. In case of the plantlets of the second regeneration, 66.66% cells showed normal somatic chromosome number as 2n=80 while the remaining 33.33% cells showed deviant chromosome numbers of 2n=70, 76, 78, 84, 86. In the plantlets of the third regeneration, cells showing normal somatic chromosome number of 2n=80 was decreased to 60% and deviant chromosome complements of 2n=70; 76; 84; 86 were observed in 40% cells analyzed.

The repetitive sequences located in the heterochromatin regions of the nuclear genome are also reported to influence chromosomal instability in tissue culture-raised plants. Therefore, in the present study, an attempt was made to observe if there was any change in the copy number of the heterochromatin repetitive sequences of the *in vitro*-raised plants of *N. khasiana* using base-specific fluorochromes *viz.*, chromomycin A3

(CMA), 4-6-diamidino-2-phenylindole (DAPI). A total of 30 cells were analyzed in both the mother plant and the plantlets of the three subsequent regenerations. In the mother plant, the number of $DAPI^+$ sites recorded was 5.33±0.73 with the range of 2-8. In case of the plantlets of the first regeneration, the number of $DAPI^+$ sites was 5.74±0.47 with the range of 2-8 which was found to be increased to 6.61±0.39 with the range of 5-12 and 6.74±0.57 with the range of 3-12 in the plantlets of the second and the third regenerations respectively. The number of CMA^+ sites observed in the mother plant was 5.11±0.47 with the range of 2-6. In the plantlets of the first regeneration, the number of CMA^+ sites observed was 5.00±0.30 with the range of 4-9 which was found to be decreased to 4.63±0.45 with the range of 1-8, and 4.16±0.47 with the range of 0-8 in the plantlets of the second and the third regenerations respectively. These results indicated that there was an increase in the number of AT base pairs and a corresponding decrease in the number of GC base pairs with the increase in the regeneration stages of the micropropagated plants in culture.

Genetic fidelity among the micropropagated plantlets of *N. khasiana* was also analyzed with the help of single primer amplification reactions (SPAR) methods using the molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and directed amplification of minisatellite DNA (DAMD). A total of 136 primers for RAPD, ISSR and DAMD were screened out of which 41 primers were finally selected for further profiling. Fourteen RAPD primers resulted in 74 clear, well-separated and reproducible bands out of which 10 bands were polymorphic exhibiting 13.51% polymorphism across all the plantlets of the three regenerations. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.94 to 1.00 among the micropropagated plantlets and the mother plant. In case of ISSR, twelve primers generated a total of 50 distinct and scorable bands of which 7 bands were polymorphic exhibiting 14% polymorphism. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.94 to 1.00 among the micropropagated plantlets and the mother plant. In DAMD profiling, 15 primers resulted in 60 uniform and scorable bands out of which 10 bands were polymorphic showing 16.66% polymorphism. The genetic distance recorded using Jaccard's coefficient of similarity ranged from 0.91 to 1.00 among the micropropagated plantlets and the mother plant. The cumulative analysis commonly regarded as SPAR was also carried out for the three molecular markers in which a total of 184 fragments were produced collectively of which 27 fragments were polymorphic indicating 14.67%. The cumulative data were also used to compute pairwise distances by Jaccard's coefficient which showed a distance range of 0.95-1.00 among the micropropagated plantlets and the mother plant.

Using RAPD marker, 4.1% polymorphism was observed in the plantlets of the first regeneration which was increased to 6.9% and 9.4% in the plantlets of the second and the third regenerations respectively. In case of ISSR, 4.3% polymorphism was observed in the plantlets of the first regeneration which was increased to 6.1% and 10% in the plantlets of the second and the third regenerations respectively. DAMD marker detected 8.47% polymorphism in the plantlets of the first regeneration which was increased to 10.10% and 13.33% in the plantlets of the second and the third regenerations respectively. However, collective data regarded as SPAR approach detected polymorphism of 4.65% in the plantlets of the first regeneration which was subsequently increased to 7.77% and 10.87% in the plantlets of the second and the third regenerations respectively.

In conclusion, from the present study it was observed that there was a subsequent increase in genetic variation from the plantlets of the first regeneration to the plantlets of the third regeneration although no perceptible difference in the general morphology of the regenerated plantlets of the three subsequent regenerations relative to their mother plant was observed. The efficient protocol described in the present study for the micropropagation of *N. khasiana* through axillary bud multiplication facilitates the rapid propagation of this rare plant species. However, in the context of providing quality planting material of *N. khasiana* germplasm it is particularly important to assess the genetic stability of the *in vitro*-raised plants. Micropropagation using axillary bud proliferation is considered to be one of the safest methods which give rise to genetically uniform and true-to-type plants. The present investigation clearly shows that this may not always be the case, which further supports the need for testing micropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the micropropagation protocol for its large scale production.

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Publications

- **Devi SP**, Rao SR, Kumaria S, Tandon P (2012) Mitotic chromosome studies in *Nepenthes khasiana*- an endemic insectivorous plant of northeast India. *Cytologia* 77(3):381-384
- **Devi SP**, Kumaria S, Rao SR, Tandon P (2013) *In vitro* propagation and assessment of clonal fidelity of *Nepenthes khasiana* Hook. f.; a medicinal insectivorous plant of India. *Acta Physiologeae Plantarum* 35: 2813-2820
- **Devi SP**, Kumaria S, Rao SR, Tandon P (2014) 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. *Gene* 538: 23-29

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- "Himalayan Biodiversity: Prospects and Challenges", 20th -21st March, 2014 held at North-Eastern Hill University, Shillong.
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- Workshop on Hands on training on "Next Generation Sequencing and its Applications" 3rd - 10th March, 2014 organized under the DBT State level Biotech Hub Project at the Department of Botany, North-Eastern Hill University, Shilllong.
- 83rd Annual Session and Symposium on "Space for Human Welfare", 5th -7th December, 2013 held at Goa University, Goa.
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- National Symposium on Plant Cell Tissue and Organ Culture: The Present Scenario and XXXI Annual Meeting of Plant Tissue Culture Association (India), 3rd-5th March 2010 held at University of Calcutta, Kolkata.
    - Presented a paper (oral) entitled "High plantlet regeneration of *Nepenthes khasiana* Hook. f. *in vitro* propagation of nodal explants".

Work experience

- Worked as Junior Research Felllow (September, 2008 - March, 2011) in DBT sponsored research project entitled 'Species recovery programme for *Nepenthes khasiana, Mantisia spathulata* and *M. wengerii*" sanctioned to Prof. Pramod Tandon [Research Grant No. BT/PR-7055/BCE/08/437/2006].
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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 Jarain, 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 I 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 (Cheek and Jebb 2009); multiplication and micropropagation (Latha

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and Seeni 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, Meghalaya, 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. veeitchii*, *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

Figs. 1–2. Chromosome complements of *Nepenthes khasiana* showing somatic chromosome number $2n$=80. (Magnification 1000×)

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 ($8x$ or $16x$), 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 ellucidate taxonomic and phylogenetic relationships among the species of the genus *Nepenthes*.

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ORIGINAL PAPER

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*.

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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

International Trade in Endangered Species of Wild Fauna and Flora) and Negative List of Exports of the Government of India (Ziemer 2010).

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; Latha and Seeni 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 (Feyissa et al. 2007; 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 (Labolene, 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 % $HgCl_2$ (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-benzyl aminopurine (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-Napthaleneacetic 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 thermocol 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-months-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 fuschin 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 ×100 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 Lamda 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 $MgCl_2$, 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.

Table 1 Effect of BAP and KN on multiple shoot formation from nodal explants of *N. khasiana* cultured in 1/2 MS medium

KN (mg/l)	BAP (mg/l)	% of response	Average shoot number	Mean shoot length (cm)
1.0	–	0.0^{b}	0.0 ± 0.0^{h}	0.0 ± 0.0^{f}
1.5	–	0.0^{b}	0.0 ± 0.0^{h}	0.0 ± 0.0^{f}
2.0	–	33.32^{c}	1.87 ± 0.65^{h}	1.62 ± 0.15^{c}
2.5	–	50.23^{c}	4.04 ± 0.72^{e}	1.29 ± 0.07^{cd}
3.0	–	41.66^{bc}	2.08 ± 0.56^{gh}	2.05 ± 0.00^{b}
–	1.0	58.34^{ac}	2.37 ± 0.42^{fg}	2.10 ± 0.04^{b}
–	1.5	66.67^{ac}	4.25 ± 0.63^{ef}	1.13 ± 0.04^{de}
–	2.0	75.13^{a}	5.04 ± 0.62^{de}	0.81 ± 0.12^{e}
–	2.5	75.52^{a}	3.54 ± 0.44^{efg}	1.02 ± 0.02^{de}
–	3.0	70.12^{a}	2.50 ± 0.34^{fg}	1.32 ± 0.07^{cd}
2.5	1.0	66.24^{a}	9.12 ± 0.25^{cd}	1.54 ± 0.07^{cd}
2.5	1.5	79.16^{a}	13.20 ± 0.20^{bc}	3.00 ± 0.03^{a}
2.5	2.0	91.68^{a}	19.16 ± 0.23^{a}	3.04 ± 0.00^{a}
2.5	2.5	83.72^{a}	14.41 ± 0.26^{b}	2.86 ± 0.11^{a}
2.5	3.0	70.00^{a}	11.29 ± 0.28^{bc}	2.40 ± 0.10^{b}

Means followed by the same letters within columns are not significantly different at the 5 % level according to Tukeys test. Data recorded after 8 weeks of culture

SE standard error

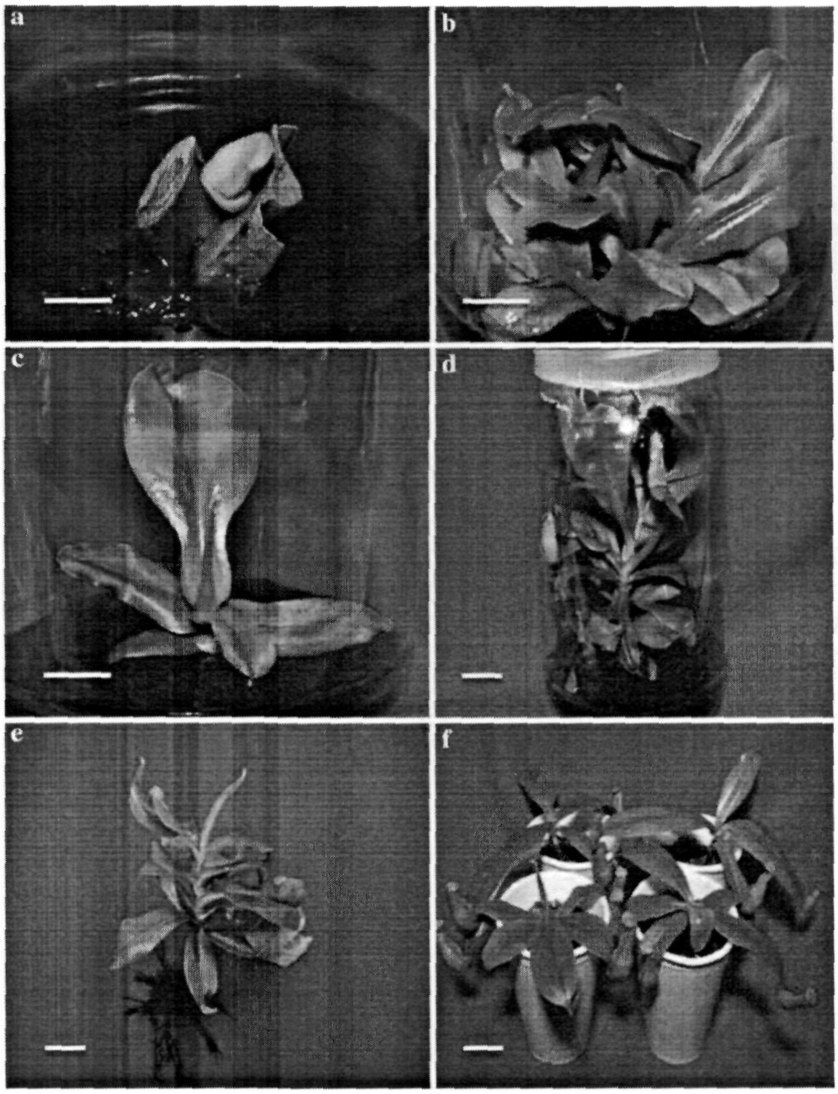

Fig. 1 Micropropagation of *Nepenthes khasiana* **a** Initiation of shoot bud in 1/2 MS + 2.5 mg/l KN + 2.0 mg/l BAP (*Bar* 1 cm). **b** Multiple shoot induction after subculture in the same medium; 5 weeks old (*Bar* 1 cm). **c** Isolated shoot before rooting (*Bar* 1 cm). **d** Plantlet with well developed pitchers (*Bar* I cm). **e** Complete plantlet with roots in MS medium supplemented with 2.0 mg/l NAA (*Bar* 1 cm). **f** Hardened plantlets (*Bar* 5 cm)

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

NAA (mg/l)	IBA (mg/l)	% of response	Average root number	Mean root length (cm)
0.5	–	54.16^{bc}	2.66 ± 0.52^{de}	1.07 ± 0.20^{cef}
1.0	–	62.50^{ac}	3.41 ± 0.57^{cd}	2.00 ± 0.32^{bce}
1.5	–	79.18^{ad}	4.41 ± 0.51^{bc}	3.39 ± 0.36^{ad}
2.0	–	95.54^{a}	9.04 ± 0.46^{a}	3.58 ± 0.16^{a}
2.5	–	70.86^{ade}	5.12 ± 0.70^{b}	2.10 ± 0.40^{cde}
–	0.5	33.35^{c}	1.50 ± 0.49^{e}	0.37 ± 0.11^{f}
–	1.0	41.12^{ce}	1.70 ± 0.43^{e}	0.91 ± 0.22^{ef}
–	1.5	50.00^{cd}	3.25 ± 0.68^{f}	2.04 ± 0.50^{e}
–	2.0	62.64^{ac}	$2.66 \pm 0.44d^{ef}$	1.21 ± 0.19^{ef}
–	2.5	45.74^{cd}	2.04 ± 0.47^{f}	1.39 ± 0.31^{ef}

Means followed by the same letters within columns are not significantly different at the 5 % level according to Tukeys test. Data recorded after 4 weeks of culture

SE standard error

Fig. 2 Chromosome complements of *Nepenthes khasiana*, 2*n* = 80 (*Bar* 10 μm)

maximum 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

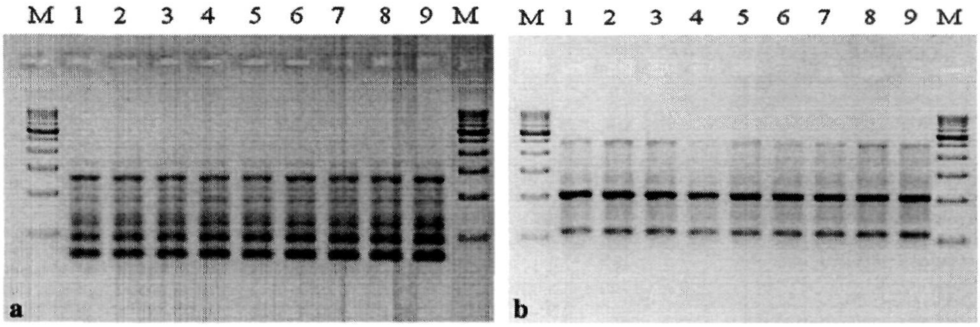

Fig. 3 RAPD profiles of *Nepenthes khasiana* with primers **a** OPK10, **b** OPK19. *Lane M* 500 bp ladder, *Lane 1* mother plant, *Lanes 2–9* micropropagated plants

Table 3 Details of RAPD primers used in detecting genetic stability in the regenerated plants of *N. khasiana*

Sl. no.	Primer code	Primer sequence (5′–3′)	No. of scorable bands per primer	No. of polymorphic band	% polymorphic bands
1	OPH-1	GGTCGGAGAA	2	0	0
2	OPH-5	AGTCGTCCCC	3	1	33.3
3	OPH-6	ACGCATCGCA	6	0	0
4	OPH-11	CTTCCGCAGT	3	0	0
5	OPK-10	GTGCAACGTG	7	0	0
6	OPK-11	AATGCCCCAG	7	0	0
7	OPK-12	TGGCCCTCAC	5	0	0
8	OPK-13	GGTTGTACCC	5	1	20
9	OPK-14	CCCGCTACAC	5	0	0
10	OPK-17	CCCAGCTGTG	9	0	0
11	OPK-18	CCTAGTCGAG	7	0	0
12	OPK-19	CACAGGCGGA	3	0	0
13	OPC-7	GTCCCGACGA	5	0	0
14	OPA-11	CAATCGCCGT	5	1	20
		Total	72	3	4.1

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

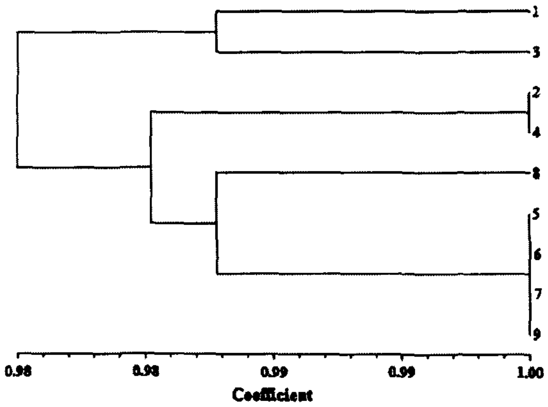

Fig. 4 Dendrogram illustrating coefficient similarities among regenerated plants and the mother plant by the UPGMA cluster analysis (NTSYS)

1.00. Relatively low genetic variation in DNA (4.1 %) observed amongst the regenerants and the mother plant may be due to various factors such as in vitro process and its duration, hormonal balance, in vitro stress, induced by added biochemicals, or other nutritional conditions, all of which are known to induce somaclonal variations (Devarumath et al. 2002). Genetic variations induced in the morphologically similar regenerants have also been reported in other plant species (Feyissa et al. 2007; Chandrika et al. 2008; Razaq et al. 2012). RAPD technique has widely been used to test the genetic stability of in vitro grown plants including *Musa paradisiaca* (Venkatachalam et al. 2007), *Dendrocalamus hamiltonii* (Agnihotri et al. 2009), *Mantisia spathulata* and *M. wengeri* (Bhowmik et al. 2009) *and Talinum triangulare* (Swarna and Ravindhran 2012).

Conclusion

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 assessment of genetic stability in the regenerants. This study also demonstrates that RAPD marker in conjunction with the cytogenetic study could be successfully employed to evaluate 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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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 $\mu mol\ m^{-2}\ s^{-1}$ 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.

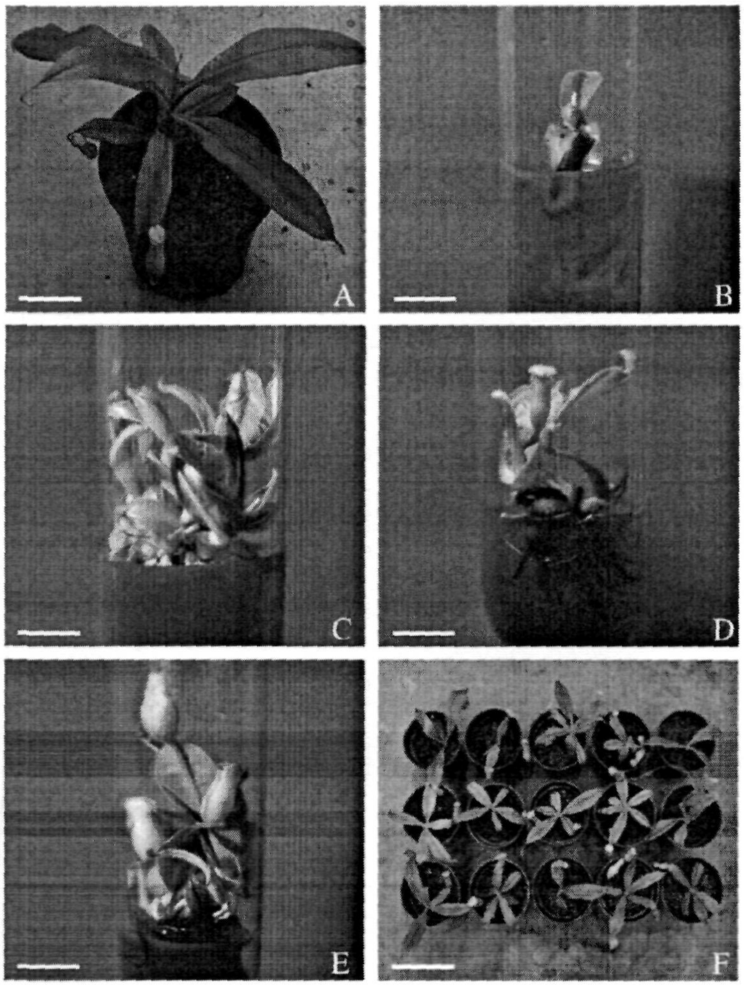

Fig. 1. Micropropagation of *Nepenthes khasiana*. (A) Mother plant (bar 5 cm). (B) Initiation of shoot bud in 1/2 MS + 2.5 mg/l KN + 2.0 mg/l BAP (bar 5 mm). (C) Multiple shoot induction after subculture in the same medium (bar 5 mm). (D) Rooted shoot in 1/2 MS + 2.0 mg/l NAA (bar 1 cm). (E) Complete plantlet with pitchers (bar 5 mm). (F) Hardened plantlets (bar 5 cm).

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 3 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 0.98 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 (8.47%) 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 49 fragments of which 3 bands were polymorphic (6.1%) 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 (7.77%) with average polymorphic bands of

Table 1
Details of RAPD, ISSR and DAMD used in the present study.

Sl. no.	Primer name	Primer sequence (5′–3′)
RAPD		
1	OPH-1	GGTCGGAGAA
2	OPH-5	AGTCGTCCCC
3	OPH-6	ACGCATCGCA
4	OPH-11	CTTCCGCAGT
5	OPK-10	GTGCAACGTG
6	OPK-11	AATGCCCCAG
7	OPK-12	TGGCCCTCAC
8	OPK-13	GGTTGTACCC
9	OPK-14	CCCGCTACAC
10	OPK-17	CCCAGCTGTG
11	OPK-18	CCTAGTCGAG
12	OPK-19	CACAGGCGGA
13	OPC-7	GTCCCGACGA
14	OPA-11	CAATCGCCGT
ISSR		
1	N1	ACACACACACACACACT
2	N2	TGTGTGTGTGTGTGTGA
3	N3	GAGAGAGAGAGAGAGAYT
4	N4	CACACACACACAGG
5	N5	CACACACACACAAC
6	N6	CACCACCACGC
7	N7	GAGGAGGAGGC
8	N8	CACACACACACAGT
9	N9	ACACACACACACACACAG
10	N10	ACACACACACACACACAA
11	N11	ACGACGACGACGACGACG
12	N12	ACACACACACACACACGA
DAMD		
1	URP1F	ATCCAAGGTCCGAGACAACC
2	URP2F	GTGTGCGATCAGTTGCTGGG
3	URP2R	CCCAGCAACTGATCGCACAC
4	URP6R	GGCAAGCTGGTGGGAGGTAC
5	URP9F	ATGTGTGCGATCAGTTGCTG
6	URP13R	TACATCGCAAGTGACACAGG
7	URP17R	AATGTGGGCAAGCTGGTGGT
8	URP25F	GGACAAGAAGAGGATGTGGA
9	URP30F	GGACAAGAAGAGGATGTGGA
10	URP32F	TACACGTCTCGATCTACAGG
11	URP38F	AAGAGGCATTCTACCACCAC
12	HBV5	GGTGTAGAGAGGGGT
13	HVR	CCTCCTCCCTCCT
14	INS	ACAGGGGTGGGG
15	YN73	CCCGTGGGGCCGCCG

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 0.91 to 1.00. A total of 184 bands were generated* collectively of which 20 fragments were polymorphic (10.87%) with an average of 0.48 polymorphic bands per primer and genetic distance recorded using Jaccard's coefficients of similarity ranged from 0.95 to 1.00 (Fig. 3C).

Table 2
Comparison of SPAR methods (RAPD, ISSR and DAMD) for the analysis of genetic variations in *all the three consecutive regenerations*, individually as well as collectively.

Regenerations	SPAR approach	No of primers used	Total bands amplified	Average bands/primer	Total no. of polymorphic bands	Average no. of polymorphic bands/primer	% of polymorphism	Distance range (Jaccard's coefficient)
1st	RAPD	14	72	5.10	3	0.21	4.1	0.98–1.00
	ISSR	12	46	3.80	2	0.16	4.3	0.96–1.00
	DAMD	15	59	3.94	5	0.33	8.47	0.94–1.00
	SPAR	41	177	4.31	10	0.24	5.65	0.98–0.99
2nd	RAPD	14	72	5.10	5	0.28	6.9	0.96–1.00
	ISSR	12	49	4.08	6	0.25	6.1	0.95–1.00
	DAMD	15	59	3.93	3	0.40	10.10	0.92–1.00
	SPAR	41	180	4.39	14	0.34	7.77	0.96–1.00
3rd	RAPD	14	74	5.28	7	0.50	9.40	0.95–1.00
	ISSR	12	50	4.16	5	0.41	10.00	0.94–1.00
	DAMD	15	60	4.00	8	0.53	13.33	0.91–1.00
	SPAR	41	184	4.48	20	0.48	10.87	0.95–1.00

4. Discussion

Somaclonal 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 (Kaeppler 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; Keyte et al., 2006; Lukens and Zhan, 2007), genome adaptation to different regulatory microelements (Bogani et al., 1996) and the presence of hot spots (Linacero 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 (Palombi 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* (Winberg et al., 1993; Zhou et al., 1997), *Piper* (Verma et al., 2004), mulberry (Bhattacharya et al., 2005), *Murraya* species (Ranade et al., 2006), *Jatropha* (Kumar et al., 2011; Ranade et al., 2008), *Sapindus* (Mahar et al., 2011), *Mantisia* species (Sharma 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 (Devarumath 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). Genomic 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 lanceolate* (Guo et al., 2006), *Dactyospermum ovalifolium* (Chandrika et al., 2008) and *Spilanthes calva* (Razaq et al., 2012) using PCR-based molecular markers. Corroboratory results using RAPD and ISSR markers for testing the clonal fidelity have also been reported in other plant species (Alizadeh and Singh, 2009, Sreedhar et al., 2007). Mishra et al. (2008) could successfully employ three single primer amplification reaction (SPAR) techniques viz. RAPD, ISSR and DAMD for the assessment of genetic stability in micropropagated plants of *Aegle marmelos*.

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 if the regenerants are cultured for a longer period of time. Micropropagation

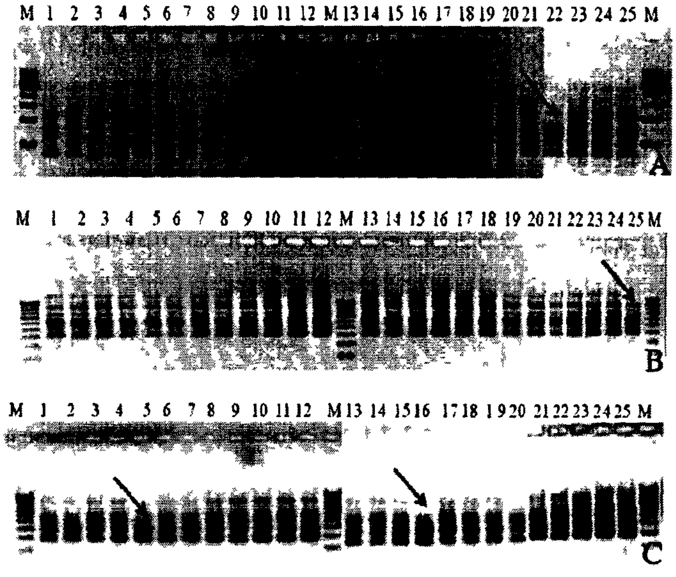

Fig. 2. SPAR profiles of *Nepenthes khasiana* obtained with RAPD primer (OPK13, Lane M 500 bp ladder; A), ISSR primer (N7; Lane M 100 bp ladder, B), DAMD primer (URP2F Lane M 500 bp ladder; C), Lane 1 mother plant, Lanes 2–9 micropropagated plants of the first regeneration, Lanes 10–17 micropropagated plants of the second regeneration, Lanes 18–25 micropropagated plants of the third regeneration.

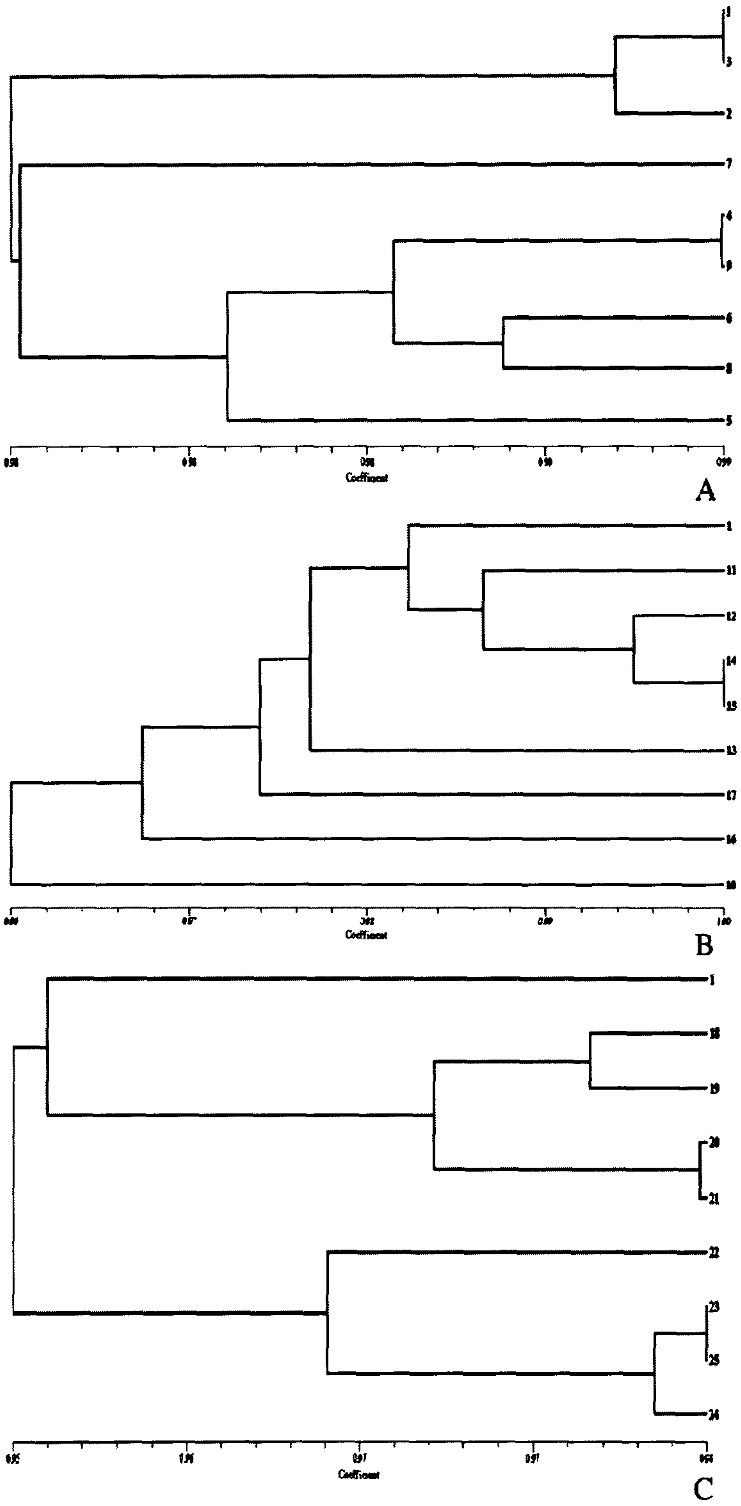

Fig. 3. UPGMA dendrogram generated for cumulative band data from the three SPAR methods (RAPD, DAMD and ISSR) illustrating coefficient similarities among regenerated plants and the mother plant (first regeneration, A, second regeneration, B, third regeneration C)

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 micropropagated plantlets periodically well before their actual planting in the field and confirming the reliability of the micropropagation protocol for its large scale production

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