

**CONSERVATION AND DNA FINGERPRINTING OF
NEPENTHES KHASIANA HOOK. F- A THREATENED
INSECTIVOROUS PLANT OF INDIA**

BY

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**THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE
DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY**

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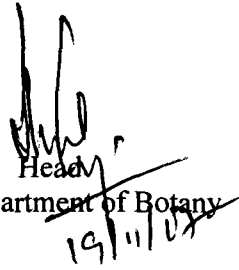
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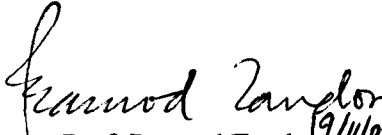
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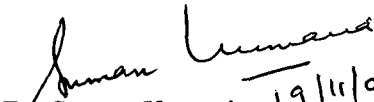
I do hereby declare that the thesis entitled '**Conservation and DNA Fingerprinting of *Nepenthes khasiana* Hook. f – A threatened insectivorous plant of India**' is a record of original and independent research work carried out by me in the Department of Botany, North- Eastern Hill University, Shillong, under the supervision of Prof. Pramod Tandon and Dr. Suman Kumaria. The work is original and no part of the thesis has been submitted for any other degree or diploma of any University.

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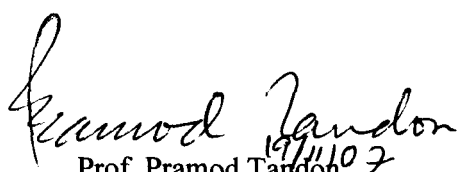
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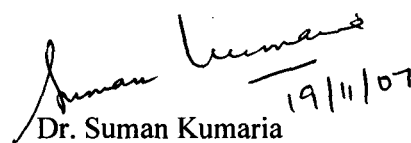
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CERTIFICATE

We certify that the thesis entitled '**Conservation and DNA Fingerprinting of *Nepenthes khasiana* Hook. f – A threatened insectivorous plant of India**', submitted by Ms. Iaibadaiahun Nongrum 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. This work has not been submitted for any degree of any other University.


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CONTENTS

CHAPTER I	Introduction	1 - 7
CHAPTER II	Review of Literature	8 - 25
CHAPTER III	<i>In vitro</i> seed germination of <i>Nepenthes khasiana</i> as influenced by different media and physical factors Introduction Materials and Methods Results Discussion	26 - 43
CHAPTER IV	<i>In vitro</i> seed germination of <i>Nepenthes khasiana</i> as influenced by growth regulators Introduction Materials and Methods Results Discussion	44 - 56
CHAPTER V	Hardening, establishment and reintroduction of <i>in vitro</i> raised plants Introduction Materials and Methods Results Discussion	57 - 67
CHAPTER VI	Analysis of genetic variability in <i>Nepenthes khasiana</i> using Random Amplified Polymorphic DNA (RAPD) Introduction Materials and Methods Results Discussion	68 - 90
CHAPTER VII	Summary	91 - 97
CHAPTER VIII	References	98 - 144

Life on earth is the result of over 3 billion years of natural selection which favoured efficiency, productivity and specialization. The living organisms are catalysts which capture and transform energy and materials, recycle wastes, create pure drinking water and drive global biogeochemical cycles to generate soil fertility. They also create and maintain an aerobic atmosphere and regulate global and local climate. Natural resources are vital for food, livelihood and environmental security. Biodiversity as one of the resource ecosystems of the environment provides basic raw materials which are primary infrastructures and requirements for life. Changes in diversity can greatly reduce sources of food, fuel, structural materials, medicinal plants and can also alter the abundance of other species that control ecosystem processes, leading to further change in community composition and vulnerability to invasion (Chapin *et al.*, 2000).

Biodiversity is not equally distributed across the different continents of the earth. Out of the 5-50 million species of the world's biota estimated so far, only 1.7 million species have been described (Groombridge and Jenkins, 2000). Forty four percent of all species of vascular plants and 35% of all species of four vertebrate groups are confined to 25 hotspots comprising only 1.4% of the land surface of the Earth (Myers *et al.*, 2000). Mittermeier *et al.* (1997) defined a centre of megadiversity as an area or a country possessing a much larger proportion of species than would be

expected by its extent, latitudinal position and other factors. On scores of species richness and endemism, these biodiverse countries are Brazil, Indonesia, Columbia, Australia, Mexico, Madagascar, Peru, China, Philippines, India, Ecuador and Venezuela (Sarukhan and Dirzo, 2001). India, one of the 12 megadiversity countries, has about 80,000 species of animals and 45,000 species of plants, which accounts for 8% of the global genetic resources (Choudhury and Murti, 2000). India has many endemic plant and vertebrate species. Among plants, species endemism is estimated at 33% with approximately 140 endemic genera but no endemic families (Botanical Survey of India, 1983). Areas rich in endemism in India are the Northeast India, southern parts of the peninsular India and the Northwest Himalayas. Northeast India alone has a remarkably rich and diverse flora. The country has about 17,000 flowering plants out of which 5000 species are found in the Northeastern region (Tandon and Kumaria, 2005). Many of these valuable plants have now become rare or endangered and some are approaching extinction.

Human dominance, intervention and uncontrolled exploitation particularly in the last 250 years since the industrial revolution have markedly reduced the diversity of species within many habitats in the biosphere leading to its accelerating extinction. There is growing concern about the depletion of the natural resources especially affecting the plant biodiversity of the ecosystem. Human development depends on plant resources for nutrition, health and other aspects leading to a better quality of life. These needs are largely met by plants taken from the wild and the demand far exceeds the resources. Studies have shown that many such plant species are in danger of extinction, while some have already become extinct. On a global basis, the IUCN has

estimated that about 12.5% of the world's vascular plants, totaling about 34,000 species are under varying degrees of threat. It is estimated that 1 in 8 of living plants is endangered (IUCN, 1998). In India, about 15-20% plant species are considered to be threatened. A great number of plant species in the Northeast India including several unique and irreplaceable varieties are becoming extinct and many more are awaiting a similar fate (Tandon and Kumaria, 2005). Furthermore, a considerable amount of genetic diversity within species that might survive is likely to be lost. Fragmentation of habitats adversely affects the fate of rare and threatened species which may be reduced to such low numbers that they may not constitute viable populations. In such populations, genetic drift and inbreeding may result in inbreeding depression, with deleterious effects on reproductive output. Thus, a combination of demographic and genetic factors may hasten the extinction of rare species from small isolated fragments (Bawa and Ashton, 1991). This has enacted a collected consciousness on the significance of biodiversity for future human developments; hence the issue of conservation and sustainable utilization of bioresources has come to the fore.

The need for conservation is exceptionally high and of paramount importance to preserve plant heritage for posterity. Conservation does not only mean the preservation of a species or habitat but also the preservation of an evolutionary lineage consisting of genetically diverse individuals. Over a long term, the ability of a species to respond adaptively to environmental changes depends on the level of genetic variability it contains (Ayala and Kiger, 1984). The amount and partitioning of genetic variation among and within populations result from the dynamic processes

of gene flow, selection, inbreeding, genetic drift and mutation (Hartl and Clark, 1994). A species without an appropriate amount of genetic diversity is unable to cope with changing environment or evolving competitors and parasites. Therefore, investigations of population genetic diversity and the structure of populations within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation and phylogenetic analysis (Schaal *et al.*, 1991a, b). A success of any genetic conservation programme is dependent on an understanding of the amount and distribution of the genetic variation present in the genepool. Availability of genetic data can help researchers assess the ability of rare plant populations to adapt to changes in their natural environment, to respond to reintroduction programmes and natural selection.

The basic requirements for normal growth of plants are space, sunlight, water, carbon dioxide, and some inorganic nutrients. A deficiency in any of these basic requirements imposes a harsh environment for the plants which in turn causes the plant to acquire special adaptations in order to survive in those conditions. In marshes and swamps, the soil is generally acidic with a low content of mineral substances vital for all green plants. Plants growing in this type of environment have adopted carnivory in order to sustain themselves. These plants preferentially occupy the mineral-poor soil and engage in capturing their animal prey. The relatively small group of these flowering plants has come to be known as "insectivorous" or "carnivorous" plants. Insectivorous plants are specialized in trapping insects and receive part of their nutrition from them. They are usually associated with rain washed, nutrient poor soils, or wet and acidic areas that are ill drained. Such wetlands

are poor in available nitrogen, hence for such plants, capturing insects is one way of getting nitrogenous compounds without manufacturing them. Carnivorous or insectivorous plants are therefore among the curiosities of nature, and are of interest to plant growers all over the world. Insectivorous plants are diverse and represent members of three orders of dicots: Nepentales, Scrophulariales, and Rosales. A majority of these plants are in the Nepentales, including the pitcher plants, the sundews, and the Venus flytrap. Others include the bladderworts and the butterworts. Insectivorous plants of India belong to mainly three families: Droseraceae, Nepenthaceae and Lentibulariaceae.

Nepenthes khasiana Hook f. (Common name: - English: Pitcher plant; Hindi: Ghatparni; Bengali: Kalaspatri; Khasi: Tiew Rakot; Jaintia: Kset phare; Garo: Memang koksi) was discovered by Sir J. D. Hooker in the year 1873. It belongs to the family Nepenthaceae with polyploid chromosome number $2n=80$. The genus *Nepenthes* comprises 86 species distributed in different areas of the world (www.joachim_nerz.de/nepintro, Jebb and Cheek, 1997). *N. khasiana* is the only species found in India (Photoplate 1.1a, b). It is restricted to the Jarain (Jaintia Hills), Lawbah (Khasi Hills) and Baghmara (Garo Hills) areas of Meghalaya, a state located in Northeast India. It is a climbing undershrub which ranges from a few centimeters to several meters in height. Two heights of the plant are noticeable in its natural habitat; those growing on rocky clefts or sandy pockets of silt attain a height of only 10-15cm, with small pitchers and under-developed tendrils whereas, those that grow along hill streams or on moist soil strata of substantial depth are tall climbers rising up to a height of 15-20 m (Bordoloi, 1977). The plant has a root system which is very

Photoplate 1.1

(a) *Nepenthes khasiana* in its natural habitat

(b) A closer view of the pitcher



superficial, penetrating only a few centimeters. A large number of roots, fibrous in nature develop from the base of the stem in a profuse manner. The stem is cylindrical, reddish in colour when young, becomes green and ultimately turns brown as it grows older. The lower portion of the leaf is modified into a thick, glossy, leathery lamina (30-40 cm by 4-9 cm). Lamina merges into the tendril portion (12-15 cm) of the leaf which helps to hold the pitchers in an upright position. Pitcher size ranges from 5-20 cm in length and 2-6 cm in diameter. Uniformly coloured green or red small pitchers are seen in dwarf varieties. Adult pitchers are green with splotching of red on the upper portion of the outer surface (Photoplate 1.1b). Flowers are arranged in inflorescence in both male and female plants. Inflorescences are lateral with a stout greenish or reddish cylindrical stalk (30-35 cm). Male flowers have many stamens, synandrous with a solid staminal column. Anthers are bilocular, opening by the longitudinal slit. Pollen grains are numerous and spherical. Female flowers have four carpels each, syncarpous, stigma is broad and dome shaped, ovary superior, four chambered with many ovules.

The brightly coloured pitchers are a fatal attraction for the insects. A honey-like substance is also secreted from the glands at the entrance of the pitcher, as well as the lower surface of the lid to attract insects. The inner surface of the pitcher is very slippery because of the presence of waxy glands. Hence once the insect is trapped, it is unable to climb out. The inner wall towards its lower half bears numerous glands which secrete Nepenthacin, a proteolytic enzyme, which helps to digest the insects.

N. khasiana is a plant which is of botanical curiosity as well as ecological importance. The presence of the pitchers renders ornamental importance to this plant;

therefore it is extensively collected for trade. It has some ethno- medicinal uses. The local physicians, known as the 'Kavirajas' prescribe the pitcher fluid for diabetic patients. Pitcher as well as pitcher powder is used to treat patients with cholera, frequent and painful urination, cysts, vaginal tumours and leprosy (Bordoloi, 1977).

The population of *N. khasiana* has dwindled in the last few decades due to deforestation and forest fires, excessive collection for trade, and 'jhum' cultivation. It is now threatened in its natural habitat. It is listed as an endangered plant in Appendix- I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Propagation of the plant is mainly vegetative from stem cuttings or rhizomatous part of the stem. Although the seed set is very high with more than 300 seeds per capsule, germination in nature is very scant. It takes as long as 223 days for the seeds to germinate in nature (Bordoloi, 1977).

Considering the status of *N. khasiana* in nature and its economic importance, the present study was undertaken with the following objectives.

1. Conservation of *N. khasiana* by large scale propagation through *in vitro* seed germination.
2. Hardening and establishing *in vitro* grown seedlings and their reintroduction to nature.
3. Study of the variation of *N. khasiana* plants collected from various locations in Meghalaya viz. Jarain, Baghmara and Lawbah using PCR based markers.

Conservation aims at maintaining the diversity of living organisms, their habitat and the interrelationships between organisms and their environment (Spellerberg and Haldes, 1992). Conservation of plant genetic resources can be accomplished by both *in situ* and *ex situ* methods (Tandon, 2004). *In situ* conservation involves protection of genetic resources in the natural environment through the protection of the environment itself. It aims to maintain and recover viable population of species in its natural habitat, to reintroduce the rare and endangered species and to recreate the requisite habitats for such plant species (Maxted *et al.*, 1997). In tropical ecosystems, like those of the Indian sub continent, *in situ* conservation is difficult because of environmental disasters like landslides, unpredictable rainfall, floods, etc., and man made disasters like forest fires, deforestation, and overexploitation for trade. *Ex situ* conservation programmes provide a higher degree of protection off-site and has become a more common mode of conservation. It comprises seed storage, tissue culture, *in vitro* storage, DNA storage, field gene banks, and cryopreserved gene banks and aims at acclimatization, rehabilitation, multiplication and judicious exploitation of plant resources. It may often be the only conservation option, for example, for species with recalcitrant seeds. Biotechnology can directly assist plant conservation programmes through *in vitro* technologies, molecular marker

technology, molecular diagnostics, and cryopreservation (Tandon, 2001; Tandon and Kumaria, 1998).

Plant tissue culture holds great potential for conservation of germplasm (Prance, 1997; Feijoo and Iglesias, 1998; Tandon and Kumaria, 1998; Lynch, 1999). It offers many unique advantages over conventional propagation methods, such as, rapid mass propagation of valuable genotypes in a limited space, expeditious release of improved varieties, production of disease-free plants, non seasonal production, germplasm conservation, etc. The method is particularly important in case of plants which are rare, threatened or critically endangered particularly for the species with reproduction problems and/or extremely reduced population. While most threatened species are rare, the converse is not necessarily true (Rabinowitz, 1981). When however, rarity is due to human influence or when naturally rare species are sensitive to human activities, the threat of extinction becomes more of a concern. The tremendous potential of tissue culture technique can be utilized for increasing the number of such plants and for immediate conservation.

The concept of *in vitro* cell culture was originally proposed during the early years of the twentieth century by the German Botanist, Gottlieb Haberlandt (1902). Blumenthal and Meyer (1924) first reported callus cultures of *Daucus carota*. The totipotency of the plant cell was completely resolved by Vasil and Hilderbrandt (1965) by their demonstration that a single isolated cell can divide and ultimately grow into a whole plant. The first successful production of haploid plants was achieved by Guha and Maheshwari (1964) in *Datura innoxia*. Plant regeneration from isolated protoplasts was first accomplished by Takebe *et al.* (1971) in *Nicotiana*

tabaccum and the first somatic hybrid was obtained from two species of *Nicotiana* (Carlson *et al.*, 1972). Composition of different culture media was formulated by different workers such as Gautheret (1942), Nitsch (1951), Heller (1953), Reinert and White (1956), Murashige and Skooge (1962), White (1963), Linsmaier and Skoog (1965), Nitsch and Nitsch (1969), Schenk and Hilderbrandt (1972) and Gamborg *et al.* (1976). Three main groups of growth regulators used in tissue culture media (cytokinins, auxins and gibberellins) have naturally occurring and synthetic members. Snow (1935) first demonstrated that indole-3-acetic acid (IAA) discovered by Went (1926) stimulated cambial activity. The possibility of cultivating plant tissues for an unlimited period, using media enriched with auxins, was announced independently by Gautheret, White and Nobecourt in 1939. Kinetin (KN) or 6-furfurylaminopurine was the first cytokinin discovered and was isolated by Miller *et al.* (1955). George and Sherrington (1984) gave an extensive account of the use of growth regulators in tissue culture.

Over the years, a large number of plants have been propagated *in vitro* using different explant sources, media and culture conditions (Pence and Soukup, 1995; Muhammad *et al.*, 2002). During the last thirty years, micropropagation has become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with those crops which are propagated vegetatively or have recalcitrant seeds that cannot be stored under conventional seed bank conditions (George and Sherrington, 1984; Dodds, 1991; George, 1993). Likewise, *in vitro* culture is being used in an increasing number of botanical gardens for the propagation and conservation of wild plant species (Fay,

1992). Considerable work has also been done on rare, threatened and endangered plants like *Costus speciosus* (Chaturvedi *et al.*, 1984), *Dysosma pleiantha* (Chuang and Chang, 1987), *Picrorhiza kurroa* (Lal *et al.*, 1988), *Saussurea lappa* (Arora and Bhojwani, 1989), *Podophyllum hexandrum* (Arumugam and Bhojwani, 1990), *Coleus forskohlii* (Sharma *et al.*, 1991), *Gentiana kurroo* (Sharma *et al.*, 1993), *Leontochir ovallei* (Lu *et al.*, 1995), *Gymnema elegans* (Komalavalli and Rao, 1997), *Coryphantha minima* (Malda *et al.*, 1999), *Rotula aquatica* (Martin, 2003), *Dioneae muscipula* (Jang *et al.*, 2003), etc. Several reviews on the use of *in vitro* propagation of endangered plants have appeared (Fay, 1992; Fay and Gratton, 1992; Pence, 1999; Tandon and Kumaria, 2005). In addition to micropropagation in its strict sense, other techniques available include *in vitro* seed germination, regeneration of plants from callus cultures, dual culture with symbiotic fungi and micrografting.

In vitro culture of seeds is a method that allows the rescue and recovery of plant species with non-germinating or poorly germinating seeds. *In vitro* seed germination has been extensively used for multiplication of a number of plant species (Mohan and Jorapur, 1984; Yam and Weatherhead, 1988; Sharma and Tandon, 1987, 1990; Kumaria and Tandon, 1991; Kondo *et al.*, 1997; Iankova *et al.*, 2001, Vargas *et al.*, 2004). Seeds are preferred to vegetative material as the source of propagation material for multiplication of rare and endangered species, as a wider genetic base can be maintained, so this would ensure genetic diversity. The application of *in vitro* seed propagation technique to rare and endangered species, which are suffering from over-collection and continuous loss of their natural habitats, undoubtedly is a powerful tool for *ex situ* biodiversity conservation (Stenberg and Kane, 1998; Gangaprasad *et al.*,

1999). Germination of seeds *in vitro* has been successfully carried out on many rare, threatened and endangered species like *Helianthemum polygonoides* (Iriando *et al.*, 1995), *Lepidium sisymbrioides* (Allen, 1998) *Ipsea malabarica* (Gangaprasad *et al.*, 1999), *Zeyhera montana* (de Sousa *et al.*, 1999), *Anoectochilus formosanus* (Shiau *et al.*, 2002), *Helianthemum bystropogophyllum* (Lopez *et al.*, 2004) *Liparis loeselii* (Illyés *et al.*, 2005) and *Arachnis labrosa* (Temjensangba and Deb, 2005). Growth of seeds and embryos *in vitro* depends on medium composition, especially on sucrose concentration and the nature and concentration of the growth regulators (Blackman *et al.* 1996). Murashige and Skoog medium (MS, 1962), first developed for proliferation of tobacco callus, has been commonly used for seed germination. Since MS medium has a relative high macro and micronutrient content, it is often used in half or quarter strengths. Illyes *et al.* (2005) reported increased germination percentage of *Liparis loeselii* seeds in reduced nutrient media.

In order to induce germination of embryos and seeds on culture medium, gibberellins (GA) and cytokinins have been extensively used (Sharma *et al.* 1996; Miyoshi and Sato, 1997). The use of *Arabidopsis* mutants with lesions in GA biosynthesis has confirmed the key role of endogenous gibberellins in the germination process (Bianco *et al.*, 1996). Blackman *et al.* (1996) showed the significant effect of GA₃ on germination of maize embryos. Van Waes and Debergh (1986) found that cytokinins were essential for the germination of *Cypripedium calceolus* and *Epipactis helleborine*, but not necessary for germination of *Dactylorhiza maculata* and *Listera ovalis*. Seed germination of *Cypripedium candidum* increased with the addition of cytokinins to the culture medium. Miyoshi and Mii (1995) also reported enhanced

seed germination of *Calanthe discolor* with pretreatment in various levels of benzyladenine (BA) or 6-benzylaminopurine (BAP), a common cytokinin used in plant tissue culture. Stewart and Kane (2006) found that zeatin and KN at 1 μ M in the nutrient medium enhanced seed germination of *Habenaria macroceratitis*.

Temperature and light are also critical factors affecting seed germination. Optimum temperature varies with ecotype; at this temperature, seeds are biochemically active and any fluctuation above and below it, retard the rate of biochemical activity, which in turn results in inhibition or slowing of the rate of germination. During the last couple of decades, considerable progress has been made towards the quantification of germination responses to temperature. Several researchers have shown that cardinal temperature and thermal time for the rate of germination depend on species and within species may vary significantly among ecotypes (Ellis *et al.*, 1986; Battaglia, 1994; Mwale *et al.*, 1994; del Monte and Tarquis, 1997). Determination of the cardinal temperature and thermal time for seed germination rate facilitate conservationists to select a suitable agro-climatic zone for introduction of species in field for regeneration and as an *in situ* conservation strategy.

Similarly, the light requirement for optimum germination varies from species to species. Stoutamire (1974) reported that a 12h light/12h dark photoperiod negatively affected germination of several temperate terrestrial orchid species. However, Arditti *et al.* (1981) reported that illumination negatively affected the germination of *Calypso bulbosa* and *Epipactis gigantean*. On the other hand, no difference was found between illumination and complete darkness in seed germination of *Goodyera*, *Piperia*, and *Platanthera* spp. In both studies the exact

length of photoperiod was not included. Subsequently, Oliva and Arditti (1984) reported that photoperiod did not have a significant effect on germination of several species of *Aplectrum*, *Spiranthes*, and *Cypripedium*. Van Waes and Debergh (1986) reported that continual darkness was more beneficial for germination of *Orchis morio* than a 14h light/10h dark photoperiod. Vargas *et al.* (2004) observed that germination percentage of the seeds of *Anthurium andreanum* was better when cultivated under continuous light conditions than those incubated in complete darkness.

The establishment of *in vitro* grown plantlets under *in vivo* conditions which involves the acclimatization of the plantlets to glasshouse conditions is the last but most crucial stage involved in successful micropropagation of plants. *In vitro* raised plants often require extensive hardening treatments to prevent high mortality after transfer to *ex vitro* conditions. Acclimatization can be started while the plantlets are still *in vitro* or when the plantlets are removed from the containers to soil. The transfer of *in vitro* raised seedlings from the culture tubes to the natural conditions requires a careful, stepwise procedure which would enable them to harden and acclimatize to the harsh outside environment therefore leading to better survival. Hardening of the plants is necessary to develop resistance against physical, chemical and biological factors at the time of transferring them into field conditions. The *in vitro* raised plantlets fail to withstand direct exposure to harsher environment outside the *in vitro* regimes due to poorly developed cuticle, stomatal apparatus, photosynthetic ability and conducting tissues (Vij, 1998). Plantlets which are cultured on agar based media, wilt rapidly on transfer to a water deficit atmosphere in the greenhouse or field conditions. The scarcity or poor availability of ground water to the plantlets coupled with the

excessive loss of water through transpiration may lead to high mortality rates when transferred, unless they are subjected to gradual decrease in humidity and increase of temperature. Bhojwani and Razdan (1983) have stressed on high humidity conditions during the initial days for successful transplantation to the field. Temperature also plays a major role in successful acclimatization of plants on transplantation. *In vitro* raised plants are subjected to optimum temperature conditions suitable for their growth and survival when in culture. On transplantation they have to adapt to the outside temperature which may be lower or higher than the optimum temperature. This may not be possible if the plants are suddenly exposed to these conditions. The shock is more severe in the cold regions where the temperature in winter drops to sub zero level. Sudden exposure to high temperature during summer may also result in low survival rate. Hence, temperature should be gradually altered to enable the plantlets to adapt to the changing environment before transferring them to the field. Bordoloi (1977) suggested that temperature requirement for *Nepenthes* should as far as practicable be regulated to 18-29°C in winter and 20-28°C in summer in glasshouses. The types of compost and soil conditions also affect the growth and survival of the *in vitro* raised plantlets. A good growing medium having properties such as maximum water holding capacity, porosity and drainage, is essential for proper growth and development of *in vitro* raised plants. Right stage of transplants, suitable compost, moisture and other physical factors greatly affect the survival rate of plants.

The recovery of threatened species generally hinges on providing suitable habitat and conditions in which they can thrive. Most biodiversity losses can be

directly attributed to habitat loss, so provision of habitat is often the key requirement for recovery of a species. The re-establishment of a large number of micropropagated plantlets into their natural habitat is of prime concern as some endangered plants may not reproduce well by traditional methods (Tandon, 2000). The development of a reliable *in vitro* protocol is of great importance for conservation of rare plant species (Bramwell, 1990) since it is difficult to generalize the cultural practices used. The threatened plants can be multiplied using tissue culture methods and exposed in *ex situ* collections to offset the pressure on the natural populations as well as for medicinal and ornamental purposes. A number of plants have been reestablished in their natural habitat using *in vitro* propagation methods (Tandon *et al.*, 1990; Tandon and Rathore, 1992, 1994; Seeni and Sabu, 1997; Ramsay and Stewart, 1998).

In recent years, the assessment of variation between plants at DNA level has achieved great importance. A success of any genetic conservation programme is dependent on an understanding of the amount and distribution of the genetic variation present in the genepool. Availability of genetic data can help researchers assess the ability of rare plant populations to adapt to changes in their natural environment, to respond to reintroduction programs and natural selection.

A range of plant characters is currently available for distinguishing closely related individuals (Newbury and Ford-Lloyd, 1993). Assessment of variation in plant species traditionally was based on differences in morphological and agronomical characteristics. Although these morphological genetic markers proved useful in several cases (Epling and Dobzhansky, 1942; Faberge, 1943), this approach has certain limitations. Typically, accessions are grown in the field or green house and

estimates of diversity are based on the range of expression of various traits among genotypes. Trait expression is often highly influenced by environmental factors. If the magnitude of environmentally induced variation is large in comparison to genetic variation, diversity estimates based on morphological data may poorly reflect actual levels of genetic diversity among accessions (Yee *et al.*, 1999). Moreover, morphological variants are not available for most species, studies utilizing morphological data are generally limited to one locus and many characters e.g. flower colour have to be scored relatively late in the life cycle (Cruzan, 1998). Karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al.*, 1993). The development of protein based markers i.e. allozymes over three decades ago greatly facilitated the study of genetic variation in plant populations. Isozymes are a class of enzymes that share a common substrate but exhibit different electrophoretic mobilities (Markert and Moller, 1959). When different electromorphs are identified by genetic analysis as allelic, they are called allozymes. Allozymes have been employed to characterize patterns of genetic variation within and among populations and to examine the processes of dispersal and the patterns of mating that influence levels of genetic differentiation (Brown, 1979; Loveless and Hamrick, 1984; Hamrick and Godt, 1989; Barrett and Kohn, 1991). These markers provide a convenient method for detecting genetic changes but are subject to ontogenic variations. Factors such as tissue specific and developmentally controlled expressions as well as environmentally induced expression can cause difficulty in detecting activity. Gene duplications can complicate both interpretation of goals and data analysis, in part because the means by which duplications occur are not always the same (Wendel and Weeden, 1989). Additionally, because allozymes

are often under functional or selective constraints, they may not be the most appropriate markers for estimation of genetic diversity in conservation studies (Mitton, 1994). They are also limited in number and only DNA regions coding for soluble proteins can be sampled (Rout *et al.*, 1998).

Recent advances in techniques for DNA analysis and subsequent data analysis have greatly increased our ability to understand the genetic relationships among organisms at the molecular level. For all the techniques that result in DNA based markers, the first step must be DNA extraction. There are many general references describing extraction methods (Draper and Scott, 1988; Boffey, 1991; Croy *et al.*, 1993; Weising *et al.*, 1995). Different protocols may be followed depending on whether the sample is to be used for hybridization studies, which usually involve microgram quantities, or amplification using polymerase chain reaction (PCR), in which case only a few nanograms of DNA may be required.

The most common DNA based techniques use Restriction Fragment Length Polymorphism (RFLP) and numerous genetic marker assays based on PCR such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP) (Karp *et al.*, 1996). RFLP which is widely used for gene mapping and studying population diversity in plant populations (Bernatsky and Tanksley, 1986; Helentjaris *et al.*, 1986) uses hybridization technology; cloned DNA sequences are labelled and used as probes to identify differences in the sizes of specific genomic DNA fragments following digestion by a restriction endonuclease. The PCR-based markers require very small amounts of DNA, are technically simpler,

cheaper, and less labour intensive than RFLPs. The speed and convenience of PCR reaction permits screening of a larger number of individuals than is possible with other types of DNA markers.

The introduction of PCR (Saiki *et al.*, 1985; Mullis and Faloona, 1987) has thus provided a powerful tool to detect polymorphism at the DNA level. PCR based markers allow the direct comparison of the genetic material of two individual plants avoiding any environmental influences on gene expression (Newbury and Ford-Lloyd, 1993). They are 100% heritable, not affected by environment and available in abundant numbers, which increases the power of discrimination. Polymorphism revealed by molecular markers can be used to construct genetic maps, to determine genetic diversity at DNA level, to determine relatedness or phylogenetic relationship among population and species and in DNA fingerprinting (Rafalski *et al.*, 1991).

Application of molecular markers as complementary approach for genetic characterization has been reported in many crops (Karp *et al.*, 1996; Nagaoka and Ogiwara, 1997; Paul *et al.*, 1997; Liu and Wendel, 2001; Dongre and Parkhi, 2005). Genetic characterization of numerous crop species has been conducted with DNA markers alone or in tandem with morphological analysis (Hill *et al.*, 1996; Sharma *et al.*, 1996). Molecular markers have been widely used to characterize clones in plants (Sheffield *et al.*, 1989; Bayer, 1990; Parker and Hamrick, 1992; Smith *et al.*, 1992; Parks and Werth, 1993; Neuhaus *et al.*, 1993; Hsiao and Rieseberg, 1994; Stiller and Denton, 1995; Waycott, 1995; Ayres and Ryan, 1997; Montalvo *et al.*, 1997; Palombi and Damiano, 2002). Molecular markers have also been extensively used to provide genetic data for protection of rare and endangered plant species like *Calamagrostis*

porteri ssp. *insperata* (Esselman *et al.*, 1999), *Eryngium alpinum* (Gaudeul *et al.*, 2000), *Metrosideros bartlettii* (Drummond *et al.*, 2000), *Podophyllum hexandrum* (Singh *et al.*, 2001), *Juniperus phoenicea* (Meloni *et al.*, 2006). These DNA markers have also enabled researchers to investigate genetic diversity among various plant species across natural populations of which some examples include *Viola pubescens* (Culley and Wolfe, 2000), *Oryza granulata* (Qian *et al.*, 2001), *Asparagus acutifolius* (Sica *et al.*, 2005), *Nymphaea odorata* (Woods *et al.*, 2005). Among these, PCR-based techniques of random multilocus analysis (RAPD, AFLP, ISSR) have been successfully used in genotyping, genome mapping and phylogenetic studies in horticultural crops such as strawberry (Korbin *et al.*, 2002), soybean (Ferreira *et al.*, 2000) and potato (Prevost and Wilkinson, 1999).

RAPD, a rapid and sensitive technique using the PCR was introduced in the early nineties (Williams *et al.*, 1990; Welsh and McClelland, 1990; Welsh *et al.*, 1991). It is based on the use of single oligonucleotide primers of arbitrary sequence that will amplify genomic DNA sequences in PCR whenever a region of homology at favourable distance and in converging orientation is found on the two DNA strands. This technique can be used to determine taxonomic identity, assess kinship relationships, detect interspecific gene flow and analyse hybrid speciation. Advantages of RAPDs include suitability for work on anonymous genomes, applicability to work where limited DNA is available, efficiency and low expense (Hadrys *et al.*, 1992). By using different primers, molecular characters can be generated that are diagnostic at different taxonomic levels. RAPD amplification products can be either variable (polymorphic) or constant (non-polymorphic). In a

RAPD analysis of several individuals within a species, and species within a genus, constant fragments diagnostic for a genus may be identified, as well as fragments which are polymorphic between species of the genus (Hadrys *et al.*, 1992). RAPDs can be applied to analyse fusion of genotypes at different taxonomic levels. At the level of the individual, RAPD markers can be applied to parentage analysis, while at the population level, they can detect hybrid populations, species or subspecies.

For species with limited population size and number, RAPD fingerprinting provides an ideal tool for detecting existing variation within and between constituting individuals (Hadrys *et al.*, 1992; Lynch and Milligan, 1994; Rieseberg and Swensen, 1996). The detection of genotype hybrids relies on the identification of diagnostic RAPD markers for the parental genotypes under investigation (Hadrys *et al.* 1992). RAPD is therefore useful in distinguishing individuals, cultivars or accessions (Karp *et al.* 1996).

RAPD markers have been used successfully to assess genetic stability among micropropagated plants of poplar (Rani *et al.*, 1995), *Zingiber officinales* Rosc. (Rout *et al.*, 1998), *Pinus thunbergii* Parl. (Goto *et al.*, 1998), *Camellia sinenses* and *C. assamica* ssp. *assamica* (Devarumath *et al.*, 2002; Mondal and Chand, 2002), *Dioscorea bulbifera* L. (Dixit *et al.*, 2003) and many other plants. In certain cases, genetic instability of plants derived from meristem cultures has been reported (Zucchi *et al.*, 2002).

It has also been shown to be an effective method for detecting polymorphism in various fruit and crop species (Virk *et al.*, 1995; Penner, 1996; Sun *et al.*, 1999;

Lowe *et al.*, 2000; Guistine *et al.*, 2002; Aras *et al.*, 2005). Devos and Gale (1992) optimized a RAPD protocol in wheat and concluded that though sensitive, RAPD finds many applications in the analysis of genotypes. Yang and Quiros (1992) used RAPD markers for the identification and classification of celery cultivars. Stiles *et al.* (1993) used RAPD markers for evaluating genetic relationships among papaya cultivars and showed that RAPD was a rapid, sensitive and precise technique for genomic analysis. The use of RAPDs in an investigation of the bush mango (*Irvingia gabonensis* and *I. wombolu*) from central/west Africa suggested that RAPD analysis can be used effectively for the initial assessment of levels and partitioning of genetic variation within species for which there is little other genetic diversity information available (Lowe *et al.*, 2000). Guistine *et al.* (2002) analysed the genetic variation of RAPD markers for North American white clover (*Trifolium repens*) collections and cultivars. Upadhyay *et al.* (2004) used RAPD markers to analyze genetic relationship among coconut accessions.

For assessment of genetic diversity, RAPDs have been used within many plant groups, including herbaceous (Dawson *et al.*, 1993; Nolan *et al.*, 1996), woody angiosperms (Schierenbeck *et al.*, 1997; Heaton *et al.*, 1999) and conifers (Bucci and Menozi, 1995; Alnutt *et al.*, 1999a, b, 2001; Gomez *et al.*, 2001).

RAPDs also provide a useful tool for molecular characterization of endemic species and for resolving relationships among populations (Abo-elwafa *et al.*, 1995; Casler *et al.*, 2003; Phan *et al.*, 2003; Fu *et al.*, 2003, Wang *et al.*, 2006; Geleta *et al.*, 2007). Ayres and Ryan (1999) analysed the genetic diversity and structure of the narrow endemic *Wyethia reticulata* and its congener *W. bolanderi* using RAPD and

allozyme techniques. RAPD was used to analyze genetic variation among and within populations of different sizes in *Ranunculus reptans* (Fischer *et al.*, 2000). Hsu *et al.* (2000) found low RAPD polymorphism within existing individuals of a single population of *Archangiopteris itoi*, a rare and endemic fern in Taiwan. RAPDs could differentiate two populations of *Podophyllum hexandrum* (Singh *et al.*, 2001). Chen *et al.* (2005) used RAPD markers to detect genetic variation in populations of *Coelonema draboides*, an endemic plant of the Qilian Mountains in the Qinghai-Tibet Plateau.

RAPD markers were shown to have been sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991; Roy *et al.*, 1992; Abo-elwafa *et al.*, 1995). Kresovich *et al.* (1992) studied RAPD assay for quick, cost effective and reliable method to estimate genetic relationship in *Brassica oleracea* and demonstrated that RAPD markers were able to detect variation at the intraspecific level. Esselman *et al.* (2000) employed RAPD markers to measure genetic diversity within and among species of *Dendroseris*, a genus endemic to the Juan Fernandez Islands, Chile.

RAPDs have been used in many threatened plant taxa for analysis of genetic variation with an aim of conserving them both *in situ* (Hogbin and Peakall, 1999; Newton *et al.*, 1999) and *ex situ* (Virk *et al.*, 1995; Gillies *et al.* 1999; Hsu *et al.*, 2000; Alnutt *et al.*, 2001; Singh *et al.*, 2001; Torres *et al.*, 2003).

RAPD markers have been frequently used to determine the genetic variability of endangered, relict and/or endemic taxa (Stewart and Porter, 1995; Bauert *et al.*,

1998; Liao and Hsiau, 1998; Las Heras *et al.*, 1999; Maki and Horie, 1999). Rossetto *et al.* 1995 used RAPDs to study the genetic variability of *Grevillea scapigera* which helped to devise conservation strategies for a sound recovery plan of this endangered plant. Genetic variation in *Rosmarinus tomentosus* an endangered species endemic to Spain was also analysed using RAPD markers (Martin and Bermejo, 2000). Fu *et al.* (2003) used RAPD markers to measure genetic diversity of *Changium smyrnoides* Wolff (Apiaceae), a genus endemic to eastern China and an endangered medicinal plant. Torres *et al.* (2003) used RAPD markers to study the genetic variability and population structure of an endangered plant, *Antirrhinum microphyllum*, a narrow endemic of central Spain known from only four populations.

The utility of PCR-based RAPD variations as phylogenetic markers has been clearly established (Thorman *et al.*, 1994; Karihaloo *et al.*, 1995; Maugham *et al.*, 1995; Spooner *et al.*, 1997; Ghislain *et al.*, 1995; Roman *et al.*, 2003). Despite questions about its reproducibility, its utility in diversity analysis, mapping and genotype identification has been exploited in many plant species (Weining and Langridge 1991; Weising *et al.*, 1995; Wenguang *et al.*, 1998; Fahima *et al.*, 1999; Harris, 1999; Colombo *et al.*, 2000; Wang *et al.*, 2002; Aga *et al.*, 2003; Fu *et al.*, 2003; Kaminski *et al.*, 2003).

Estimations of genetic diversity are very important in programmes for the conservation of biodiversity. In some situations, such as when the habitat is being rapidly destroyed, it is futile to be concerned with long-term goals such as genetic variability analysis (Haig, 1998) and it would be better to concentrate resources on the effective protection of the environment instead of conducting genetic studies.

However, genetic diversity is important for the population to be able to face future environmental changes and to ensure a long-term response to selection (Gilpin and Soule, 1986; Hunter, 1996; Frankham *et al.*, 2002; Fernandez *et al.*, 2004). Genetic markers are assumed to reflect adaptive genetic variation that is important to the evolutionary potential of the species (Hunter, 1996; Frankham *et al.*, 2002) and consequently, the selection of useful molecular markers is necessary to conduct these studies. RAPD may be just such a technique as it could readily be used to select priority areas for conservation, to design and monitor management strategies in protected areas and to plan conservation strategies for a wide range of plant and animal genetic resources.

CHAPTER III: *IN VITRO* SEED GERMINATION OF *NEPENTHES KHASIANA* AS INFLUENCED BY DIFFERENT MEDIA AND PHYSICAL FACTORS

Introduction

Seed imbibition and germination are basic processes in the growth and development of higher plants (Bewley and Black, 1994; Kigel and Galili, 1995; Lang, 1996; Bewley, 1997). Germination is regarded as 'events which commence with the uptake of water by a quiescent dry seed and terminates with the elongation of the embryonic axis' (Bewley, 1997). The visual cue for the completion of germination and the beginning of seedling establishment is the appearance of the radicle. Germination is considered as a triphasic event comprising an initial period of water uptake, followed by a lag phase during which all the metabolic events leading up to radicle emergence occur and ending with the elongation of the embryonic axis and radicle emergence (Bewley and Black, 1982). Seed germination and its regulation have important implications in the fields of horticulture, agriculture, human nutrition, ecology, and conservation biology. Seeds show remarkable variation in germination (Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1994; Kigel and Galili, 1995; Lang, 1996). The diversity of seed germination is, in part, due to great selective pressure placed on seed quiescence, germination, and seedling establishment in nature (Yang *et al.*, 1999). A completely non-dormant seed has the capacity to germinate

over the wide range of normal physical environmental factors possible for the genotype (Finch-Savage and Leubner-Metzger, 2006). Besides the basic requirements for water, oxygen and an appropriate temperature the seed may also be sensitive to light and/or nitrate.

In vitro seed germination is a concerted biotechnological approach for mass propagation and conservation of many rare and endangered plants. It allows the rescue and recovery of plant species with non-germinating or poorly germinating seeds. Seeds are preferred to vegetative material as the source of propagation material for multiplication of rare and endangered species as they are heterozygous so a wider genetic base can be maintained which would ensure genetic diversity. *In vitro* seed germination has been extensively used for multiplication of a number of plant species (Mohan and Jorapur, 1984; Yam and Weatherhead, 1988; Sharma and Tandon, 1987, 1990; Kumaria and Tandon, 1991; Kondo *et al.*, 1997; Iankova *et al.*, 2001, Vargas *et al.*, 2004). The application of *in vitro* seed propagation technique to rare and endangered species, which are suffering from over-collection and continuous loss of their natural habitats, undoubtedly is a powerful tool for *ex situ* biodiversity conservation (Stenberg and Kane, 1998; Gangaprasad *et al.*, 1999). Germination of seeds *in vitro* has been successfully carried out for many rare, threatened and endangered species (Iriondo *et al.*, 1995; Allen, 1998; Gangaprasad *et al.*, 1999; de Sousa *et al.*, 1999; Shiau *et al.*, 2002; Lopez *et al.*, 2004; Illyés *et al.*, 2005; Temjensangba and Deb, 2005). Growth of seeds and embryos *in vitro* depends primarily on medium composition (Blackman *et al.*, 1996). Physical factors such as

pH, temperature and light are also critical factors affecting germination of seeds. These requirements vary from species to species.

The propagation of *N. khasiana* is mainly vegetative as seed germination is very poor in nature. Moreover, the seeds take as long as 223 days to germinate in nature (Bordoloi, 1977). Although multiplication of *N. khasiana* through enhanced axillary branching *in vitro* has been tried earlier (Rathore *et al.*, 1991; Tandon and Rathore, 1994; Latha and Seeni, 1994), the method does not provide maintenance of variability required for conservation strategy. Hence, propagation of this rare and endangered plant through *in vitro* seed germination would fulfill the demand of plants on large scale for conservation.

Materials and Methods

Seed source

Mature seeds were obtained from the plants growing in nature from Jarain, Jaintia Hills, Meghalaya, India. Immediately after collection, capsules were wrapped in blotting sheets and allowed to dry at 4°C for two weeks. The seeds were then transferred to polythene bags, sealed and stored at 4°C in darkness. Moisture content of the seeds was estimated just after collection following the International Seed Testing Association protocol (ISTA, 1985):

$$\text{Moisture Content} = \frac{W_2 - W_1}{W_2} \times 100$$

Where, W_1 and W_2 are the fresh weight and dry weight of the seeds respectively.

One hundred seeds were taken per set and 10 replicates were used.

Effect of different media

The effect of three basal media i.e., Murashige and Skoog (MS, 1962), Gamborg *et al.* (B₅, 1968) and Nitsch and Nitsch (NN, 1969) on *in vitro* seed germination was assessed. Half and one-fourth strengths of the major and minor salts of each of the media tried were also used (vitamins and sucrose were incorporated in full) (Table 3.1, 3.2). Additional adjuvants like ascorbic acid (50.0 mg l⁻¹) and citric acid (10.0 mg l⁻¹) were added to the medium (Rathore *et al.*, 1991). Agar (0.8%) was added to the media and the pH was adjusted to 5.8 with 0.1N NaOH before autoclaving. The media were dispensed into 150 ml conical flasks and autoclaved at 1.06 Kgcm⁻² and 121°C for 15 min.

The rest of the steps for culture of seeds were carried out aseptically in the transfer table of the laminar flow cabinet. Before using the transfer table, the laminar flow chamber was thoroughly scrubbed with cotton soaked in 70% ethanol. The ultra violet (UV) tube light of 30 watt was switched on for 15-20 min after which the transfer area was left as such for about 4-5 min with the flow of the ultra filtered sterile air to strain out particles as small as 0.3 mm providing a sterile atmosphere for work. The velocity of the air flow which is 27 ± 3 m/min helped in preventing airborne contamination. Forceps, needles, surgical blades, etc. were sterilized by dipping in 70% alcohol and flamed before use for proper sterilization. Sterile media were dispensed as 15 ml aliquots into 60 mm diameter petri plates (Tarsons). Seeds were surface sterilized with 15% sodium hypochlorite (4% w/v available chlorine) for 5min and rinsed thoroughly 3-4 times with sterile distilled water. These were then dried by placing on sterilized filter paper before inoculation. An average of 30 surface

Table 3.1. Composition of culture media used (concentration in mg l⁻¹)

Components	MS	½ MS	¼ MS	B ₅	½B ₅	¼B ₅	NN	½ NN	¼ NN
Macronutrients									
NH ₄ NO ₃	1650	825	412.5	-	-	-	720	360	180
KNO ₃	1900	950	475	2500	1250	625	950	475	237.5
CaCl ₂ .2H ₂ O	440	220	110	150	75	37.5	166	83	41.5
MgSO ₄ .7H ₂ O	370	185	92.5	250	125	62.5	185	92.5	46.25
KH ₂ PO ₄	170	85	42.5	-	-	-	68	38	19
(NH ₄) ₂ SO ₄	-	-	-	134	67	33.5	-	-	-
NaH ₂ PO ₂ .H ₂ O	-	-	-	150	75	37.5	-	-	-
Micronutrients									
KI	0.83	0.415	0.2075	0.75	0.375	0.1875	-	-	-
H ₃ BO ₃	6.2	3.1	1.55	3	1.5	0.75	10	5	2.5
MnSO ₄ . 4H ₂ O	22.3	11.1	5.575	-	-	-	-	-	-
MnSO ₄ . H ₂ O	-	-	-	10	5	2.5	18.9	9.45	4.725
ZnSO ₄ . 7H ₂ O	8.6	4.3	2.15	2	1	0.5	10	5	2.5
Na ₂ MoO ₄ .2H ₂ O	0.25	0.125	0.0625	0.25	0.125	0.0625	25	12.5	6.25
CuSO ₄ . 5H ₂ O	0.025	0.0125	0.00625	0.025	0.0125	0.00625	0.025	0.0125	0.00625
CoCl ₂ . 6H ₂ O	0.025	0.0125	0.00625	0.025	0.0125	0.00625	-	-	-
FeSO ₄ . 7H ₂ O	27.8	13.9	6.95	27.80	13.9	6.95	27.8	13.9	6.95
Na ₂ EDTA. 2H ₂ O	37.3	18.65	9.325	37.30	18.65	9.325	37.3	18.65	9.325
Organic constituents									
Inositol	100	100	100	100	100	100	100	100	100
Nicotinic acid	0.5	0.5	0.5	-	-	-	5	5	5
Pyridoxine HCl	0.5	0.5	0.5	-	-	-	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	0.1	10	10	10	0.5	0.5	0.5
Glycine	2	2	2	2	2	2	2	2	2
Folic acid	-	-	-	-	-	-	0.5	0.5	0.5
Biotin	-	-	-	-	-	-	0.5	0.5	0.5
Sucrose	3%	3%	3%	2%	2%	2%	2%	2%	2%

sterilized seeds was placed per plate with the help of the sterilized forceps. Ten replicate plates were inoculated per medium type and each was sealed with a single layer of parafilm (Pechiney plastic packaging, Menasha, W.I) to avoid media dehydration and contamination during the culture period. The cultures were incubated at $25 \pm 2^\circ\text{C}$ under white fluorescent light under $150 \mu\text{molesm}^{-2}\text{s}^{-1}$ with a 12h photoperiod per day. Seeds were observed daily for germination. Seeds were considered to have germinated with the emergence of the radicle. The percentage seed germination was recorded every 30 days of culture. The data obtained was subjected to statistical analysis using Student's 't' test.

Effect of different physical factors

The seeds were surface sterilized as described and inoculated on $\frac{1}{4}$ MS medium (vitamins and sucrose were incorporated in full) without growth regulators. Ascorbic acid (50.0 mg l^{-1}) and citric acid (10.0 mg l^{-1}) were added in the medium. The cultures were subjected to different physical factors such as temperature (0, 8, 16, 24, 32°C), light intensity (0, 50, 100, 150, $200 \mu\text{molesm}^{-2}\text{s}^{-1}$), photoperiod (0, 6, 12, 18, 24 h day) and pH of the medium (3.8, 4.8, 5.8, 6.8 and 7.8). The percentage germination was recorded at monthly intervals and the data was subjected to statistical analysis using Student's 't' test.

Results

The moisture content of the collected seeds was found out to be 10 - 12 %.

Effect of different media

Seed germination commenced within 30 days after inoculation on MS, $\frac{1}{2}$ MS, B₅ and $\frac{1}{2}$ B₅ media with the highest percentage and early germination (17%) recorded on $\frac{1}{2}$ B₅ (Table 3.3, Photoplate 3.1). Seeds took more time to germinate on the other media tried. By 60 days of culture, more seeds had germinated on the different media and highest germination (42%) was recorded on $\frac{1}{2}$ MS medium followed by $\frac{1}{2}$ B₅ (41%). Seed germination did not remain constant till 90 days of culture. After 90 days, the best germination of *N. khasiana* seeds was recorded on $\frac{1}{4}$ MS (70%) followed by $\frac{1}{2}$ MS (47%) and $\frac{1}{2}$ B₅ (44%). This was significantly higher when compared with all the other media tried. NN medium was not found to be much helpful in germination of *N. khasiana* seeds. A maximum of only about 29% of the seeds germinated in $\frac{1}{2}$ NN after 90 days of culture. The seeds cultured on full strength MS, B₅ and NN showed only 4%, 7% and 20% germination respectively. In all these media, there was no further increase in germination percentage after 60 days of culture. Comparatively, a large number of seeds still germinated even after 60 days in $\frac{1}{4}$ MS, $\frac{1}{4}$ B₅ and $\frac{1}{4}$ NN. After 90 days, no further increase in germination percentage was recorded in any of the media tried.

Effect of temperature

When the cultured seeds were incubated at different temperatures, no initiation of germination was observed within 30 days of culture (Table 3.4; Photoplate 3.2).

Table 3.3. Effect of different media on seed germination

Media	Average Germination (%)		
	(Days)		
	30	60	90
MS	2 ± 1.9 ^a	4 ± 0.8 ^a	4 ± 0.8 ^a
½ MS	7 ± 0.5 ^a	42 ± 1.7 ^b	47 ± 2.3 ^b
¼ MS	-	36 ± 1.6 ^c	70 ± 1.8 ^c
B ₅	6 ± 1.1 ^a	7 ± 1.9 ^a	7 ± 1.9 ^a
½ B ₅	17 ± 7.4 ^a	41 ± 1.1 ^{bd}	44 ± 4.4 ^{bd}
¼ B ₅	-	27 ± 3.6 ^c	34 ± 6.1 ^c
NN	-	20 ± 3.8 ^{ef}	20 ± 3.8 ^f
½ NN	-	23 ± 6.6 ^{efg}	29 ± 4.8 ^{eg}
¼ NN	-	1 ± 0.8 ^h	14 ± 1.8 ^h

± S. E.

In each column significant differences are indicated with different letters ($\alpha = 0.05$)

- No germination

Photoplate 3.1

Seed germination in different media after 90 days

(a) MS (b) $\frac{1}{2}$ MS (c) $\frac{1}{4}$ MS (d) B₅ (e) $\frac{1}{2}$ B₅ (f) $\frac{1}{4}$ B₅ (g) NN (h) $\frac{1}{2}$ NN (i) $\frac{1}{4}$ NN

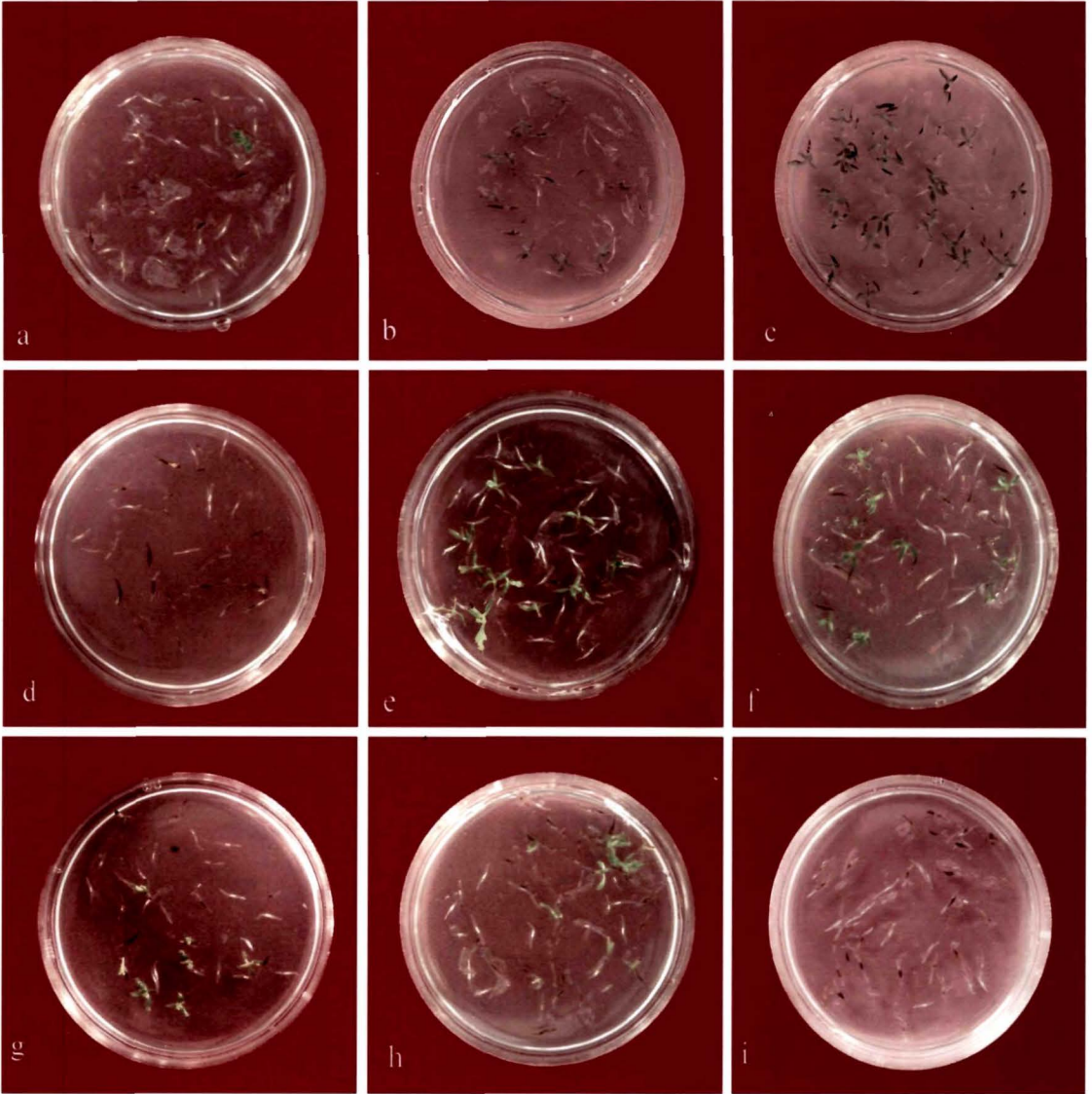


Table 3.4. Germination of seeds inoculated in ¼ MS medium as influenced by different physical factors

	Treatments	Average germination (%)		
		(Days)		
		30	60	90
Temperature (°C)	0	-	-	-
	8	-	-	-
	16	-	13 ± 2.9 ^b	15 ± 3.8 ^b
	24	-	36 ± 1.6 ^a	67 ± 1.5 ^a
	32	-	20 ± 3.3 ^b	20 ± 3.3 ^b
pH	3.8	-	34 ± 2.2 ^a	57 ± 1.9 ^a
	4.8	-	37 ± 3.8 ^a	60 ± 1.9 ^{abc}
	5.8	-	34 ± 3.8 ^a	68 ± 2.2 ^{bd}
	6.8	-	31 ± 2.9 ^a	57 ± 6.1 ^{acde}
	7.8	-	14 ± 1.1 ^b	49 ± 1.3 ^{ae}
Light Intensity (µmolesm ⁻² s ⁻¹)	0 - 2	-	-	1 ± 1.1 ^a
	50	-	3 ± 1.9 ^{ab}	3 ± 1.9 ^a
	100	-	14 ± 4.8 ^{bcd}	35 ± 1.3 ^b
	150	-	30 ± 5.7 ^{cd}	68 ± 3.0 ^c
	200	20 ± 0.0	23 ± 3.8 ^d	25 ± 2.9 ^d
Photoperiod (h day)	0	-	-	1 ± 1.1 ^a
	6	-	2 ± 1.1 ^a	2 ± 1.1 ^a
	12	-	30 ± 5.1 ^b	66 ± 3.3 ^b
	18	-	68 ± 1.1 ^c	70 ± 1.1 ^{bc}
	24	-	51 ± 4.0 ^d	67 ± 2.9 ^c

± S. E.

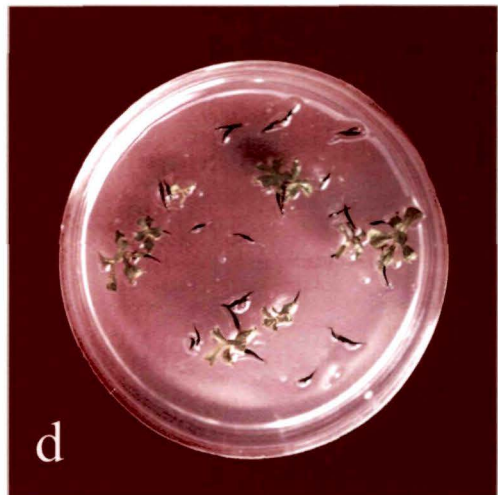
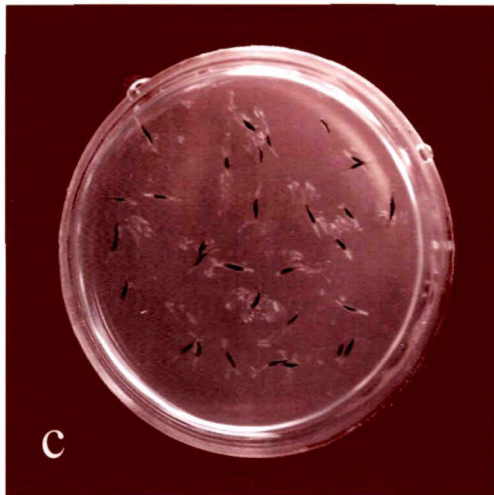
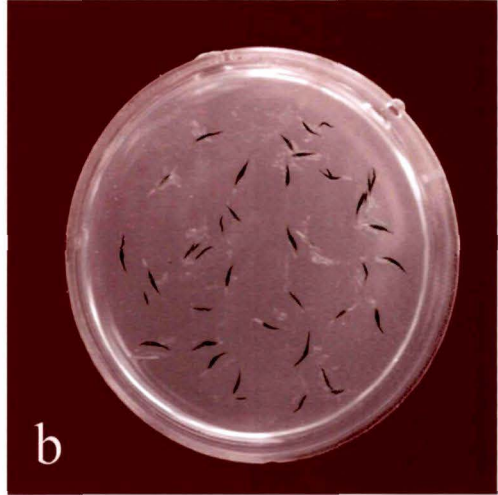
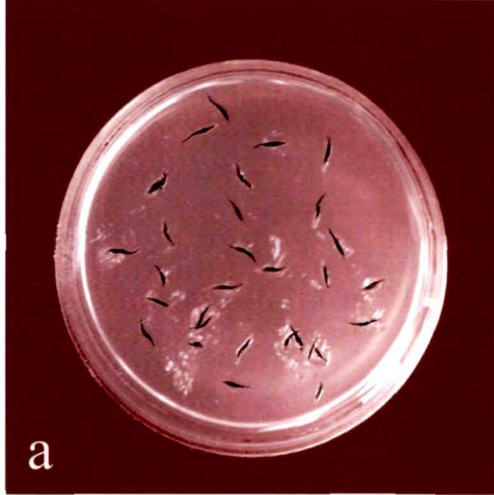
Under each column and treatment, same letters indicate insignificant difference ($\alpha = 0.05$)

- No germination

Photoplate 3.2

Seed germination of *N. khasiana* in ¼ MS medium at different temperature after 90 days of culture

(a) 0 °C (b) 8 °C (c) 16 °C (d) 24 °C (e) 32 °C



However, observations taken after 60 days showed that some of the seeds incubated at 16°C, 24°C and 32°C had germinated with maximum being 36% at 24°C. Maximum germination of 67 % was achieved after 90 days of culture at the same temperature. This increase of germination was significantly higher when compared to those incubated at both 16°C and 32°C. Only about 15% and 20% seed germination were observed for seeds incubated at 16°C and 32°C respectively. The seeds which were incubated at lower temperatures 0°C and 8°C did not germinate even after 90 days of culture.

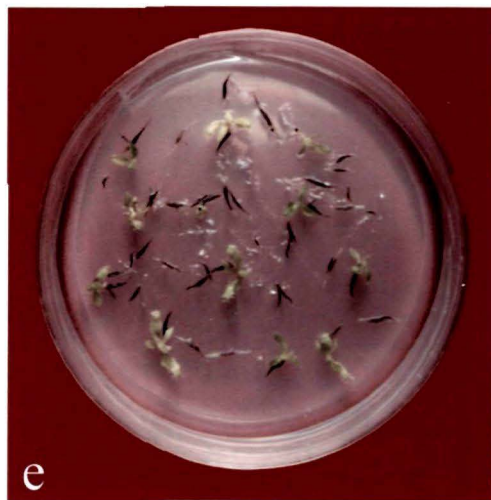
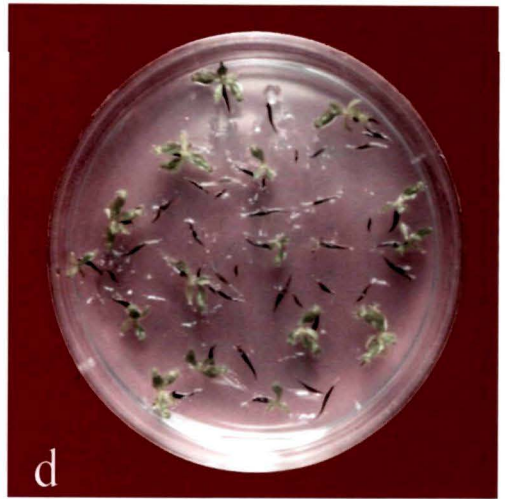
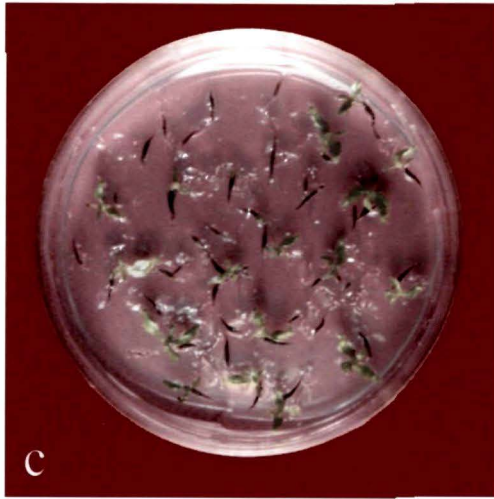
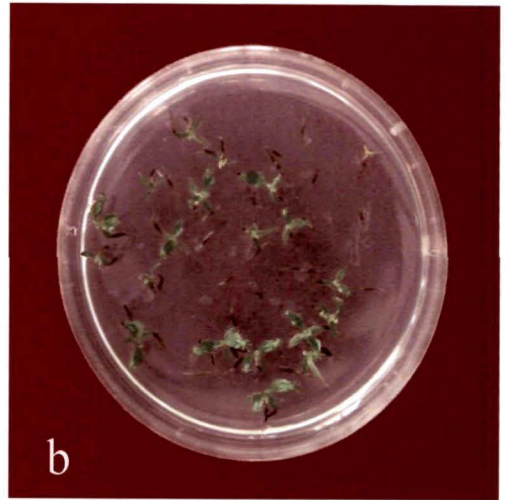
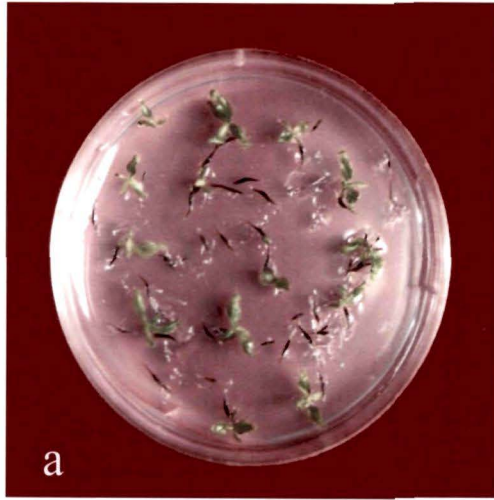
Effect of pH

The germination of seeds was observed at all the different pH ranges. The seeds started to show initial signs of germination in different pH only after 30 days of culture (Table 3.4). Records taken after 60 days of culture showed that all pH treatments supported initiation of seed germination. There was no significant difference between the germination percentages at pH 3.8, 4.8, 5.8 and 6.8 with germination percentage ranging between 31% and 37%. At pH 7.8, only about 14% of the seeds were observed to have germinated after this period. Observations taken after 90 days revealed a substantially good number of germinated seeds under varying pH levels with best germination percentage of 68% recorded at pH 5.8 followed by 60% at pH 4.8. However, there were no significant differences in seed germination at other pH ranges (Photoplate 3.3).

Photoplate 3.3

Seed germination of *N. khasiana* in $\frac{1}{4}$ MS medium of different pH after 90 days of culture

(a) 3.8 (b) 4.8 (c) 5.8 (d) 6.8 (e) 7.8



Effect of light intensity

Seeds were incubated under different light intensities to determine the optimum intensity required for germination of *N. khasiana* seeds. Within 30 days of culture, only light intensity of $200 \mu\text{molesm}^{-2}\text{s}^{-1}$ supported early initiation of germination where about 20% of the inoculated seeds germinated (Table 3.4). None of the seeds incubated under other light intensities germinated within this period. After 60 days, records indicated that all light intensities except $0 - 2 \mu\text{molesm}^{-2}\text{s}^{-1}$ enhanced seed germination with best being $150 \mu\text{molesm}^{-2}\text{s}^{-1}$ showing about 30% germination. After 90 days of culture, even at $0 - 2 \mu\text{molesm}^{-2}\text{s}^{-1}$, about 1% of the seeds germinated. Only 3% of the seeds germinated under $50 \mu\text{molesm}^{-2}\text{s}^{-1}$. Best germination percentage of 68% was observed at $150 \mu\text{molesm}^{-2}\text{s}^{-1}$ which was significantly higher when compared with all other treatments (Photoplate 3.4).

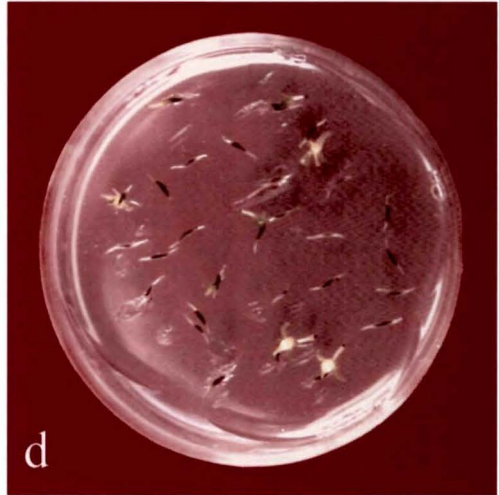
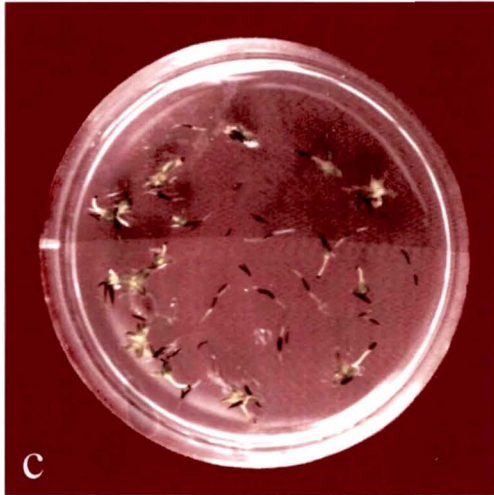
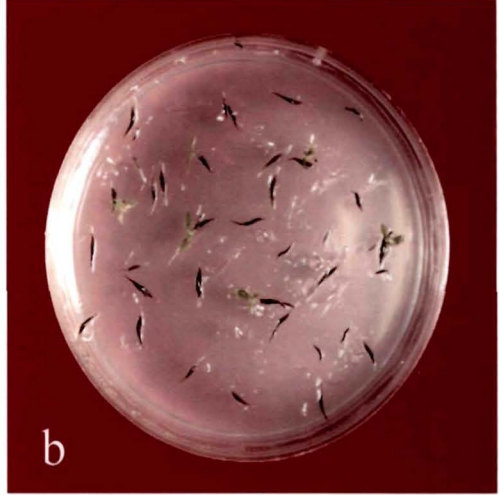
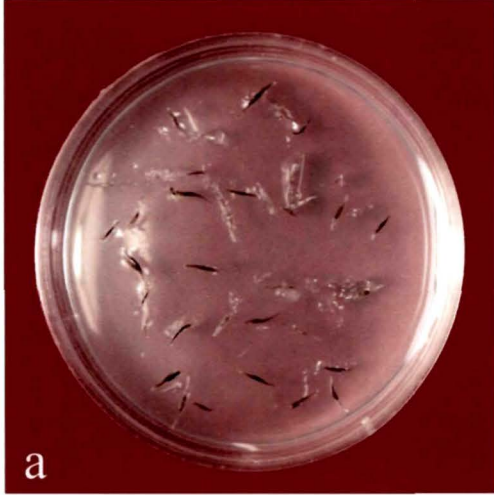
Effect of photoperiod

Seeds were incubated at $150 \mu\text{molesm}^{-2}\text{s}^{-1}$ light intensity for different photoperiod to determine the optimum photoperiod requirement for their germination. None of the seeds germinated within 30 days of culture (Table 3.4). Records taken after 60 days of culture revealed that in the dark, no germination occurred. Under different photoperiods, germination occurred with the maximum being 68% under 18h photoperiod followed by 51% under 24h and 30% under 12h photoperiod (Photoplate 3.5). Only 2% of the seeds germinated under 6h photoperiod after 60 days of culture. When observed after 90 days of culture, 1% germination was recorded in darkness and 2% of the seeds were seen to have germinated under 6h photoperiod.

Photoplate 3.4

Seed germination of *N. khasiana* in ¼ MS medium at different light intensities after 90 days of culture

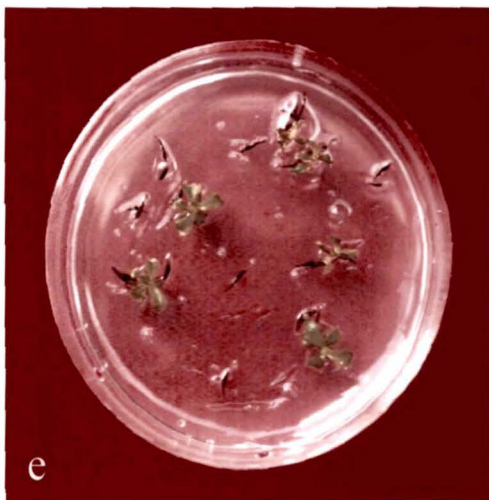
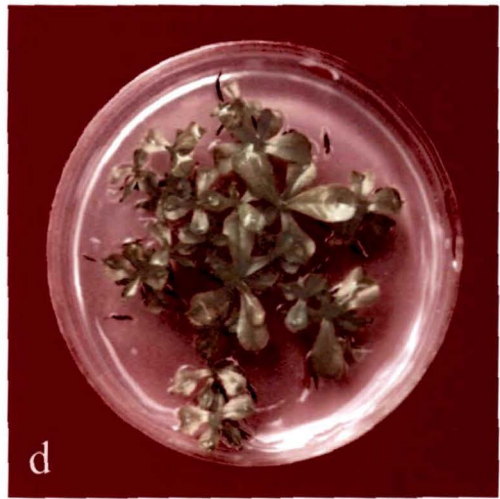
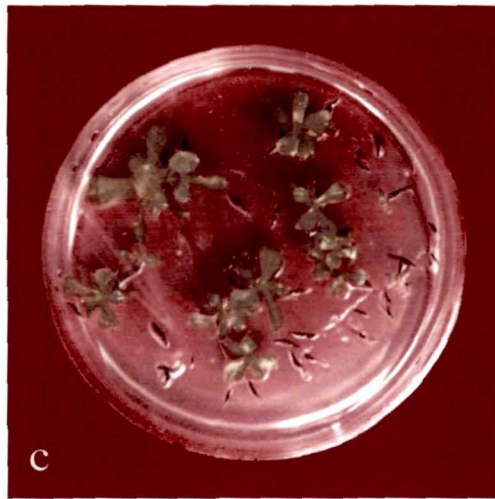
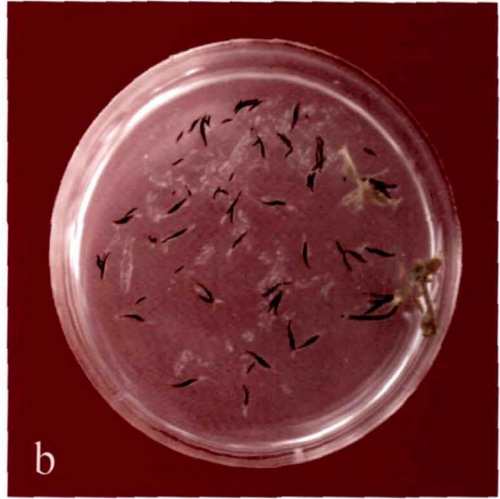
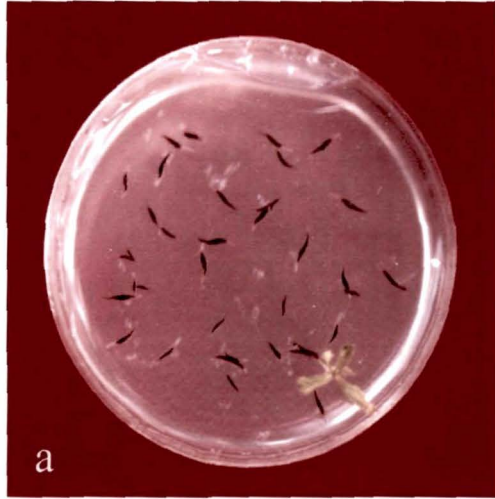
(a) 50 $\mu\text{molesm}^{-2}\text{s}^{-1}$ b) 100 $\mu\text{molesm}^{-2}\text{s}^{-1}$ (c) 150 $\mu\text{molesm}^{-2}\text{s}^{-1}$ (d) 200 $\mu\text{molesm}^{-2}\text{s}^{-1}$



Photoplate 3.5

Seed germination of *N. khasiana* in $\frac{1}{4}$ MS medium at different photoperiod after 100 days of culture

(a) 0 h (b) 6 h (c) 12 h (d) 18 h (e) 24 h



With increase of hours of light per day, the germination percentage increased. The maximum overall germination was found to be 70% under 18h photoperiod after 90 days of culture. Seeds incubated under 12h and 24h photoperiod also showed a substantially good percentage of germination i.e. 66% and 67% respectively after 90 days of culture. There was no further increase of germination percentage after this time period.

Discussion

As *in vitro* cultures are normally axenic, it is necessary to eliminate possible contaminants from the surface of the explants. Such contaminants include fungal, bacterial and algal spores. Surface sterilization of *N. khasiana* seeds was accomplished by treating with 15% Sodium Hypochlorite (4% w/v available chlorine) for 5 min and rinsing thoroughly 3-4 times with sterile distilled water. Various sterilants are used for surface sterilization of explants, the most commonly used compound being sodium hypochlorite. Other sterilants used include calcium hypochlorite, mercuric chloride and hydrogen peroxide. A wetting agent such as Tween 80 is often included to improve contact between the sterilant and the plant tissue. In general, seeds can be subjected to more stringent conditions than most vegetative material (George, 1993). The selection of suitable sterilizing agent depends upon factors like nature of explants, extend of surface microflora and the sensitivity of explant tissues to various sterilizing agent, treatment time, concentration of sterilizing agent, etc. These are usually determined by trial and error methods. The treatment time and concentration of sterilants vary considerably with different species or nature of explants used (Sakamura and Suga, 1989; Nadgauda *et al.*, 1997).

The composition of culture media is one of the most important criteria for the successful establishment of cultures (McCown and Sellmer, 1987). In the present study, seed germination of *N. khasiana* is significantly affected by the type of the culture media used. Seed germination was initiated in MS and B₅ media within 30 days of culture. This suggests that media constituents in any form and concentration did not affect the initiation of seed germination in *N. khasiana*. However, observations recorded at 60 and 90 days of culture showed that the seeds cultured on full strength media did not germinate further after 60 days of culture. But, subsequent increase of germination percentage with time was found in reduced strengths of nutrient media, suggesting that *N. khasiana* seeds rely on lower concentration of nutrients to support enhanced germination. After 90 days, the best germination percentage of *N. khasiana* seeds was recorded on ¼ MS followed by ½ MS and ½ B₅. All media used in the present study contained a nitrogen source but the form and concentration in which it was present differed among the media. While inorganic nitrogen source was reduced in the reduced nutrient media tried, vitamins and sucrose were incorporated in full strength. Ammonium content was highest in MS, and may have contributed to the maximum overall germination rate. It is a well known fact that the rapid assimilation and utilization of ammonium nitrogen in most plants provides for less inhibition of germination as compared to nitrate forms which accumulates in toxic quantities (Raghavan and Torrey, 1964). Nitrogen, phosphorus, potassium, calcium, magnesium and sulphur have long been known to be indispensable for plant growth, and chlorine and sodium are normally included, although their role is less clear (George and Sherrington, 1984). Nearly all media contain salts of these elements, but the concentrations of these salts used vary between formulations. The

form of nitrogen appears to be more critical than for most other elements. Brown *et al.* (1995) reported that across all the plant species, half of the media used for induction of seed germination are MS based media. Half strength MS medium could promote the seed germination of *Haemaria discolor* (Chou and Chang, 1999; Chang and Chou, 2001; Shiau *et al.*, 2005) and *Anoectochilus formosanus* (Shiau *et al.*, 1995; Shiau *et al.*, 2002). NN medium was not found helpful in germination of *N. khasiana* seeds. Gamborg *et al.* (1976) reported that micronutrients such as boron, manganese, zinc, molybdenum, copper, cobalt, and iron are required in plant tissue culture media. All these micronutrients are found in MS and B₅ while cobalt is not present in NN media, which could have been the reason for *N. khasiana* seeds not responding to this medium.

Minor minerals are essential for the normal physiological functioning of plants. For example, iron, manganese, zinc, molybdenum and copper are essential for chlorophyll synthesis and chloroplast function, boron is involved in meristematic activity, and cobalt is the metal component of Vitamin B₁₂. Iron is often used in a chelated form to keep it in solution. Other elements used in media include aluminium, nickel and iodine (George and Sherrington, 1984). Nearly all plant tissue cultures are grown on media containing a sugar as an energy source, as very few cell and tissue lines had been shown to be fully autotrophic *in vitro*, i.e., they are not capable of satisfying their own carbohydrate requirements by photosynthesis. Sucrose is the most frequently used sugar, but glucose and other sugars are also used. Most media contain small quantities of other organic compounds, including vitamins, myo-inositol and amino acids. In general, intact plants synthesize these compounds themselves, but

these are beneficial or necessary in at least some tissue cultures. Myo-inositol and thiamine are normally considered to be the two most important compounds in this group.

Explant browning and media staining are major obstacles common in initial establishment of cultures. In the present study, the addition of adjuvants like ascorbic acid and citric acid to the medium were helpful in checking phenolic exudates and browning of the medium at the time of germination (Rathore *et al.*, 1991). Browning of cultures has been reported to occur through action of copper containing enzymes such as polyphenol oxidases and tyrosinase. The oxidation of polyphenols by polyphenolic oxidases inhibits enzyme activity by killing the explants (Lerch, 1981). To reduce phenol induced browning, explants in a number of plant species have been pretreated with polyvinylpyrrolidone (PVP) (Rao, 1986; Amin and Jaiswal, 1987; Gautham *et al.* 1993; Matthew, 1993). In several cases, browning could be reduced by maintaining the cultures in darkness for an initial period of one week followed by frequent subculture (Hu and Wang, 1983; Amin and Jaiswal, 1987).

Since different plants are adapted to variable habitats, optimal *in vitro* culture conditions suitable for one species may differ for other species. Some of such culture conditions are physical factors like temperature, light and pH. Although the role of these physical factors is important, their effects have not been explored in germination of *N. khasiana*. The present study determined the conditions most suitable for germination of *N. khasiana* seeds. For every plant species, there is an optimal soil temperature for germination, and at that temperature, the maximum number of seeds germinate and in less time than that at any other temperature. As the temperature

declines or increases from the optimal temperature, two things happen at the same time. While the percentage of seeds to germinate decreases, the number of days to germination increases. In the present study, no germination occurred at 0°C and 8°C but with the increase in temperature to 24°C, germination increased. Above this temperature, which is the optimum for seed germination of *N. khasiana*, the percentage of germination decreased. The minimum temperature required for seed germination for most plant species lies between 16-24°C. But in case of *N. khasiana* seeds, at 16°C only 15% germination was recorded. About 20% of the seeds which had been kept at 32°C germinated. However, this was significantly lower than that recorded at 24°C. This indicates that both low and high temperatures inhibited germination of *N. khasiana* seeds. Similar results were obtained by Norsworthy and Oliviera (2005) wherein about 70% of the seeds of *Cassia occidentalis* germinated at temperatures ranging from 12.5°C to 30°C with highest being at 25°C. Hanson and Johnson (2005) also reported 25°C as the optimum temperature for germination of *Panicum virgatum* seeds. Burke *et al.* (2002) reported a maximum of about 42% germination in *Dactyloctenium aegyptium* at a constant temperature of 30°C. Seeds of this species were reported to have germinated at constant temperatures ranging from 15°C to 40°C. However with alternating temperatures of 20°C and 35°C, germination could be increased to 94%. Both alternate (25°C/35°C) and constant (23±2°C) temperatures, along with a 16h photoperiod, promoted *in vitro* germination of *Passiflora caerulea* (Severin *et al.*, 2003). For many species, alternating temperatures are optimal for germination (Baskin and Baskin, 1998). But in the case of *N. khasiana*, alternate temperature treatment was not required as a constant temperature of 24°C could induce 70% of the seeds to germinate.

The results obtained in the present study showed that the rate of germination was not significantly affected by the pH of the medium. The germination of seeds was observed in all the different pH treatment and was initiated after 30 days of culture. Records taken after 60 days of culture showed that all pH treatments supported initiation of seed germination. There was no significant difference between the germination percentages at pH 3.8, 4.8, 5.8 and 6.8 with germination percentage ranging between 31% and 37%. At pH 7.8, only about 14% of the seeds were observed to have germinated after this period. Observations taken after 90 days revealed a substantially good number of seed germination in all treatments of pH. Highest germination percentage of 68% was recorded at pH 5.8 followed by 60% at pH 4.8. Requirements in terms of pH of the medium also differ for different species. Singh *et al.* (1975) reported better seed germination for *Iseilema antheophorodes* and *Sehima nervosum* in acidic pH. Burke *et al.* (2002) reported that highest germination percentage of *Dactyloctenium aegyptium* was at pH 4.0 and 5.0 and germination decreased as pH increased. However, Freeman (1973) reported highest germination of *Fouquieria splendens* at pH 7.5. Mitchell *et al.* (2000) reported lowest percentage germination of *Viola cornuta* at pH 2.0 and highest at pH 9.0. Most of the seeds of *Cassia occidentalis* germinated at pH 6.0 and germination percentage decreased at lower and higher pH levels (Norsworthy and Oliviera, 2005). In case of *N. khasiana*, acidic pH did not have any significant effect on either overall germination percentage or rate of germination. However, higher pH affected the seed germination. The germination declined with the increase of pH to 7.8. As *N. khasiana* is known to grow in acidic soil, these findings suggest that the germination behavior of the seeds in nature may be dependent on other factors.

In the present study, among all light treatments, seed germination was significantly higher at 150 $\mu\text{molesm}^{-2}\text{s}^{-1}$ light intensity. Light intensities lower and higher than this intensity did not enhance *in vitro* seed germination. However, high light intensity of 200 $\mu\text{molesm}^{-2}\text{s}^{-1}$ induced earlier seed germination. Since the medium was always the same, this acceleration of germination has to be attributed to the effect of high light intensity. However, the overall germination percentage of the seeds exposed to this light intensity was significantly reduced. Seeds of different species respond differently to different light treatments. *N. khasiana* showed considerable preference for light for seed germination as there was only 1% germination in darkness. Lower intensity of 50 $\mu\text{molesm}^{-2}\text{s}^{-1}$ was not helpful as seeds showed poor germination. Photoperiod of 6h also was not sufficient to induce seed germination as only 2% of the seeds germinated. Longer photoperiod (12-24h) could not accelerate germination time however they supported higher germination capacity.

The seeds of some plants require long exposure to light for germination (Isikawa and Shimogawara, 1954; Njoku, 1959; Edwards 1975; Okusanya 1978; 1979). Seeds of *Anthurium andreanum* are reported to germinate under continuous light conditions (Vargas *et al.*, 2004). However, seed germination of some other plants increases under complete darkness compared to illumination (Oliva and Arditti, 1984; Van Waes and Debergh, 1986). Time period of exposure to light also play an important role in germination of seeds. Oliva and Arditti (1984) reported that photoperiod did not have a significant effect on germination of several species of *Aplectrum*, *Spiranthes*, and *Cypripedium*. Stoutamire (1974) reported that a 12h light/12h dark photoperiod negatively affected germination of several temperate

terrestrial species. Van Waes and Debergh (1986) reported that continual darkness was more beneficial for germination than a 14h light/10h dark photoperiod. For example, under continual darkness 60% germination of seeds was observed for *Orchis morio*. Light intensities of $1.2 \mu\text{molesm}^{-2}\text{s}^{-1}$, $9.0 \mu\text{molesm}^{-2}\text{s}^{-1}$, and $30.4 \mu\text{molesm}^{-2}\text{s}^{-1}$ reduced germination to 8.4%, 3.4%, and 0%, respectively. Rasmussen and Rasmussen (1991) examined the roles of photoperiod duration, light intensity, and light quality in the symbiotic germination of *Dactylorhiza majalis*. Cultures were subjected to continual darkness, 8h light/16h dark, and 16h light/8h dark photoperiods. Short and long-day illumination inhibited germination, while continual darkness was optimal. Temperature may also affect the effective length of the light period required for germination of some species. For example, low temperatures reduced the light requirement of *Tsuga canadensis* (Stearns and Olson, 1958). The need for a light period in the germination of *Betula pubescens* was replaced by prechilling, seedcoat removal, or the use of an oxygen enriched atmosphere (Black and Wareing, 1955). Therefore, generalizations regarding the effects of light on the germination of seeds are not possible.

Successful germination of *N. khasiana* was achieved in the present study. Effects of different media composition and culture conditions were studied to ascertain the conditions most suitable for germination of the seeds. Literature survey shows that seeds take as long as 223 days to germinate in nature with scant germination (Bordoloi, 1977). This shows that the seeds have to remain dormant in the soil for three to four months before they could germinate. The seeds mature and fall to the ground in the months of November - December and germination does not

occur till February or March of the following year. One could hypothesize that seeds of *N. khasiana* require cold stratification to overcome dormancy in nature. In the present study, seeds collected from the natural habitat were stored at 4°C before keeping for germination. Therefore, the requirement for chilling treatment of *N. khasiana* seeds were met, which along with the provision of other necessary requirements enhanced seed germination *in vitro*. Seeds of many plants require a period of cold stratification before they germinate. Effective temperatures for stratification are from 0 – 10°C with 5°C often being optimum (Stokes, 1965). Once stratified, the temperatures experienced by seeds may also affect their ability to germinate (Matsuo and Kubota, 1988); however, if they experience an appropriate thermal range, they respond to temperature by showing differences in their germination rate (Bewley and Black, 1982). *In vitro* conditions were helpful in enhancing seed germination of *N. khasiana* species and significantly reducing the time required for germination to occur. Controlled germination regimes and *in vitro* culture methods have been reported to improve germination activity. Hall *et al.* (1998) confirmed that controlled laboratory conditions could improve germinability of alfalfa seeds from 40% in the field to 86%. In many cases, growth chamber conditions that provide an optimal controlled environment are sufficient to improve germination capacity (Dilday *et al.* 1994; Blackman *et al.* 1996; Hall *et al.* 1998). To improve germinability and seedling emergence, seeds of some plant species having hard seed coat are either scarified mechanically (Acharya *et al.*, 1999; Bringmann *et al.*, 2002), chemically by immersion in sulphuric acid (Ruiz and Devesa, 1998; El Hamdouni *et al.*, 2001) or by a treatment involving several freeze-thaw (-80°C/+20°C) cycles (Hall *et al.*, 1998). Iriondo *et al.* (1995) reported high germination percentage when seeds

of *Helianthemum polygonoides* were pretreated by immersion in water at 90°C and soaked for 24 h as the water cooled down. In many species, warm temperature (c. 25°C) pretreatment of hydrated seeds facilitates their germination in cooler temperatures concomitant with the autumn to spring period for example, in *Eranthis hyemalis* (Frost-Christensen, 1974), *Hyacinthoides non-scripta* (Thompson and Cox, 1978), *Erythronium albidum* (Baskin and Baskin, 1985). Warm temperatures are one of a number of environmental factors that influence seed development on the parent plant with respect to dormancy status. Lower dormancy levels are generally observed when plants are exposed to one or a combination of the following conditions: short days, red light, drought, high nitrogen and warm temperatures (Fenner, 1991).

The enhanced germination of *N. khasiana* through *in vitro* culture is useful in securing healthy seedlings and the germinated seeds are a good source of explant material for subsequent mass micropropagation.

CHAPTER IV: *IN VITRO* SEED GERMINATION OF *NEPENTHES KHASIANA* AS INFLUENCED BY GROWTH REGULATORS

Introduction

Plant growth regulators are essential in all physiological and developmental processes occurring during plant growth. They are endogenous or naturally-occurring compounds that are produced or synthesized in one part of the plant and causes a change in physiology, growth or development in another part of the plant. They are usually present at very low concentrations in the plants (Moore, 1979). They influence the development, differentiation and growth of cells and tissues. Growth regulators affect flowering, seed development, seed dormancy, seed germination and seedling growth. The concentration of growth regulators required for plant responses are very low (10^{-6} to 10^{-5}). Therefore, the effects of growth regulators and their relationship to plant physiology were not known till the late 1970s. Much of the earlier work involved the study of plants that were deficient in the growth regulators or the use of tissue cultured plants to study the effect of different ratios of growth regulators provided to them. Presently, different growth regulators are being used in *in vitro* plant development including vegetative propagation, micropropagation, flower induction, somatic embryogenesis, growth enhancement/ retardation, seed production, dormancy break, etc. *In vitro* seed germination can be manipulated by the addition of various growth regulators in the medium. *In vitro* seed germination is influenced

differentially by different auxins, cytokinins and gibberellins and the results are usually inconsistent and inconclusive.

Cytokinins have been isolated from seeds of a number of species (Thomas, 1977). Particular cytokinins have specific roles in the various physiological processes in plants. Although cytokinins are generally considered to be involved in nutrient mobilization, it has yet to be established if they have more specific functions during the germination process which also embodies considerable nutrient mobilization. KN has been reported to alleviate both innate and salinity-induced seed dormancy in many halophytes (Khan and Ungar, 1997; Khan *et al.*, 2004). However, Gulzar and Khan (2002) found that KN did not affect germination in some halophytic grasses. KN was also reported to prolong the viability of recalcitrant seeds, which was interpreted as possible protection of cell membranes against oxidative stress (Chaitanya and Naithani, 1998). However, application of various forms of exogenous cytokinins, including BAP, decreased the final seed germination of *Tagetes minuta* (Taylor *et al.*, 2005), indicating that inhibitory concentrations were achieved with exogenous application. Van Waes and Debergh (1986) found that cytokinins were essential for the germination of *Cypripedium calceolus* and *Epipactis helleborine*, but not necessary for germination of *Dactylorhiza maculata* and *Listera ovalis*. Therefore, exogenous cytokinins have differential effects on seed germination in different species. Modern analytical methods have shown their very active metabolism in all phases of germination, from imbibition to radicle emergence and the start of seedling establishment (Stirk *et al.*, 2005). There are many reports of cytokinins being directly involved in post-germination events such as root and hypocotyl growth and

chlorophyll synthesis (Singh and Sawhney, 1992). Gibberellins are phytohormones involved in promotion of stem elongation, mobilization of food reserves in seeds and other processes. Its absence results in the dwarfism of some plant varieties. GA₃, which was the first gibberellin to be structurally characterised, is synthesized in the embryo, germinating seeds and the roots. The use of *Arabidopsis* mutants with lesions in GA₃ biosynthesis has confirmed the key role of endogenous gibberellins in the germination process (Bianco *et al.*, 1996).

Various investigations regarding the effect of auxins on plant tissue culture established the fact that auxins IAA and α - naphthaleneacetic acid (NAA) stimulate growth of root, shoot and proliferation of tissue. Incorporation of IAA was found effective in seed germination of several plant species (Prasad and Mitra, 1975; Vij *et al.*, 1991). Enhanced germination of seeds has been reported in medium containing NAA (Das and Ghosal, 1989). Its positive effects on seedling development have also been well documented (Hazarika and Sharma, 1995). However, Kumaria (1991) reported that incorporation of NAA in the medium inhibited both seed germination and seedling growth of *Dendrobium fimbriatum* var. *occulatum*. In studies of plant tissue culture, 2,4 - dichlorophenoxyacetic acid (2,4-D) has been reported to induce callusing at very low concentrations (Biondi and Thorpe, 1982; Negrutia *et al.*, 1978; Cornijo-Martin *et al.*, 1979). It has been shown to either inhibit germination or stimulate callusing of seeds (Mitra, 1986).

Interactions between auxins, cytokinins and gibberellins may result in enhanced seed germination but the effects of these combinations vary with the hormones used, their concentration and plant species.

Materials and Methods

Effects of auxins (IAA, NAA and 2,4-D), cytokinins (KN, BAP) and GA₃ incorporated singly and in combination in the medium at different concentrations (0-10 mg l⁻¹) on seed germination of *N. khasiana* were examined. The optimal medium (1/4 MS basal medium) was used in all treatment combinations (vitamins and sucrose were incorporated in full). Ascorbic acid (50.0 mg l⁻¹) and citric acid (10.0 mg l⁻¹) were incorporated in the medium. Agar (0.8%) was added to the medium and the pH was adjusted to 5.8 with 0.1N NaOH before autoclaving. The different growth regulators except GA₃ were added to the medium before being dispensed into conical flasks. Autoclaving was done at 1.06 Kg cm⁻² and 121°C for 15 min.

In the laminar flow chamber, the autoclaved media was dispensed into 60 mm petriplates. GA₃ was filter sterilized using Millipore filters (0.2 µm pore size) and added to the flasks containing autoclaved medium before dispensing into the petriplates. Thirty seeds were inoculated in each plate. Ten replicate plates were inoculated per treatment and each plate was sealed with a single layer of parafilm (Pechiney plastic packaging, Menasha, W.I) to avoid media dehydration and contamination during the culture period. The cultures were incubated at 25±2°C under white fluorescent light under 150 µmoles m⁻² s⁻¹ with a 12h photoperiod per day. Seeds were observed daily for germination. Seeds were considered to have germinated with the emergence of the radicle. The percentage seed germination was recorded every 30 days of culture. The data obtained was subjected to statistical analysis using Student's 't' test.

Results

Effect of growth regulators incorporated singly

Records taken after 30 days of culture showed that initiation of germination did not start in ¼ MS medium devoid of growth regulators within this time. Seeds cultured in medium containing auxins (IAA, NAA and 2, 4-D) singly also did not show any signs of germination within 30 days of culture. After 60 days, maximum germination percentage recorded was in the medium supplemented with 0.5 mg l⁻¹ NAA (53%) followed by that supplemented with 0.5 mg l⁻¹ IAA (43%) (Table 4.1). These values were comparatively higher than that of the control (36%). However, the overall germination percentage in these two formulations did not increase beyond 63% when recorded after 90 days of culture. Seeds inoculated in medium consisting of 2.5 mg l⁻¹ IAA (Photoplate 4.1) and 2.5 mg l⁻¹ NAA (Photoplate 4.2) singly also showed a good response wherein overall germination percentage was 43%. Seeds cultured in the medium incorporated with 2,4- D did not show good response wherein a maximum of only 34% of the seeds germinated in low concentration of 2,4-D (0.5mg l⁻¹). Higher concentration of this growth regulator in the medium was inhibitory for both seed germination and growth of the plant (Photoplate 4.2, 4.3). Higher concentrations of auxins in the medium were found to be inhibitory for seed germination. It was observed that with the increase in auxin concentration there was a relative decrease in germination percentage. In the medium devoid of growth regulators, a maximum of 67% of the seeds germinated. Seeds cultured in medium containing cytokinins responded within 30 days within which seeds started germinating. Records taken after 30 days showed a high germination percentage (17%

Table 4.1. Seed germination in ¼ MS medium supplemented with IAA, NAA and 2,4-D

Auxins	Concentration (mg l ⁻¹)	Average Germination (%) (Days)		
		30	60	90
IAA	0.5	-	43 ± 4.8	63 ± 6.6 ^a
	2.5	-	23 ± 7.6	43 ± 5.9 ^b
	5.0	-	13 ± 2.9	13 ± 2.9 ^b
	10.0	-	3 ± 3.0	3 ± 3.0 ^b
NAA	0.5	-	53 ± 8.7	63 ± 5.5 ^a
	2.5	-	33 ± 8.1	43 ± 5.5 ^b
	5.0	-	17 ± 4.7	17 ± 4.7 ^b
	10.0	-	7 ± 3.8	7 ± 3.8 ^b
2,4-D	0.5	-	34 ± 0.7	34 ± 0.7 ^b
	2.5	-	8 ± 0.6	8 ± 0.6 ^b
	5.0	-	8 ± 1.4	8 ± 1.4 ^b
	10.0	-	4 ± 0.8	4 ± 0.8 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

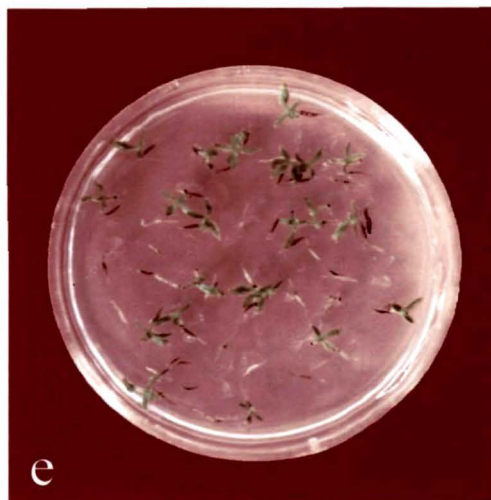
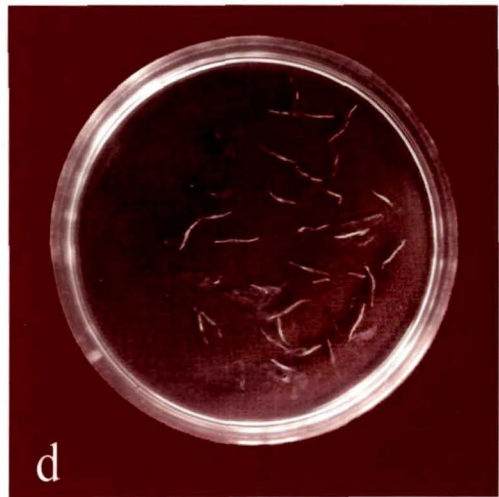
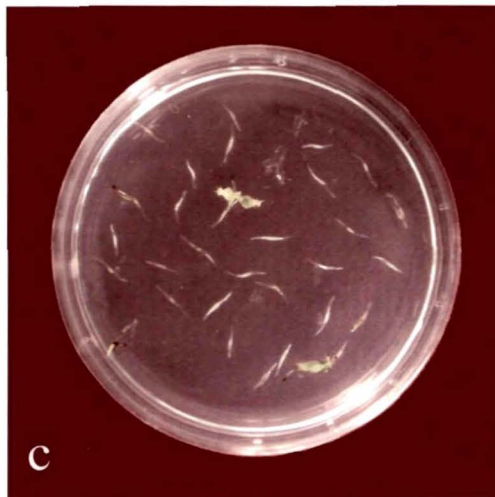
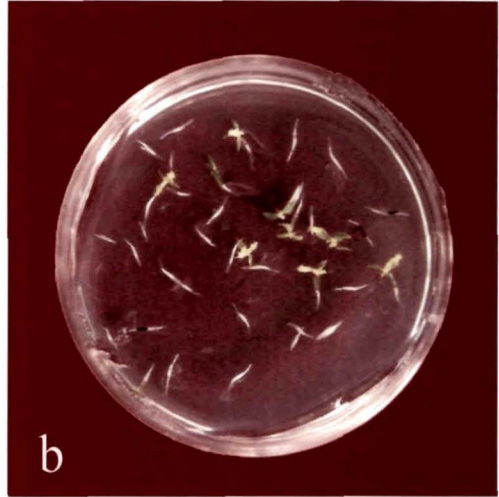
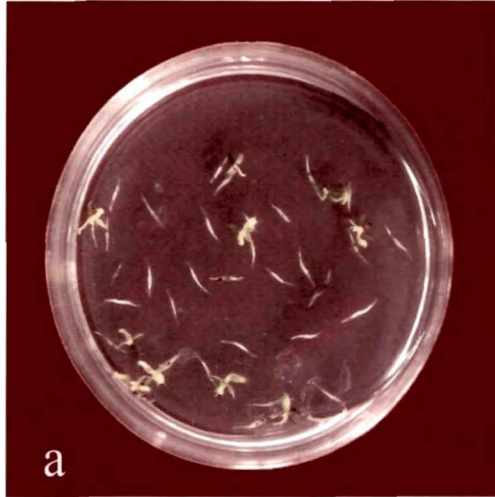
Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Photoplate 4.1

Seed germination of *N. khasiana* after 90 days of culture in ¼ MS medium incorporated with different concentrations of IAA

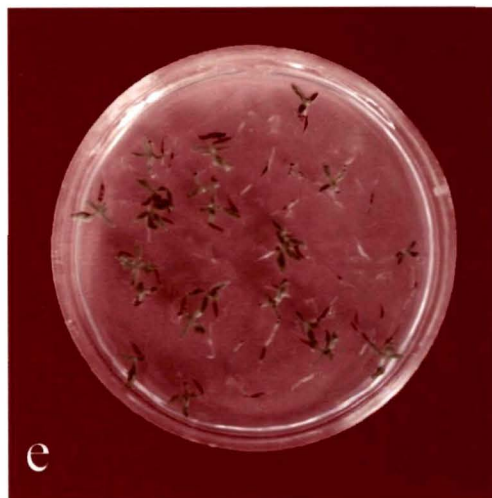
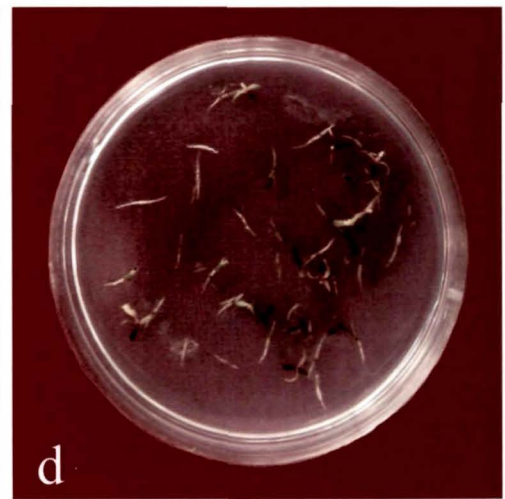
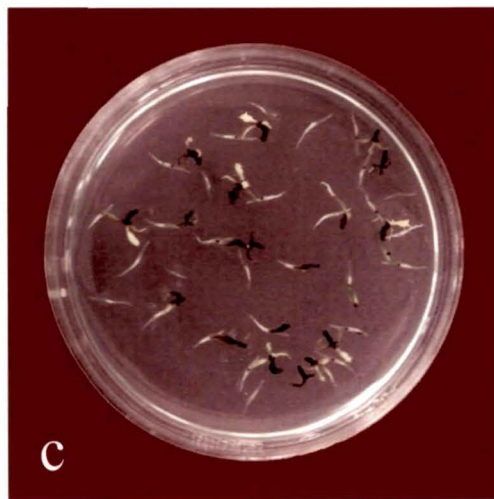
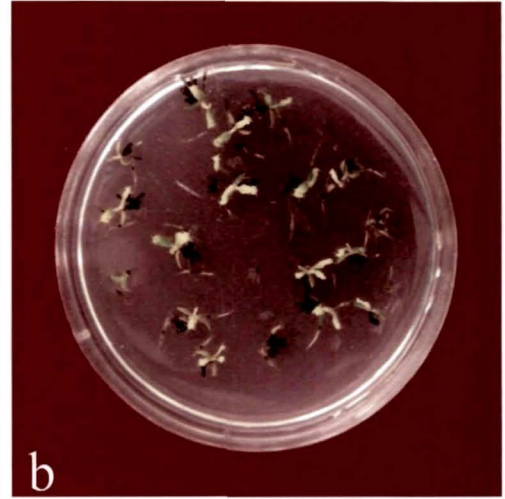
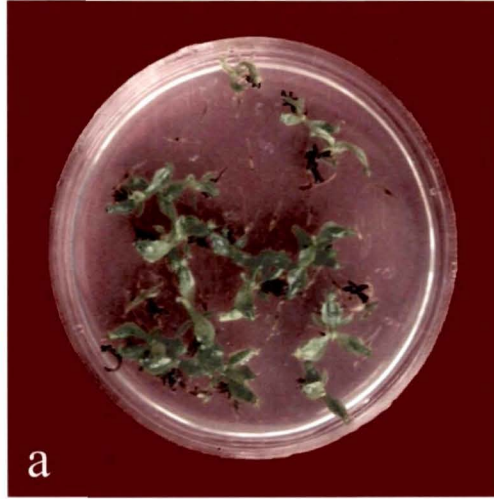
(a) 0.5 mg l⁻¹ (b) 2.5 mg l⁻¹ (c) 5.0 mg l⁻¹ (d) 10.0 mg l⁻¹ (e) Control



Photoplate 4.2

Seed germination of *N. khasiana* after 90 days of culture in ¼ MS medium incorporated with different concentrations of NAA

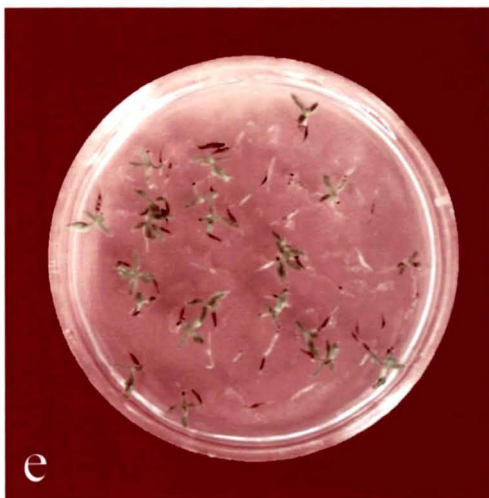
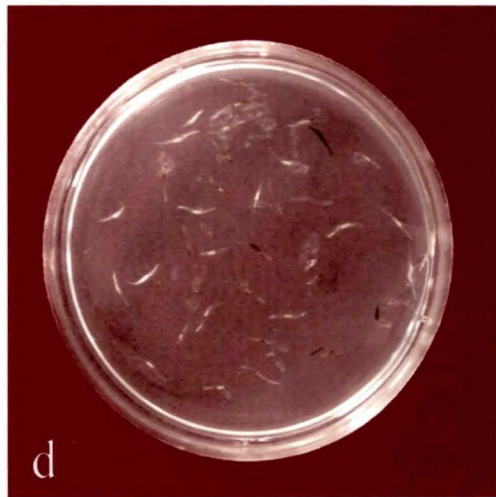
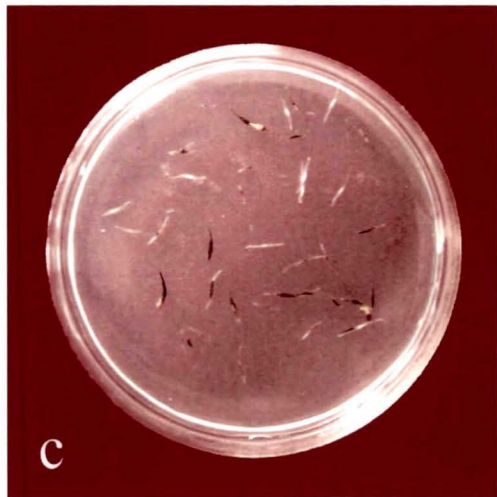
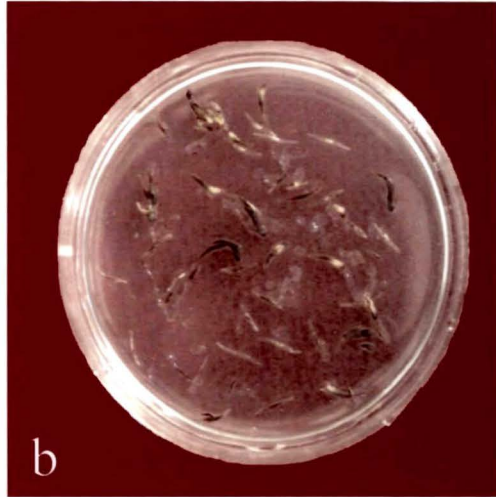
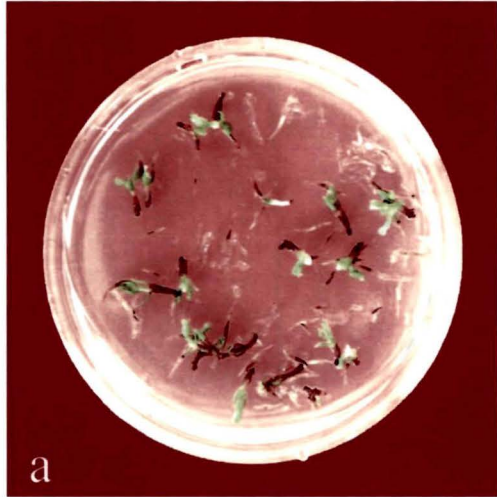
(a) 0.5 mg l⁻¹ (b) 2.5 mg l⁻¹ (c) 5.0 mg l⁻¹ (d) 10.0 mg l⁻¹ (e) Control



Photoplate 4.3

Seed germination of *N. khasiana* after 90 days of culture in $\frac{1}{4}$ MS medium incorporated with different concentrations of 2,4-D

(a) 0.5 mg l^{-1} (b) 2.5 mg l^{-1} (c) 5.0 mg l^{-1} (d) 10.0 mg l^{-1} (e) Control



to 56%) of the seeds cultured in medium containing KN and BAP singly (Table 4.2). GA₃ containing medium revealed only 3% to 17% of germinating seeds within this time period. With time, more seeds germinated and readings taken after 60 days showed that a higher number of seeds had germinated in the medium containing different concentrations of BAP and GA₃ singly when compared to the one containing KN. Maximum number of seeds germinated within 60 days of culture and records taken after 90 days did not show any significant increase in percentage germination (Photoplate 4.4, 4.5, 4.6). None of the growth regulators when incorporated singly showed any significant effect in overall germination percentage from the control (67%). However, incorporation of cytokinins and GA₃ in the medium did elicit early initiation of germination.

Effect of growth regulators in combination

Different combinations of growth regulators (IAA, NAA, BAP, KN and GA₃) in varying concentrations were added to ¼ MS medium to study their combined effect on *in vitro* germination of *N. khasiana* seeds. When KN was combined with NAA in the medium, seeds started germinating within 30 days of culture in all combinations except in medium containing high concentrations of NAA (Table 4.3). About 22% of seeds germinated in medium containing 5.0 mg l⁻¹ KN and 0.5 mg l⁻¹ NAA in combination whereas in other combinations, only about 2% to 7% of the seeds germinated within this time period. Records taken after 60 days showed a range of 33% (10.0 mg l⁻¹ KN + 10.0 mg l⁻¹ NAA) to 61% (10.0 mg l⁻¹ KN + 5.0 mg l⁻¹ NAA) germination in the different combinations. Among the different combinations, medium containing 5.0 mg l⁻¹ each of KN and NAA revealed a maximum germination

Table 4.2. Seed germination in ¼ MS medium supplemented with KN, BAP and GA₃

	Concentration (mg l ⁻¹)	Average Germination (%) (Days)		
		30	60	90
KN	0.5	20 ± 5.5	54 ± 1.1	59 ± 4.0 ^a
	2.5	33 ± 8.2	59 ± 4.0	59 ± 4.0 ^a
	5.0	56 ± 3.6	57 ± 1.9	57 ± 1.9 ^a
	10.0	17 ± 4.7	54 ± 1.1	54 ± 1.1 ^a
BAP	0.5	53 ± 1.9	63 ± 4.7	67 ± 2.9 ^a
	2.5	50 ± 1.1	63 ± 2.0	63 ± 2.0 ^a
	5.0	50 ± 5.6	63 ± 3.1	63 ± 3.1 ^a
	10.0	40 ± 6.0	60 ± 2.6	60 ± 2.6 ^a
GA ₃	0.5	3 ± 2.9	67 ± 2.9	68 ± 3.0 ^a
	2.5	17 ± 5.9	68 ± 3.0	69 ± 3.0 ^a
	5.0	7 ± 4.7	60 ± 2.9	67 ± 2.9 ^a
	10.0	-	46 ± 2.4	50 ± 2.6 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

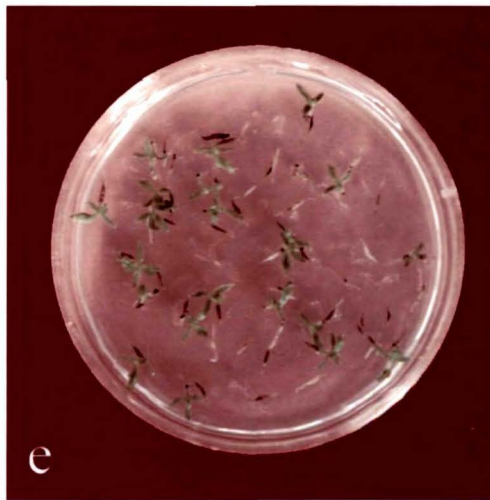
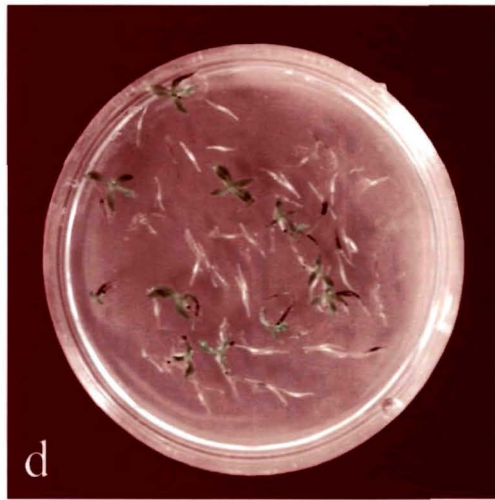
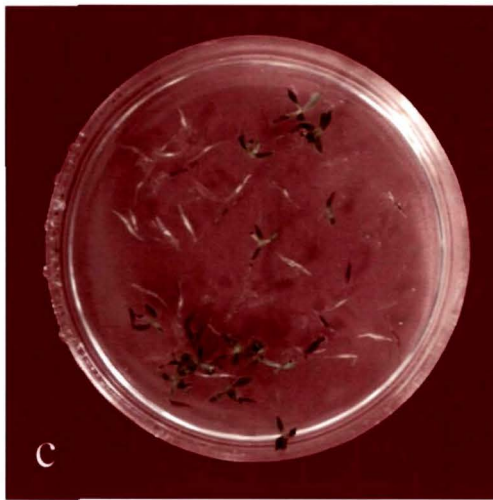
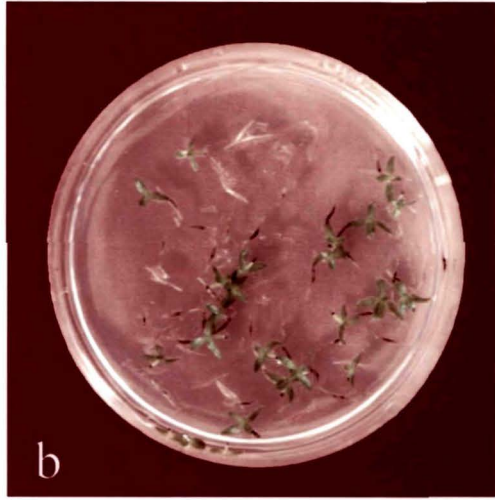
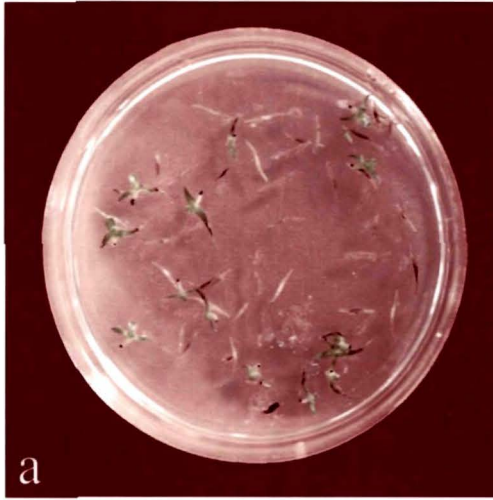
Significant differences between treatment and control are indicated with different letters
($\alpha = 0.05$)

- No germination

Photoplate 4.4

Seed germination of *N. khasiana* after 90 days of culture in $\frac{1}{4}$ MS medium incorporated with different concentrations of KN

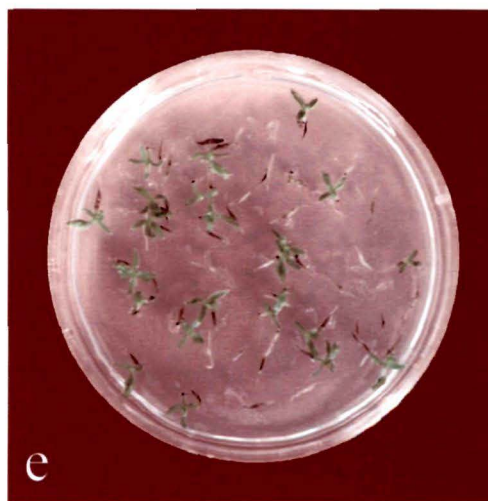
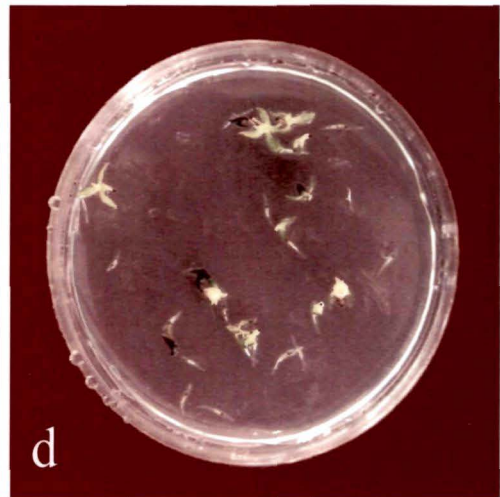
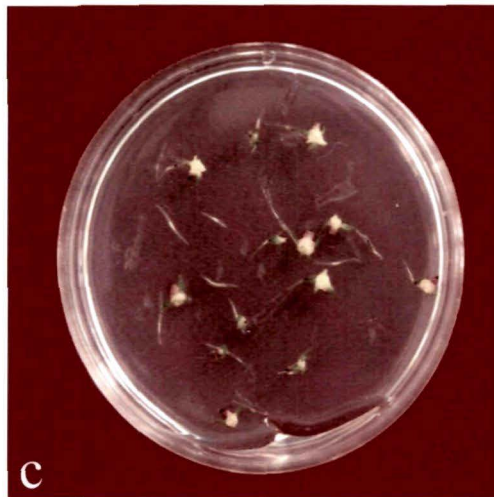
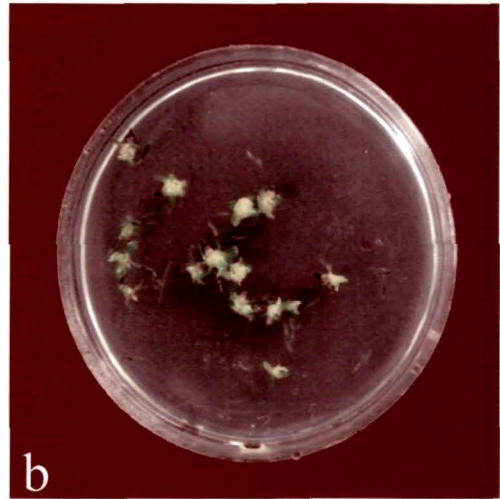
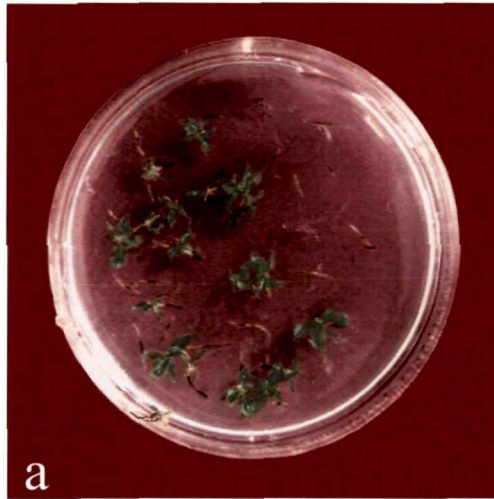
(a) 0.5 mg l⁻¹ (b) 2.5 mg l⁻¹ (c) 5.0 mg l⁻¹ (d) 10.0 mg l⁻¹ (e) Control



Photoplate 4.5

Seed germination of *N. khasiana* after 90 days of culture in ¼ MS medium incorporated with different concentrations of BAP

(a) 0.5 mg l⁻¹ (b) 2.5 mg l⁻¹ (c) 5.0 mg l⁻¹ (d) 10.0 mg l⁻¹ (e) Control



Photoplate 4.6

Seed germination of *N. khasiana* after 90 days of culture in ¼ MS medium incorporated with different concentrations of GA₃

(a) 0.5 mg l⁻¹ (b) 2.5 mg l⁻¹ (c) 5.0 mg l⁻¹ (d) 10.0 mg l⁻¹ (e) Control

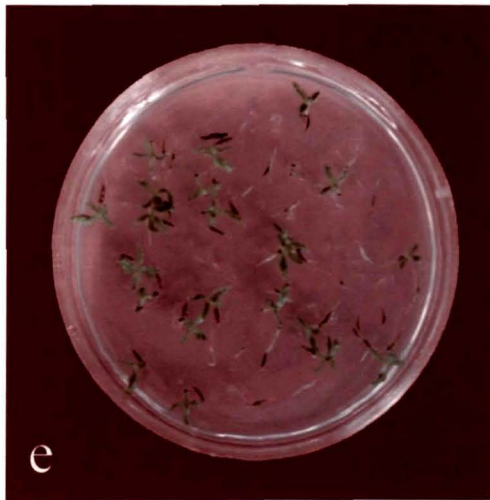
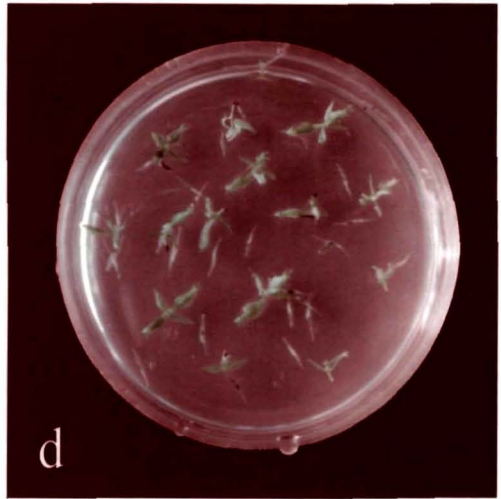
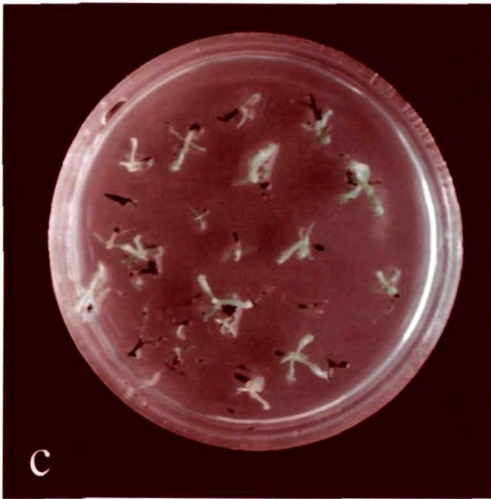
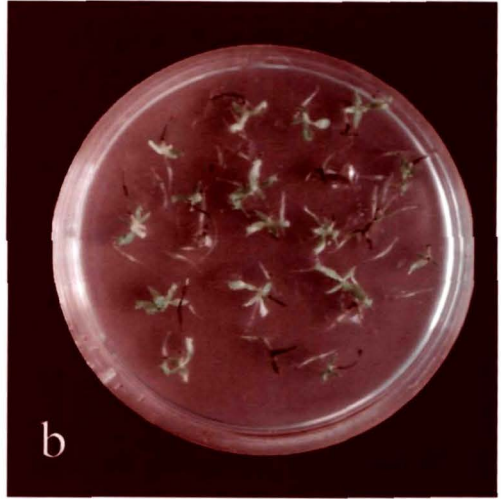
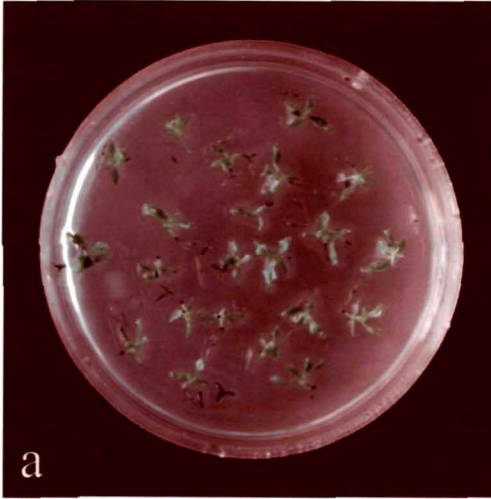


Table 4.3. Seed germination in ¼ MS medium supplemented with KN and NAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
KN	NAA	30	60	90
0.5	0.5	5 ± 3.3	57 ± 5.5	57 ± 5.5 ^b
0.5	2.5	2 ± 1.6	58 ± 2.6	58 ± 2.6 ^a
0.5	5.0	-	43 ± 6.1	53 ± 3.3 ^b
0.5	10.0	-	40 ± 5.5	52 ± 3.1 ^b
2.5	0.5	5 ± 3.3	53 ± 2.0	60 ± 2.0 ^a
2.5	2.5	3 ± 1.9	60 ± 4.8	60 ± 4.8 ^a
2.5	5.0	3 ± 3.3	67 ± 4.5	67 ± 4.5 ^a
2.5	10.0	2 ± 1.6	47 ± 6.2	58 ± 2.6 ^b
5.0	0.5	22 ± 2.7	60 ± 2.0	63 ± 2.9 ^a
5.0	2.5	8 ± 5.2	57 ± 4.0	64 ± 4.2 ^a
5.0	5.0	5 ± 3.3	53 ± 3.3	68 ± 4.8 ^a
5.0	10.0	-	35 ± 3.9	47 ± 2.4 ^b
10.0	0.5	7 ± 3.1	44 ± 7.3	44 ± 7.3 ^b
10.0	2.5	7 ± 3.1	48 ± 3.1	53 ± 3.3 ^b
10.0	5.0	7 ± 3.1	61 ± 7.1	63 ± 7.6 ^a
10.0	10.0	-	33 ± 6.9	45 ± 1.0 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

percentage of 68% when recorded after 90 days (Table 4.3). In all other treatments of NAA and KN in combination in the medium, seed germination was not much affected by the growth regulators. A combination of KN and IAA also induced earlier seed germination, wherein 2% (0.5 mg l^{-1} KN + 10.0 mg l^{-1} IAA) to 47% (5.0 mg l^{-1} KN + 0.5 mg l^{-1} IAA) of seeds germinated within 30 days of culture (Table 4.4). Although more number of seeds germinated with time, records taken after 90 days of culture did not show significant increase in germination percentage when compared with the control. When BAP was combined with NAA in the medium, a maximum of only about 5% seeds germinated in medium containing 5.0 mg l^{-1} BAP and different concentrations of NAA within 30 days of culture (Table 4.5). Although germination started late, the combination of these two growth regulators (BAP and NAA) supported overall increase in germination. The best overall germination percentage recorded was 68% in the medium containing 5.0 mg l^{-1} each of BAP and NAA) after 90 days of culture. The seeds responded well to the medium having other concentrations of BAP and NAA also, wherein about 25% (0.5 mg l^{-1} BAP + 10.0 mg l^{-1} NAA) to 68% (5.0 mg l^{-1} BAP + 5.0 mg l^{-1} NAA) of seeds germinated after 90 days. The seeds cultured in medium having BAP and IAA in different combinations germinated within 30 days of culture with a range of 3% (10.0 mg l^{-1} BAP + 10.0 mg l^{-1} IAA) to 43 % (0.5 mg l^{-1} BAP + 0.5 mg l^{-1} IAA) (Table 4.6). Records taken after 60 days and 90 days showed an increase in germination with time. Maximum germination percentage (70%) was recorded in the medium containing 0.5 mg l^{-1} BAP and 2.5 mg l^{-1} IAA. GA_3 in combination with auxins (IAA, NAA) and cytokinins (KN, BAP) inhibited early initiation of seed germination. In all the cases, germination was observed after 30 days of culture. Records of germination in medium containing GA_3

Table 4.4. Seed germination in ¼ MS medium supplemented with KN and IAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
KN	IAA	30	60	90
0.5	0.5	23 ± 1.6	42 ± 4.5	47 ± 3.3 ^b
0.5	2.5	20 ± 3.3	40 ± 4.8	58 ± 7.9 ^a
0.5	5.0	13 ± 4.2	30 ± 2.0	42 ± 6.4 ^b
0.5	10.0	2 ± 1.6	15 ± 4.8	40 ± 4.8 ^b
2.5	0.5	25 ± 5.2	43 ± 7.6	45 ± 8.1 ^b
2.5	2.5	27 ± 5.5	47 ± 6.7	55 ± 3.3 ^b
2.5	5.0	17 ± 3.7	53 ± 3.3	65 ± 4.0 ^a
2.5	10.0	3 ± 1.6	17 ± 2.6	40 ± 7.1 ^b
5.0	0.5	47 ± 4.2	58 ± 5.8	63 ± 3.3 ^a
5.0	2.5	30 ± 6.2	48 ± 7.1	52 ± 8.0 ^a
5.0	5.0	18 ± 5.5	43 ± 7.1	47 ± 6.7 ^b
5.0	10.0	7 ± 1.6	22 ± 5.6	35 ± 5.2 ^b
10.0	0.5	40 ± 5.5	55 ± 4.2	58 ± 2.6 ^b
10.0	2.5	18 ± 5.5	52 ± 7.6	52 ± 7.6 ^a
10.0	5.0	13 ± 5.0	38 ± 4.2	42 ± 5.2 ^b
10.0	10.0	8 ± 2.6	31 ± 6.4	31 ± 6.4 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Table 4.5. Seed germination in ¼ MS medium supplemented with BAP and NAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
BAP	NAA	30	60	90
0.5	0.5	-	47 ± 5.6	59 ± 8.3 ^a
0.5	2.5	-	46 ± 4.6	50 ± 7.9 ^a
0.5	5.0	-	28 ± 7.1	40 ± 4.0 ^b
0.5	10.0	-	25 ± 4.1	25 ± 4.1 ^b
2.5	0.5	3 ± 1.9	48 ± 6.2	60 ± 7.1 ^a
2.5	2.5	-	60 ± 5.8	60 ± 5.8 ^a
2.5	5.0	-	54 ± 8.8	55 ± 9.3 ^a
2.5	10.0	-	54 ± 4.1	54 ± 4.1 ^b
5.0	0.5	5 ± 3.3	53 ± 7.3	60 ± 6.6 ^a
5.0	2.5	5 ± 2.0	58 ± 5.2	61 ± 4.1 ^a
5.0	5.0	5 ± 2.0	68 ± 3.4	68 ± 3.5 ^a
5.0	10.0	5 ± 2.0	57 ± 5.0	57 ± 5.0 ^a
10.0	0.5	-	18 ± 2.5	36 ± 4.1 ^b
10.0	2.5	-	18 ± 3.1	52 ± 6.6 ^a
10.0	5.0	-	42 ± 6.4	63 ± 5.1 ^a
10.0	10.0	-	37 ± 4.7	41 ± 4.7 ^a
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Table 4.6. Seed germination in ¼ MS medium supplemented with BAP and IAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
BAP	IAA	30	60	90
0.5	0.5	43 ± 6.6	68 ± 4.8	68 ± 4.8 ^a
0.5	2.5	40 ± 8.0	67 ± 5.6	70 ± 3.0 ^a
0.5	5.0	17 ± 5.8	53 ± 5.6	58 ± 4.5 ^a
0.5	10.0	12 ± 4.2	18 ± 5.5	42 ± 3.7 ^b
2.5	0.5	38 ± 1.0	60 ± 6.1	60 ± 6.1 ^a
2.5	2.5	38 ± 1.0	53 ± 4.2	57 ± 5.5 ^a
2.5	5.0	35 ± 6.6	50 ± 5.2	50 ± 5.2 ^b
2.5	10.0	8 ± 6.4	33 ± 8.7	47 ± 4.2 ^b
5.0	0.5	38 ± 6.2	53 ± 3.3	57 ± 6.6 ^a
5.0	2.5	38 ± 5.8	50 ± 6.9	54 ± 5.6 ^a
5.0	5.0	25 ± 3.7	43 ± 7.1	47 ± 4.2 ^b
5.0	10.0	10 ± 3.1	42 ± 5.2	45 ± 6.2 ^b
10.0	0.5	32 ± 4.8	37 ± 2.0	37 ± 2.0 ^b
10.0	2.5	38 ± 5.6	40 ± 5.5	40 ± 5.5 ^b
10.0	5.0	12 ± 4.9	20 ± 3.3	20 ± 3.3 ^b
10.0	10.0	3 ± 2.0	13 ± 3.3	13 ± 3.3 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

and IAA showed a range of 11% (10.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ IAA) to 57% (5.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ IAA) of germinated seeds after 90 days of culture (Table 4.7). In all other treatments of GA₃ and IAA in the medium, seed germination was much less when compared to the control. GA₃ and NAA in different combinations in the medium also had an inhibitory effect on germination of *N. khasiana* seeds wherein only about 22% (0.5 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ NAA) to 62% (5.0 mg l⁻¹ GA₃ + 2.5 mg l⁻¹ NAA) of seeds germinated after 90 days of culture (Table 4.8). In all the other treatments of GA₃ and NAA in the medium, seed germination was much less when compared to the control. A range of 39% (10.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ KN) to 63% (2.5 mg l⁻¹ GA₃ + 2.5 mg l⁻¹ KN) of the seeds germinated in the medium incorporated with GA₃ and KN in combination after 90 days of culture (Table 4.9). In all other treatments of GA₃ and KN in the medium, seed germination percentage was significantly lower than that of the control. Incorporation of GA₃ and BAP in the medium also did not help in increasing percentage germination wherein a maximum of only about 27% (10.0 mg l⁻¹ GA₃ + 10.0 mg l⁻¹ BAP) to 60% (5.0 mg l⁻¹ GA₃+5.0mg l⁻¹ BAP) of the seeds germinated when recorded after 90 days of culture (Table 4.10).

Discussion

The germination of seeds of *N. khasiana* was affected differentially by the incorporation of growth regulators in the medium. The role of various hormones in seed germination has been reported with inconclusive results - sometimes enhancing germination, other times inhibiting and still other times showing no effect. This

Table 4.7. Seed germination in ¼ MS medium supplemented with GA₃ and IAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
GA ₃	IAA	30	60	90
0.5	0.5	-	41 ± 2.2	43 ± 3.3 ^b
0.5	2.5	-	40 ± 3.6	47 ± 3.3 ^b
0.5	5.0	-	29 ± 5.8	47 ± 3.3 ^b
0.5	10.0	-	14 ± 1.1	34 ± 2.9 ^b
2.5	0.5	-	48 ± 1.6	50 ± 1.9 ^b
2.5	2.5	-	48 ± 4.0	52 ± 2.9 ^b
2.5	5.0	-	31 ± 2.0	50 ± 3.3 ^b
2.5	10.0	-	23 ± 5.8	43 ± 3.3 ^b
5.0	0.5	-	53 ± 5.0	57 ± 5.2 ^a
5.0	2.5	-	53 ± 5.0	53 ± 5.0 ^a
5.0	5.0	-	38 ± 1.1	52 ± 6.7 ^b
5.0	10.0	-	34 ± 1.4	43 ± 2.0 ^b
10.0	0.5	-	8 ± 1.1	11 ± 2.2 ^b
10.0	2.5	-	32 ± 2.9	40 ± 3.3 ^b
10.0	5.0	-	48 ± 2.9	54 ± 4.0 ^a
10.0	10.0	-	38 ± 2.9	43 ± 2.3 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Table 4.8. Seed germination in ¼ MS medium supplemented with GA₃ and NAA in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
GA ₃	NAA	30	60	90
0.5	0.5	-	22 ± 3.4	22 ± 3.4 ^b
0.5	2.5	-	48 ± 5.1	48 ± 5.1 ^b
0.5	5.0	-	43 ± 1.0	43 ± 1.0 ^b
0.5	10.0	-	27 ± 1.2	27 ± 1.2 ^b
2.5	0.5	-	51 ± 2.9	51 ± 2.9 ^b
2.5	2.5	-	52 ± 4.0	52 ± 4.0 ^a
2.5	5.0	-	57 ± 3.3	57 ± 3.3 ^b
2.5	10.0	-	57 ± 2.7	57 ± 2.7 ^b
5.0	0.5	-	57 ± 6.6	57 ± 6.6 ^a
5.0	2.5	-	62 ± 1.6	62 ± 1.6 ^a
5.0	5.0	-	58 ± 5.2	58 ± 5.2 ^a
5.0	10.0	-	49 ± 2.2	49 ± 2.2 ^b
10.0	0.5	-	37 ± 1.9	37 ± 1.9 ^b
10.0	2.5	-	46 ± 3.3	46 ± 3.3 ^b
10.0	5.0	-	46 ± 3.3	46 ± 3.3 ^b
10.0	10.0	-	35 ± 4.0	35 ± 4.0 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Table 4.9. Seed germination in ¼ MS medium supplemented with GA₃ and KN in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
GA ₃	KN	30	60	90
0.5	0.5	-	47 ± 2.6	52 ± 1.6 ^b
0.5	2.5	-	42 ± 1.8	48 ± 1.1 ^b
0.5	5.0	-	41 ± 1.6	41 ± 1.6 ^b
0.5	10.0	-	38 ± 1.6	38 ± 1.6 ^b
2.5	0.5	-	49 ± 4.8	49 ± 4.8 ^b
2.5	2.5	-	62 ± 3.5	63 ± 3.3 ^a
2.5	5.0	-	41 ± 2.9	49 ± 5.8 ^b
2.5	10.0	-	33 ± 2.9	41 ± 2.9 ^b
5.0	0.5	-	40 ± 1.1	48 ± 4.0 ^b
5.0	2.5	-	50 ± 1.9	56 ± 1.1 ^b
5.0	5.0	-	51 ± 2.9	52 ± 6.2 ^b
5.0	10.0	-	41 ± 1.1	50 ± 3.3 ^b
10.0	0.5	-	38 ± 2.8	39 ± 2.8 ^b
10.0	2.5	-	39 ± 1.1	40 ± 3.3 ^b
10.0	5.0	-	49 ± 2.9	49 ± 2.9 ^b
10.0	10.0	-	49 ± 2.2	49 ± 2.2 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

Table 4.10. Seed germination in ¼ MS medium supplemented with GA₃ and BAP in combination

Concentration (mg l ⁻¹)		Average Germination (%) (Days)		
GA ₃	BAP	30	60	90
0.5	0.5	-	35 ± 1.5	35 ± 1.5 ^b
0.5	2.5	-	46 ± 5.0	49 ± 5.5 ^a
0.5	5.0	-	43 ± 1.9	43 ± 1.9 ^b
0.5	10.0	-	23 ± 1.9	33 ± 5.7 ^b
2.5	0.5	-	39 ± 6.7	42 ± 6.7 ^b
2.5	2.5	-	49 ± 2.2	54 ± 2.9 ^b
2.5	5.0	-	46 ± 6.1	50 ± 5.0 ^a
2.5	10.0	-	31 ± 4.8	31 ± 4.8 ^b
5.0	0.5	-	42 ± 4.4	47 ± 2.0 ^b
5.0	2.5	-	46 ± 3.0	52 ± 6.7 ^a
5.0	5.0	-	54 ± 6.7	60 ± 6.9 ^a
5.0	10.0	-	29 ± 2.0	32 ± 2.2 ^b
10.0	0.5	-	49 ± 2.2	57 ± 1.9 ^b
10.0	2.5	-	48 ± 1.1	53 ± 3.3 ^b
10.0	5.0	-	26 ± 2.9	29 ± 5.0 ^b
10.0	10.0	-	18 ± 1.1	27 ± 3.3 ^b
Control		-	36 ± 2.8	67 ± 2.3 ^a

± S. E.

Significant differences between treatment and control are indicated with different letters ($\alpha = 0.05$)

- No germination

inconsistent response to exogenous hormone has been shown to vary from genus to genus and even species to species in plants.

In the present study, addition of auxins singly in different concentrations in the medium was not helpful in either increasing the germination or in inducing early seed germination. This suggests that the cells might be possessing endogenous auxin at the level needed for this purpose (Das *et al.*, 2005). However, germination percentage of the seeds decreased with increasing auxin concentration in the medium. One can therefore hypothesize that lower levels of auxins are better for germination of *N. khasiana*. A perusal of literature reveals that similar results were obtained by De *et al.* (2006) where *in vitro* germination of seeds of *Dendrobium chrysanthum* took place in presence of low concentration of NAA whereas high concentration of this hormone in the medium showed inhibitory effect on the germination. The effect of 2,4-D for seed germination of *N. khasiana* was inhibitory in all concentrations. The inhibitory effect of 2,4-D on plant development has been reported by many workers (Kumaria and Tandon, 1991; Kalita, 1999; Talukdar, 2001).

In the present study, the rate of seed germination increased significantly with the addition of cytokinins and GA₃ in the medium. In order to induce germination of embryos and seeds on culture medium, GA₃ and cytokinins have been extensively used (Sharma *et al.*, 1996; Miyoshi and Sato, 1997). Cytokinins are plant hormones implicated in the regulation of various processes of growth and development. However, among their multiple activities, the effects of cytokinins on seed germination stand apart from their role in shoot morphogenesis. Modern analytical methods have shown their very active metabolism in all phases of germination, from

imbibition to radicle emergence and the start of seedling establishment (Stirk *et al.*, 2005). Cytokinins are generally considered to have a permissive role in germination as they can alleviate the effect of germination inhibitors such as Abscisic acid (ABA) and methyl jasmonate (Singh and Sawhney, 1992; Bialecka and Kepczynski, 2003). Exogenous cytokinins have various effects on seed germination in different species. Their promotive effects are reported to be mostly related to the alleviation of stress factors (Khan and Ungar 1997; Gadallah and El-Enany, 1999; Gulzar and Khan, 2002; Khan *et al.*, 2004; Atici and Battal, 2005). Van Waes and Debergh (1986) found that a cytokinin was essential for the germination of *Epipactis helleborine*, but not necessary for germination of *Dactylorhiza maculata* and *Listera ovalis*. Cytokinins were also able to accelerate the germination rate and increase the final germination percentage of *Lotus corniculatus* (Nikolic *et al.*, 2006). Cytokinin requirement has been demonstrated for *Cypripedium reginae* (Harvais, 1982), *C. calceolus* (Van Waes and Debergh, 1986) and *C. candidum* (De Pauw *et al.*, 1995). Tomita (2002) reported although germination of *Cypripedium macranthos* occurred without the addition of cytokinins to the medium but in the presence of two of three cytokinins tested, germination significantly improved. Shiau *et al.* (2005) also reported that seeds of *Haemaria discolor* could be germinated on medium devoid of cytokinin. This may be because of the presence of sufficient level of endogenous cytokinin required for initial stages of germination.

Of the two cytokinins, BAP was more effective than KN in accelerating seed germination rate. The dominancy of BAP over KN has been reported in *Plumbago rosea* (Harikrishnan and Hariharan, 1996), *Vitex negundo* (Sahoo and Chand, 1998),

Tabebuia spp. (Rajani and Urs., 1998), *Gymnema elegans* (Komalavalli and Rao, 2000), *Bombax ceiba* (Singh and Chand, 2000) and *Gerbera jamesonii* (Ray *et al.*, 2005). There are different reasons for the preferred use of BAP as cytokinin. Slow degradation of BAP is one of the reasons and it can be autoclaved without loss of its activity (Daga, 1994). The seeds of *Anthurium andreanum* showed enhanced germination on MS medium supplemented with BAP (Vargas *et al.*, 2004). Germination of seeds of *Citrus reticulata* was also found to be better on MS medium supplemented with BAP (Hassanein and Azooz, 2004). Stewart and Kane (2006) reported a positive effect on seed germination percentage at lower concentrations of KN (1 μ M) and at higher concentrations of BAP (3 μ M - 10 μ M) in the medium. Exogenous BAP has been reported to accelerate DNA synthesis by shortening the starting time of the S-phase of the mitotic cell cycle after imbibition in maize seed axes (Stirk *et al.*, 2005). It had been suggested that BAP accelerated the transport of proteins into the nuclei and thus shortened the time to the completion of the first cell division cycle (Herrera-Teigeiro *et al.* 1999; Stirk *et al.*, 2005). With the addition of exogenous BAP, enhanced DNA polymerase activity and protein kinase activity has also been reported (Stirk *et al.*, 2005). De Pauw *et al.* (1995) suggested that the requirement for cytokinins in the germination medium might be related to utilization of storage lipid mobilization. Dimalla and van Staden (1977) reported that cytokinins assist in lipid mobilization in seeds with high levels of lipid reserves. Germination ceases if lipids are not utilized (Manning and van Staden, 1987). Pretreatment with plant growth regulators especially cytokinin has often been reported to be beneficial for increasing rates of seed germination. Miyoshi and Mii (1995) reported enhanced seed germination of *Calanthe discolor* with pretreatment in various levels of BAP.

GA₃ is a very potent hormone whose natural occurrence in plants controls their development. Cerabolini *et al.* (2004) reported that *Physoplexis camosa* seeds germinated in sterile *in vitro* conditions, in which germination percentages were extremely low (1.7%) without a supply of GA₃. However, with the addition of 100 mg l⁻¹ GA₃, 90% germination was obtained, but the germination decreased with increase in GA₃ concentration. Blackman *et al.* (1996) also reported significant effect of GA₃ on germination of maize embryos. Since GA₃ regulates growth, applications of very low concentrations can have a profound effect. That gibberellins play an important role in enhancing seed germination has long been recognized and documented. However, some workers also reported insignificant or inhibitory effect of GA₃ on seed germination. No significant effect of GA₃ treatments was observed on *in vitro* germination of *Pistacia atlantica* seeds (Chaabouni and Gouta, 2002). The highest germination of *Lychnis senno* reached only 19.4% when seeds were treated with 250 mg l⁻¹ GA₃ (Chen *et al.*, 2006). Total germination of *Primula glaucescens* seeds was also not affected by GA₃ concentration however, doses of 10 - 500 mg l⁻¹ acted equally to prompt earlier and more rapid germination (Cerabolini *et al.*, 2004). In most cases, gibberellins affect both capacity and speed of germination (Lang, 1965). However in some cases, its effect is restricted to the speed and not the capacity of germination (Volodin, 1960). Our findings are in conformity of this. Although the exogenous supply of GA₃ to the medium induced earlier seed germination, it did not help in increasing the germination percentage. In order to reduce the time and to increase the rate of germination to obtain a massive production of seedlings of the same age, El Hamdouni *et al.* (2001) used various concentrations of H₂SO₄ and H₂O₂ to scarify achenes of strawberry (*Fragaria x ananassa* Duch.). Peacock and Hummer

(1996) studied the effect of liquid nitrogen and H₂SO₄ on the germination of six species of *Rubus*. Whereas the liquid nitrogen treatment did not have a significant effect on germination percentage compared to controls, the H₂SO₄ increased significantly this rate in four species. In the present study, no such treatments were needed as the incorporation of growth regulators (cytokinins and GA₃) singly in the medium was enough to accelerate the rate of seed germination.

It was also observed in the present study that the presence of different growth regulators in combination did not promote seed germination capacity of *N. khasiana*. In all the cases, the overall germination percentages were lower than the control. Similarly, combination of auxins and cytokinins was not found to be essential for optimum shoot proliferation of *Agave parrasana* (Santacruz-Ruvalcaba *et al.*, 1999). However, some combinations of auxins and cytokinins in the medium accelerated the germination process. George (1993) stated that the combined effect of auxin and cytokinin is essential in *in vitro* multiplication of several plant species. But the effects of these combinations are inconclusive as they vary with the growth regulators, their concentrations and the concerned plant species. In the present study, cytokinins (BAP and KN) when combined with IAA was more beneficial for earlier seed germination than when combined with NAA. Similarly, beneficial effects of BAP and IAA on shoot bud induction and multiplication has been reported earlier in *Eremostachys superba* (Sunnichan and Shivanna, 1998), *Fritillaria unibracteata* (Gao *et al.*, 1999), *Capsicum annum* (Sobhakumari and Lathakumari, 2003) and *Salvia memorosa* (Skaia and Nska, 2004).

CHAPTER V: HARDENING, ESTABLISHMENT AND REINTRODUCTION OF *IN VITRO* RAISED PLANTS

Introduction

The overall success of conservation of rare and endangered plants through *in vitro* culture depends on their successful transplantation into the field and subsequent transfer to their natural habitat. The transfer of *in vitro* raised seedlings from the culture tubes to the natural conditions requires a careful, stepwise procedure which would enable them to harden and acclimatize to the harsh outside environment thereby leading to better survival. Hardening of the plants is necessary to develop resistance against physical, chemical and biological factors at the time of transferring *in vitro* raised plantlets to field conditions. The *in vitro* raised plantlets fail to withstand direct exposure to harsher environment outside the *in vitro* regimes due to poorly developed cuticle, stomatal apparatus, photosynthetic ability and conducting tissues (Vij, 1998). Plantlets which are cultured on agar based media, wilt rapidly on transfer to a water deficit atmosphere in the greenhouse or field conditions. Therefore, 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 days (Bhojwani and Razdan, 1983) after which gradual decrease in humidity (70-60%) is required. The scarcity or poor availability of ground water to the plantlets coupled with the excessive loss of water through transpiration may lead to high mortality rates

when transferred unless they are subjected to gradual decrease in humidity and increase of temperature.

Temperature also plays a major role in successful acclimatization of plants on transplantation. *In vitro* raised plants are subjected to optimum temperature conditions suitable for their growth and survival under controlled conditions of growth. On transplantation they have to adapt to the outside hostile temperature which may be lower or higher than the optimum temperature. This may not be possible if the plants are suddenly exposed to these conditions. The shock is more severe in the cold regions where the temperature in winter drops to sub zero levels. Sudden exposure to high temperature during summer may also result in low survival rate. Hence, temperature should be gradually altered to enable the plantlets to adapt to the changing environment before transferring them to the field. Bordoloi (1977) suggested that temperature requirement for *Nepenthes* in glasshouses should as far as practicable be regulated to 18-29°C in winter and 20-28°C in summer.

The types of compost and soil conditions are very critical for the growth and survival of the *in vitro* raised plantlets. A good growing medium having properties such as resistance to organic decomposition and porosity to ensure adequate aeration for root respiration and to allow drainage of excess water is essential for proper growth and development of *in vitro* raised plants. Compost which is easily available and less costly would be more beneficial.

Preconditioning of *in vitro* cultured plantlets before acclimatization to the field has been useful for successful acclimatization of plantlets. Preconditioning

by addition of high concentration of sucrose was reported to influence the *in vivo* rooting and establishment of cuttings (Wainwright and Scarce, 1989). Hazarika *et al.* (2000, 2001) also reported that *in vitro* preconditioning of citrus microshoots with sucrose concentrations of 3% was found optimum for subsequent *ex vitro* survival and growth. They also reported that preconditioning of microshoots with paclobutrazol influence higher *ex vitro* survival by intensifying internode length, thickening of root and reducing leaf dehydration by regulating the stomatal function and increasing epicuticular wax per unit area of leaf, besides chlorophyll synthesis.

The soil collected from the natural habitat has been analysed for total nitrogen content. Nitrogen (N) is absorbed from soil and used by plants in its inorganic form, as either nitrate (NO_3^-) or ammonium (NH_4^+). Both forms occur naturally in soils and are common components of inorganic fertilizers. Nitrate is the principal form in which nitrogen is taken up by plants, due to its mobile nature and greater abundance than ammonium. However, inorganic nitrogen represents only 2 to 5% of the total nitrogen in the soil. Most soil nitrogen is bound to organic matter and not readily available to plants. Total nitrogen is a measure of both inorganic and organic forms of nitrogen and is expressed as a percentage. Levels of nitrogen vary with temperature and moisture, that is, nitrogen increases with cooler temperatures and more moisture.

Right stage of transplants, suitable compost, moisture and other physical factors greatly affect the survival rate of plants on transfer. In the present chapter, successful hardening of the *in vitro* raised seedlings of *N. khasiana*, their establishment under glasshouse conditions and their successful reintroduction into nature have been studied.

Materials and Methods

For hardening, 120 days old healthy *in vitro* grown seedlings of 3-4 cm in size with well developed roots were used. Plastic cups (8 cm in diameter) were used as pots to contain the substratum. Small holes were pierced at the bottom of the cups with the help of dissecting needles. The seedlings were carefully removed from the culture tubes/ flasks by using forceps and these were placed on a tray containing pure water. The agar adhering to the roots was gently washed off with the help of soft brushes so as not to damage the roots. The seedlings were dried on a filter paper and then transferred to the cups containing the potting mixtures.

Different substrata were tested to *in vitro* raised seedlings. The cups were filled with different substrata to 3/4th of their capacity. The different potting mixtures tried were:

1. Soil obtained from the natural habitat
2. Soil, powdered charcoal, brick pieces (2:1:1)
3. Soil, pebbles (2:1)
4. Soil (pebbles to 1/3rd of the cup)
5. Sand (pebbles to 1/3rd of the cup)
6. Soil, sand (1:1) (pebbles to 1/3rd of the cup)
7. Powdered charcoal (pebbles to 1/3rd of the cup)
8. Soil, powdered charcoal (1:1) (pebbles to 1/3rd of the cup)
9. Chopped sphagnum, powdered charcoal (1:1) (pebbles to 1/3rd of the cup)

A single seedling was planted in each of the plastic cups. Water was sprayed to moisten the compost. The cups were covered with pierced polythene sheets to maintain humidity and were transferred to the glasshouse for hardening. The average minimum and maximum temperature of the glasshouse at the time of transplantation were 18°C and 25°C respectively and the humidity ranged from 70 to 80%. Ten times diluted MS nutrient salt solution (ca. 2-3 ml) was sprayed on the potting mixture every morning in the first week and then every alternate morning for two weeks. After a week, the polythene sheets were removed from the cups. The percentage survival of the seedlings was recorded after 90 days of transfer and other growth parameters such as seedling length, pitcher size, etc. were recorded after 180 days of transfer.

Well established seedlings after four to five months of transfer to cups, were transferred to the natural habitat (Jarain). The survival percentage was recorded after one month of transfer.

Soil was collected from two sites each from the natural habitats (Jarain and Lawbah) of *N. khasiana*. Air-dried soil samples were analyzed for total nitrogen content using the PE 2400 Series II CHNS/ O Analyzer at the Regional Sophisticated Instrumentation Centre, North Eastern Hill University, Shillong.

Results

Out of the various potting mixtures tried, soil from the natural habitat was found to be the best substratum for the survival and healthy growth of the seedlings with 97% survival, best seedling length (14.3 cm) and pitcher size (5.5 cm). The seedlings transferred to the compost containing pebbles and powdered charcoal also

gave encouraging results with 94% survival followed by the ones transferred to the compost containing a combination of soil and powdered charcoal (1:1) and soil + pebbles (2:1) (Table 5.1). Sand alone and in combination with soil was not found promising for seedling survival as they resulted in only 73% and 63% survival respectively. The compost comprising of chopped sphagnum and powdered charcoal was not found suitable for transplantation. Herein, only 55% of the total seedlings transferred survived.

Seedling length, number and size of pitchers were recorded after 180 days of transfer. The best seedling length (14.3 cm) and pitcher size (5.5 cm) were obtained in those seedlings which were growing in soil whereas the highest number of pitchers (5.0) was recorded in the ones hardened in the mixture of soil and pebbles. Seedlings growing in compost consisting of soil and powdered charcoal yielded the least number of pitchers (2.3), but the pitcher size and seedling length were moderate when compared with those growing in other substrata. On an average, about 3 pitchers developed in seedlings growing in composts consisting of soil from the natural habitat, soil + charcoal + brick pieces (2:1:1), soil and pebbles (2:1), soil (pebbles to 1/3rd of cup) , soil + sand (1:1) (pebbles to 1/3rd of cup), powdered charcoal (pebbles to 1/3rd of cup) and chopped sphagnum + powdered charcoal (1:1) (pebbles to 1/3rd of cup). About 4 pitchers developed in seedlings growing in substratum consisting of sand and pebbles when observed after 180 days of transfer. Size of the pitchers ranged from 1.6 cm (chopped sphagnum + powdered charcoal) to 5.5 cm (soil from the natural habitat). Pitchers of about 4 cm in length developed in seedlings growing in soil and pebbles, sand and pebbles and soil + sand (pebbles to 1/3rd of cup). Seedlings

Table 5.1. The response of *in vitro* grown seedlings to different potting mixtures

	Substratum	Survival (%)*	No. of pitchers/plant**	Pitcher Size(cm)**	Seedling Length(cm)**
1	Soil	97 ± 5.7	3.6 ± 0.24	5.5 ± 0.25	14.3 ± 0.54
2	Soil + charcoal + brick pieces (2:1:1)	81 ± 4.6	3.5 ± 0.28	3.6 ± 0.08	11.3 ± 0.68
3	Soil + pebbles (2:1)	90 ± 5.1	5.0 ± 1.29	4.0 ± 0.07	7.6 ± 0.23
4	Soil (pebbles to 1/3 rd of cup)	87 ± 4.0	3.3 ± 0.25	4.5 ± 0.13	6.8 ± 0.59
5	Sand (pebbles to 1/3 rd of cup)	73 ± 4.0	4.2 ± 0.2	4.9 ± 0.10	8.2 ± 0.43
6	Soil+ Sand (pebbles to 1/3 rd of cup)	63 ± 4.6	3.5 ± 0.22	4.5 ± 0.11	8.7 ± 0.59
7	Powdered charcoal (pebbles to 1/3 rd of cup)	94 ± 5.7	3.0 ± 0.00	2.5 ± 0.11	14.0 ± 0.4
8	Soil + powdered charcoal (1:1) (pebbles to 1/3 rd of cup)	93 ± 5.7	2.3 ± 0.25	3.2 ± 0.15	11.0 ± 0.57
9	Chopped sphagnum + powdered charcoal (1:1)(pebbles to 1/3 rd of cup)	55 ± 4.6	3.6 ± 0.28	1.6 ± 0.12	6.13 ± 0.82

± S.E.

* Data recorded after 90 days. Average of 50 seedlings

** Data recorded after 180 days. Average of 50 seedlings

transferred to powdered charcoal (pebbles to 1/3rd of cup) developed smaller pitchers of about 2.5 cm in length. Pitchers of about 3 cm in length were obtained in seedlings growing in soil + charcoal + brick pieces (2:1:1) and soil + powdered charcoal when recorded after 180 days of transfer. Best seedlings in terms of length were obtained in soil from the natural habitat followed by powdered charcoal (pebbles to 1/3rd of cup), soil + charcoal + brick pieces (2:1:1) and soil + powdered charcoal. Seedlings grew up to a length of only about 8 cm in sand alone and in combination with soil when recorded after 180 days of transfer. Seedlings of only about 6-7 cm developed in substratum consisting of chopped sphagnum + powdered charcoal (1:1) (pebbles to 1/3rd of cup), soil (pebbles to 1/3rd of cup) and soil + pebbles (2:1).

In 4-5 weeks, the seedlings were hardened and established in the cups. The plants have been maintained in the glasshouse for over two years (Photoplate 5.1). Hundred percent of the plants transferred to the natural habitat survived when recorded after one month of transfer.

Analysis of nitrogen content of the soil showed that percentage nitrogen of the soil collected from the natural habitat (Jarain and Lawbah) was lower than that from the control. The nitrogen content of soil collected from Jarain was found to be about 0.71% where density of the plants was 2.0 individuals/sqm and 1.07% where the plant density was 1.5 individuals/sqm. The soil from Lawbah was found to contain 1.09% nitrogen where the plant density was 2.0 individuals/sqm whereas total nitrogen was not detected in the site where plant density was 2.7 individuals/sqm (Table 5.2).

Photoplate 5.1

(a) *In vitro* raised seedlings in plastic cups after 120 days of transfer

(b) Seedlings transferred to earthen pots after 180 days



Table 5.2. Analysis of soil collected from different sites for nitrogen content and density of individuals

Site	Sample	% N	Average Density (individuals / sqm)
Shillong	S	1.46	0
Lawbah	L1	ND	2.7
	L2	1.09	2.0
Jarain	J1	0.71	2.0
	J2	1.07	1.5

Discussion

The successful transplantation of seedlings depends primarily on the suitable size of seedlings and the conditions to which they are exposed during transplantation. Healthy and vigorously growing seedlings with well developed roots were found to be most suitable for transfer to cups. It is a well known fact that hardiest and vigorous seedling is less susceptible to diseases and mechanical injuries. Soil obtained from the natural habitat was found to be the best substratum for hardening of the plants. Analysis of the soil obtained from the natural habitat revealed that it was deficient in nitrogen. As *Nepenthes* are known to grow in nitrogen deficient soils, this soil was therefore helpful for maximum survival of the *in vitro* raised seedlings. When soil was mixed with pebbles in the ratio of 2:1 as well as when pebbles were kept in the lower 1/3rd portion of the cup, the percentage survival of the seedlings decreased which could be due to the excess drainage of water and nutrients. Powdered charcoal alone and in combination with soil exhibited results with about 93% and 94% survival of the seedlings respectively. This may be because charcoal adsorbed the excess nutrients provided in the initial days of transplantation therefore suggesting that powdered charcoal is beneficial for *N. khasiana* and could be used instead of soil from the natural habitat for successful hardening of this species. Sand was not found suitable for hardening which could be because it could not retain the amount of water needed by the growing plants. The least number of seedlings survived in the compost consisting of chopped sphagnum and powdered charcoal, although powdered charcoal alone was beneficial. This reflects that chopped sphagnum was not helpful which could have been because of its ability to retain the moisture and absorb the tiny

amounts of nitrates and phosphates very efficiently. As *N. khasiana* is known to grow in nutrient deficient soil, excess nutrients could have hampered the survival of the seedlings.

Best pitcher size was obtained in seedlings growing in soil followed by the other substrata viz. sand, soil and pebbles and substratum containing soil and sand. This is not surprising as the substrata containing soil from the natural habitat are nitrogen deficient and pitchers are known to develop so as to trap the insects to supplement nutrient deficiency. Seedlings growing in the substrata containing charcoal however developed smaller pitchers. Smallest size pitcher was obtained in compost containing chopped sphagnum. This could again be because of the retention of nutrients supplied in the initial days of transplantation by the sphagnum. Maximum seedling length was obtained in the seedlings growing in soil from the natural habitat. In comparison, plantlets growing on compost containing pebbles to 1/3rd of the cup showed stunted growth. Sand alone and in combination also was not much helpful for the growth of the seedlings in terms of its length. These findings are in agreement with the growth conditions of the plant in nature where plants growing on moist soil substrata were found to be taller than those growing on rocky clefts or sandy pockets (Bordoloi, 1977).

Drilling of holes at the bottom of the pots was essential to facilitate drainage and prevent extra water logging. Survival of transferred plantlets largely depends on their ability to carry out photosynthesis and withstand water loss. Plantlets cultured *in vitro* are highly susceptible to desiccation once transferred to soil. *In vitro* plantlets have the characteristics of less or no photogenic pigments, malfunctioning of stomata

and marked decrease in epicuticular waxes that leads to the desiccation and hence death of the plants (Bhojwani and Dhawan, 1989). Covering the pots with polythene sheets aided in maintaining the humidity which was essential for the plantlets in the initial stage of hardening to prevent desiccation. Maintenance of high humidity is necessary for some time after transplantation, for the continued turgidity of the tissue-cultured leaves, until the freshly acclimatized leaves develop (Donnelly and Vidaver, 1984). The leaves from *in vitro* developed plants are less able to control stomatal transpiration than normal leaves especially at Relative Humidity (RH) less than 90%. Exposure to higher light intensities, while maintaining a high RH is reported to stimulate faster development of acclimatized leaves (Sutter *et al.*, 1988). Partial defoliation of the plantlets at the time of transplantation is also reported to be beneficial in certain cases (Bhojwani, 1980; Tisserat, 1981). But in the present study, there was no need to remove the leaves as they were few in number. Feeding the plants with diluted MS nutrient salt proved to be beneficial for the plants as it provided the essential nutrients required for gradual adaptation of the plantlets to the outside environment. Similar reports of supplying diluted nutrient salt solution to the potted plants have been reported (Kumaria, 1991; Kumaria and Tandon, 1994; Bhat and Dhar, 2000). Plantlets hardened and established in the cups are being maintained in the glasshouse for over two years.

The recovery of threatened species generally hinges on providing suitable habitat and conditions in which they can thrive. Most biodiversity losses can be directly attributed to habitat loss, so provision of habitat is often the key requirement for recovery of a species. Therefore, the reintroduction of *N. khasiana* into its natural

habitat was done. Records taken after one month of transfer revealed that all the plants reintroduced survived. Analysis of the soil from the natural habitat established that the soil is indeed nitrogen deficient. The density of *N. khasiana* was related to soil fertility, as expressed by nitrogen percentage analysis, of the sites occupied by species. Carnivorous plants grow well when the nutrient stress is high and where light is abundant (Brewer, 2002). They are almost entirely restricted to habitats such as bogs, where soil nutrients are extremely limiting, but where sunlight and water are readily available. Being carnivorous allows *N. khasiana* to grow better when the soil contains less nitrogen or phosphorus. In particular, an increased supply of nitrogen and phosphorus makes photosynthesis more efficient which would not be helpful for carnivorous plants. One hundred percent survival of the reintroduced plants is therefore not surprising since the reduced nutrient content of the soil is beneficial for its growth and development.

CHAPTER VI: ANALYSIS OF GENETIC VARIABILITY IN *NEPENTHES KHASIANA* USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

Introduction

The assessment of variation between plants at DNA level has achieved great importance in recent years. Polymorphism revealed by molecular markers can be used to construct genetic maps and to determine genetic diversity at DNA level and relatedness/ phylogenetic relationship among populations and species. Availability of such data can help researchers assess the ability of rare plant populations to adapt to changes in their natural environment and to respond to reintroduction programmes and natural selection. DNA based markers allow the direct comparison of the genetic material of two individual plants avoiding any environmental influences on gene expression (Newbury and Ford-Lloyd, 1993). The most common techniques use RFLP and numerous genetic marker assays based on PCR such as RAPD, SSR and AFLP (Karp *et al.*, 1996).

RAPD, a rapid and sensitive technique using the PCR was introduced in the early nineties (Williams *et al.*, 1990; Welsh and McClelland, 1990; Welsh *et al.*, 1991). This technique uses short synthetic deoxyribonucleotides of random sequences as primers for PCR. While PCR products are obtained from random regions of the genome, they are specific and reproducible since they are primed from specific DNA

sequences within the genome. In his approach, Williams *et al.* (1990) used 10-base synthetic oligonucleotides with a GC content of 50-60% wherein the number of potential 10-base primers was very large so that numerous polymorphisms between even closely related organisms could be obtained. RAPD technique does not require DNA probes or prior sequence information. The primers are homologous to the random target sites in the genome. This results in amplifying regions of the genome that are flanked by the specific priming sites. Co-migrating fragments between accessions is assumed to represent homologous regions of the genome. The RAPD procedure is simple, largely automatable, require only small amounts of DNA and can be performed without the use of radioactivity (Karp *et al.*, 1996).

The genetic structure of plant populations reflects the interactions of different processes including long-term evolutionary history of the species (shifts in distribution, habitat fragmentation, population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin, 1985; Schaal *et al.*, 1998). All these factors can lead to complex genetic structuring within populations, which is often difficult to resolve. A species without an appropriate amount of genetic diversity is thought to be unable to cope with changing environment or evolving competitors and parasites. Nevertheless, the development of different DNA markers has provided powerful tools for the investigations of genetic variation within a species and can facilitate understanding of such complexities (Mitton, 1994). Molecular analysis of intraspecific variation, in particular, may find application in resolving disputes of taxonomic identities, relations and authentication of the species, developing a comprehensive database of genetic variability in the species for future reference and

protection of genetic diversity of the species. Conservation biologists often use information about the distribution of genetic variation within and among populations to set conservation priorities and plan management strategies (Schonewald-Cox *et al.*, 1983; Hamrick *et al.*, 1991; Frankel *et al.*, 1995; Schmidt and Jensen, 2000).

Genetic diversity is important for the population to be able to face future environmental changes and to ensure a long-term response to selection (Gilpin and Soule, 1986; Hunter, 1996; Frankham *et al.*, 2002; Fernandez *et al.*, 2004). For species with limited population size and number, RAPD fingerprinting provides an ideal tool for detecting existing variation within and between constituting individuals (Hadrys *et al.*, 1992; Lynch and Milligan, 1994; Rieseberg and Swensen, 1996). RAPD markers have been used successfully to assess genetic stability among micropropagated plants of poplar (Rani *et al.*, 1995), *Zingiber officinales* Rosc. (Rout *et al.*, 1998), *Pinus thunbergii* Parl. (Goto *et al.*, 1998), *Camellia sinenses* and *C. assamica* ssp. *Assamica*, (Devarumath *et al.*, 2002; Mondal and Chand, 2002), *Dioscorea bulbifera* L. (Dixit *et al.*, 2003) and many other plants. In certain cases, genetic instability of plants derived from meristem cultures has been reported (Zucchi *et al.*, 2002). It has also been shown to be an effective method for detecting polymorphism in various crop species (Virk *et al.*, 1995; Penner, 1996; Sun *et al.*, 1999). For assessment of genetic diversity, RAPDs have been used within many plant groups, including herbaceous (Dawson *et al.*, 1993; Nolan *et al.*, 1996), woody angiosperms (Schierenbeck *et al.*, 1997; Heaton *et al.*, 1999) and conifers (Bucci and Menozi, 1995; Alnutt *et al.*, 1999, 2001). The RAPDs also provide a useful tool for molecular characterization of endemic species and for resolving relationships among

populations (Abo-elwafa *et al.*, 1995; Casler *et al.*, 2003; Phan *et al.*, 2003; Fu *et al.*, 2003). They were shown to have been sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991; Roy *et al.*, 1992; Abo-elwafa *et al.*, 1995). RAPDs have been used in many threatened plant taxa for analysis of genetic variation with an aim of conserving them both *in situ* (Hogbin and Peakall, 1999; Newton *et al.*, 1999) and *ex situ* (Virk *et al.*, 1995; Hsu *et al.*, 2000; Alnutt *et al.*, 2001; Singh *et al.*, 2001; Torres *et al.*, 2003). RAPD markers have been frequently used to determine the genetic variability of endangered, relict and/or endemic taxa (Stewart and Porter, 1995; Bauert *et al.*, 1998; Liao and Hsiao, 1998; Las Heras *et al.*, 1999; Maki and Horie, 1999). RAPD markers can be of great value in the measurement of intraspecific variations as revealed in *Brassica oleracea* (Kresovich *et al.*, 1992), *Lycopersicon esculentum* (Williams and St Clair, 1993), *Brassica juncea* (Jain *et al.*, 1994), *Erodium paularense* (Martin *et al.*, 1997), *Rosmarinus tomentosus* (Martin and Bermejo, 2000), *Pinus halepensis* (Gomez *et al.*, 2001), *Changium smyrnoides* (Fu *et al.*, 2003), *Ginkgo biloba* (Fan *et al.*, 2004), *Melia volkensii* (Runo *et al.*, 2004), *Coelonema draboides* (Chen *et al.*, 2005), *Neolitsea sericea* (Wang *et al.*, 2005), *Stipa krylovii* (Wang *et al.*, 2006), etc.

Material and Methods

Survey and Collection of Plant Material

A survey was made for collection of *N. khasiana* from three locations of Meghalaya namely Jarain (Jaintia Hills District), Baghmara (South Garo Hills District) and Lawbah (East Khasi Hills District) (Fig. 6.1, Table 6.1). Tender, young and fully expanded healthy leaves of plants were collected from these populations. To

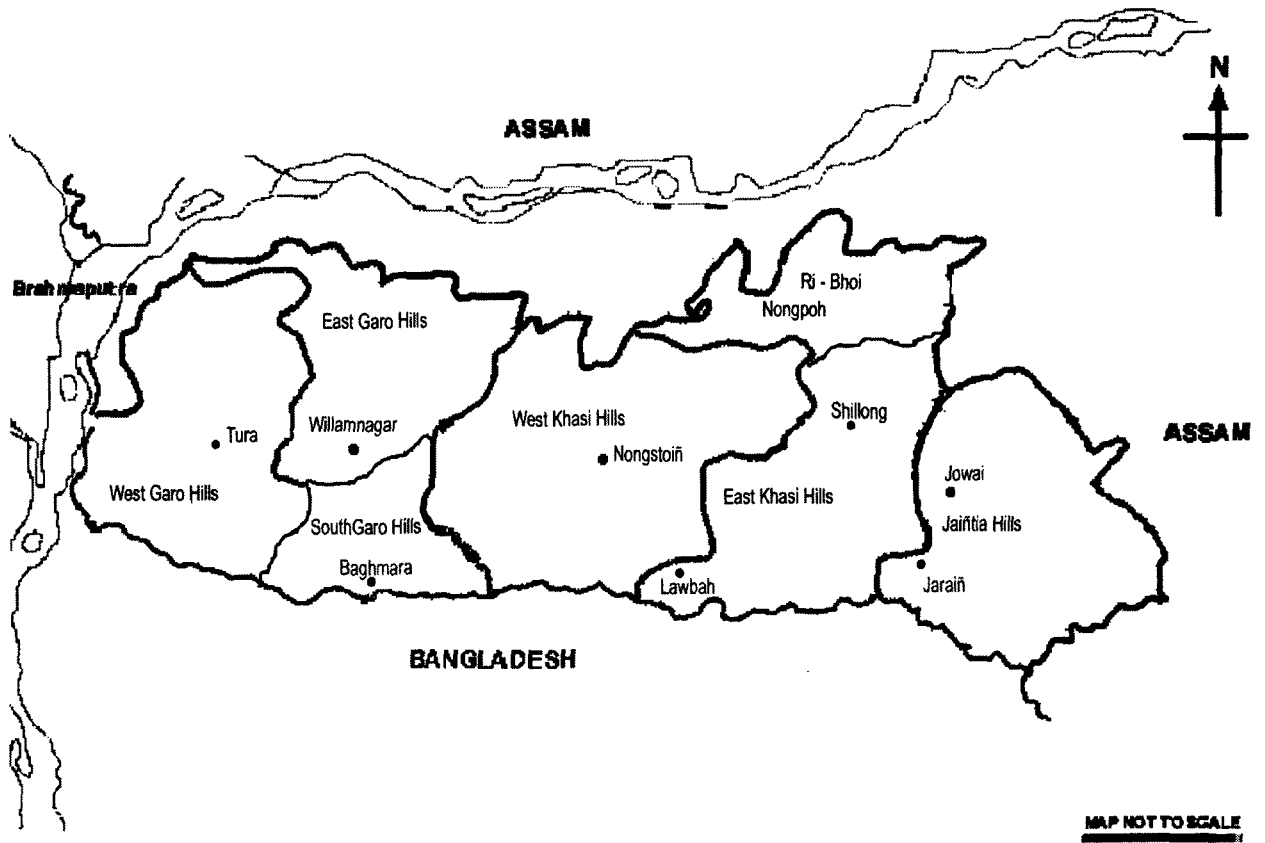


Fig. 6.1. Map of Meghalaya showing the different zones of collection

Table 6.1. Different regions of Meghalaya for collection of *N. khasiana*

Region	Altitude	Longitude	Latitude
Jarain	760m	92° 8' 31"E	25° 20' 14"N
Baghmara	60m	90° 38' 38"E	25° 12' 6"N
Lawbah	760m	91° 31' 57"E	25° 14' 21"N

assess the genetic variation between tall (15-20 m) and short (10-15 cm), and male and female plants, individuals from one population (Baghmara) were selected (Table 6.2). For intrapopulation variability studies, a total of 24 individuals (8 individuals from each population) were used for the experiment (Table 6.3). The leaves were wiped with moistened tissue paper, wrapped in aluminium foil and stored in liquid nitrogen.

Chemicals

Molecular Biology Grade Chemicals used in the present study were obtained from Sigma Chemical Corporation, USA. Taq DNA polymerase, Taq Buffer, dNTPs, MgCl₂, Ribonuclease A and Proteinase K were obtained from Bangalore Genei, Bangalore, India. All the random primers used were obtained from Operon Technologies Inc., Alameda, California. Other chemicals used were of Analytical Reagent Grade and obtained from Merck, HiMedia, and Sisco Research Laboratories (SRL).

Isolation of genomic DNA

Total genomic DNA was extracted from frozen (liquid nitrogen) young leaves of *N. khasiana* following the modified CTAB method described by Porebski *et al.* (1997). Two gram of the leaves were weighed and ground to fine powder in liquid nitrogen using pestle and mortar. The powder was transferred to a 50 ml centrifuge (Falcon) tube containing 10 ml CTAB extraction buffer (Table 6.4). This was then incubated at 60°C in the water bath for 1h with an occasional mixing. After the incubated period was over, the tubes were allowed to cool for 4-6 min. A mixture of

Table 6.2. Samples collected from Baghmara

Lane No. in the gel	Samples
1	TM1
2	TM2
3	TF1
4	TF2
5	SM1
6	SM2
7	SF1
8	SF2

T: Tall plants; S: Short Plants, M: Male; F: Female

Table 6.3. Samples collected from populations of different locations

Location	Lane No. in the gel	Samples
Jarain	1	J1
	2	J2
	3	J3
	4	J4
	5	J5
	6	J6
	7	J7
	8	J8
Baghmara	10	B1
	11	B2
	12	B3
	13	B4
	14	B5
	15	B6
	16	B7
	17	B8
Lawbah	19	L1
	20	L2
	21	L3
	22	L4
	23	L5
	24	L6
	25	L7
	26	L8

Table 6.4. Solutions for DNA Extraction

2 M Tris HC (pH 8)	
0.5 M EDTA (pH 8)	
TE buffer	10 mM Tris HCl + 0.1 mM EDTA
Ribonuclease A (10 mgml ⁻¹)	
Proteinase K (1 mgml ⁻¹)	
CTAB Extraction buffer	100 mM Tris buffer + 1.4 M NaCl + 20 mM EDTA + 2% Cetyltrimethyl ammonium bromide (CTAB) + 0.3% β -Mercaptoethanol * + 1% PVP *
3 M Sodium acetate (pH 5.2)	
5 M NaCl	
Phenol saturated with Tris	
Chloroform: Octanol (24:1)	
Chloroform:isoamyl alcohol (24:1)	
Ethanol	

* Added to the tube just before adding the sample

10 ml of chloroform: octanol (24:1) was added to the tube, balanced and mixed on a rocker for 10 min to form an emulsion. Centrifugation was done at 3000 rpm for 10 min at room temperature (25°C) using REMI C-24 Cooling Centrifuge. The upper layer was aspirated using Pasteur pipettes and transferred to new 50 ml tubes. Chloroform: octanol extraction was repeated once more to remove cloudiness. After the second aspiration, DNA was precipitated by adding 0.5 volume of 5 M NaCl and 2.5 volume of chilled 95% ethanol to the tube. The DNA was pelleted down by centrifuging at 3000 rpm for 15 min. The pellet was washed with 70% alcohol for 20-30 min at room temperature. The pellet was then dried at 37°C and dissolved in 2 ml TE buffer. Five microlitre of RNase A (10 mgml⁻¹) was added to the solution and incubated at 37°C for 1h. After the incubation period, 5 µl Proteinase K (1mgml⁻¹) was added and the solution was kept at 37°C for another hour. After incubation, the contents of the tube were transferred to a 12 ml centrifuge tube. Equal volume of phenol (tris saturated) was added to the tube, mixed on rocker for 10 min and centrifuged for 10 min at 3000 rpm. After centrifugation, the upper aqueous layer was aspirated into a fresh tube. This step was repeated again once. The second aspiration was followed by the partial phenol step where equal volume of phenol: chloroform (1:1) was added before centrifugation. After aspirating the upper layer, equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube, balanced, mixed and centrifuged. This step was repeated twice. The upper layer was aspirated into a new tube and to it was added 1/10th volume Na-acetate and 2.5 volume chilled ethanol to precipitate DNA. The tube with all its contents was kept at -20°C for 20-30 min. The DNA was pelleted and washed with 70% alcohol at room temperature for 30 min. The

DNA was air dried and finally dissolved in TE buffer in an eppendorf tube and stored at 4°C till further use.

Quality check of isolated 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 0.8 g agarose in 1X TAE (Table 6.5). Five microlitre of ethidium bromide (10 mgml⁻¹ stock) was added to the solution. 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 1 h at room temperature after which the comb was removed. The gel tray was placed into the submarine tank already filled with 1X TAE. Two microlitre of the isolated DNA sample was mixed with 7 µl sterile distilled water and 1 µl 10X loading dye. The mix was then loaded into the wells of the gel. Electrophoresis was done at 5 voltscm⁻¹. The run was stopped when the bromophenol blue dye reached 2/3rd of the gel. The gel was then observed in the gel documentation system (UVP Gel Doc) and photographed.

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 and 280 nm in a UV/Vis 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 standard ratio i.e. $A_{260} : A_{280}$ of a preparation of pure sample of DNA is equal to 1.8.

Table 6.5. Solutions for Quality Check of isolated DNA

1X TAE Buffer (pH 8)	Tris buffer + 0.5 M EDTA + Glacial acetic acid
6X Loading dye	0.25% Bromophenol blue 0.25% Xylene cyanol FF 40% Sucrose
10X Loading dye	0.4%Bromophenol blue 0.4% Xylene cyanol FF 66% Sucrose
Ethidium bromide	10 mgml ⁻¹

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:

$$\text{Concentration of DNA } (\mu\text{g}\mu\text{l}^{-1}) = \frac{A_{260} \times 50 \times \text{Dilution factor}}{1000}$$

(For double stranded DNA, 1 O.D. at 260 nm = 50 $\mu\text{g}\mu\text{l}^{-1}$ DNA)

A 100 μl (10 $\text{ng}\mu\text{l}^{-1}$) working DNA stock solution was prepared by diluting in ultra pure water and stored at 4°C until use.

Standardisation of RAPD protocol and primer screening

Different concentrations of genomic DNA (10 ng, 20 ng, 30 ng, 40 ng), MgCl_2 (1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM) and Taq DNA polymerase (0.5U, 0.7U, 1.0U) were tested (other constituents in the reaction mixture were kept at same concentration) in various combinations to ascertain the most suitable conditions for obtaining good and analyzable amplification products. RAPD analysis was performed using randomly chosen arbitrary 10-mer primers of A, C, K, H and I series, obtained from Operon Technologies Inc., Alameda, California. To obtain the best profiles, initially one hundred primers were tested. Of these, the primers which produced good analyzable amplification products were selected.

PCR parameters and RAPD fingerprinting

The PCR reaction was conducted following the protocol of Williams *et al.* (1990) with slight modifications. Amplifications were done in 25 μ l reaction mixture containing 2.5 μ l of 10X assay buffer, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 15ng primer, 1.5 mM MgCl₂, 0.5U Taq DNA Polymerase and 20 ng of template DNA. DNA amplification was performed in a thermal cycler (Applied Biosystems Gene Amp PCR System) programmed for 45 cycles as follows:

1 cycle of	4.5 min at 94°C
	1 min at 34°C
	2 min 72°C

Followed by 44 cycles, each of	1 min at 94°C
	1 min at 34°C
	2 min at 72°C

Final extension of	15 min at 72°C
--------------------	----------------

After completion of PCR, 2 μ l of 10X loading dye was added to each of the samples.

The amplification products were size separated by electrophoresis in 1.2% agarose gels containing 0.05 μ g μ l⁻¹ ethidium bromide in 0.5X TBE buffer. Hind III digested λ -DNA was loaded in one of the lanes to serve as molecular size marker. The gel was observed on the gel documentation system (UVP Gel Doc) and photographed.

Data Analysis

Amplicons were scored as discrete variables, using 1 to indicate presence and 0 for absence, and a binary data matrix was prepared. The binary data was converted into similarity matrix using Jaccard Similarity Coefficient (Jaccard, 1908).

$$J = n_{xy} / (n_t - n_z)$$

Where n_{xy} is the number of bands common to the two samples compared, n_t is the total number of bands present in all samples and n_z is the number of bands not present in the two samples compared but found in the other samples (Sneath and Sokal, 1973).

A dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested Clustering) option in the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System), version 2.02K program.

The degree of polymorphism was quantified using Shannon's diversity index:

$$H_o = - \sum p_i \ln p_i$$

Where p_i is the relative frequency of RAPD marker i (King and Schaal, 1989)

H_o can be calculated and compared for different populations

Let, $H_{pop} = 1/n \sum H_o$ be the average diversity where n is the number of populations

Let $H_{sp} = - \sum p \ln p$ be the diversity calculated from frequency p in all the populations considered together

The proportion of diversity present within populations (H_{pop}/H_{sp}) can be compared with that between populations $\{(H_{sp} - H_{pop})/ H_{sp}\}$

Results

Isolation of genomic DNA

In all the samples used for extraction of DNA, the yield of was good with less shearing.

Quality check of isolated DNA

Purity of the isolated DNA was found to be close to the standard ratio of pure DNA. For all the samples the ratio at $A_{260} : A_{280}$ was found to be between 1.2 and 2.2.

Standardisation of RAPD protocol and primer screening

Out of the different concentrations of genomic DNA (10ng, 20ng, 30ng, 40ng), $MgCl_2$ (1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM) and Taq DNA polymerase (0.5U, 0.7U, 1.0U) tested (other constituents in the reaction mixture were kept at same concentration) in various combinations, it was found that a combination of 20 ng of template DNA, 1.5 mM $MgCl_2$ and 0.5U Taq DNA polymerase were the most suitable for obtaining good and analyzable amplification products. To obtain the best profiles, initially one hundred primers were tested. Of these, 10 primers (Table 6.6) produced good analyzable amplification products and were selected.

RAPD fingerprinting

Variability within individuals of a single population

For analysis of variability of *N. khasiana*, ten primers were used for studying the RAPD banding patterns between tall (15-20 m) and short (10-15 cm), and male and female plants from one population (Baghmara). Of the 10 primers used, 6 primers showed polymorphism (Table 6.7; Photoplate 6.1, 6.2). Four of the primers namely

Table 6.6. List of primers selected for RAPD Fingerprinting

Primer	Sequence 5' to 3'
OPC-15	GACGGATCAG
OPH-03	AGACGTCCAG
OPH-13	GACGCCACAC
OPH-20	GGGAGACATC
OPK-04	CCGCCCAAAC
OPK-07	AGCGAGCAAG
OPK-16	GAGCGTCGAA
OPK-17	CCCAGCTGTG
OPK-18	CCTAGTCGAG
OPK-19	CACAGGCGGA

Table 6.7. Polymorphism generated by the RAPD primers in the Baghmara individuals

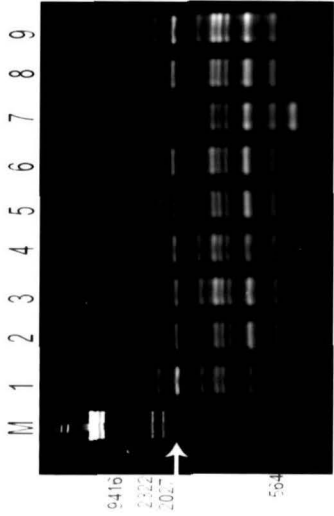
Primer	Sequence 5' to 3'	Total No. of bands	Monomorphic bands	Polymorphic bands	% Polymorphism
OPC-15	GACGGATCAG	4	2	2	50
OPH-03	AGACGTCCAG	5	4	1	20
OPH-13	GACGCCACAC	6	6	0	0
OPH-20	GGGAGACATC	3	3	0	0
OPK-04	CCGCCCAAAC	3	3	0	0
OPK-07	AGCGAGCAAG	4	3	1	25
OPK-16	GAGCGTCGAA	4	3	1	25
OPK-17	CCCAGCTGTG	4	2	2	50
OPK-18	CCTAGTCGAG	4	4	0	0
OPK-19	CACAGGCGGA	4	3	1	25
Total		41	33	8	Avg 24

Photoplate 6.1

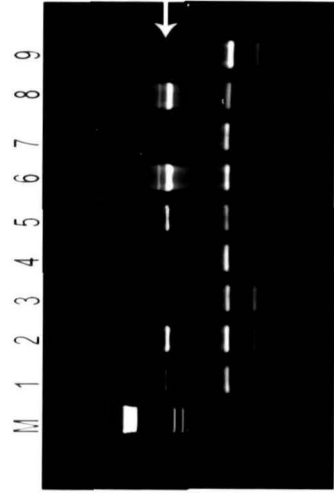
RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

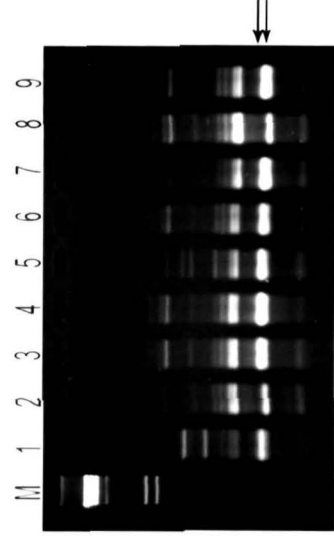
**Lane 1: TM1, Lane 2: TM2, Lane 3: TF1, Lane 4: TF2, Lane 5: SM1, Lane 6: SM2,
Lane 7: SF1, Lane 8: SF2, Lane 9: Bulk**



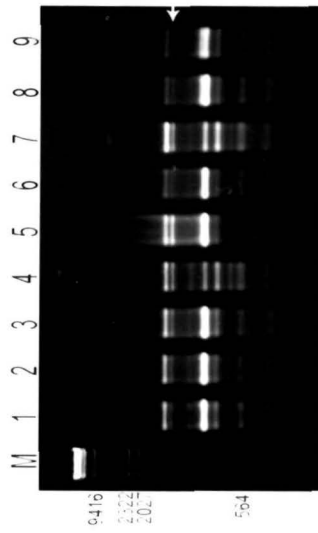
OPH-03



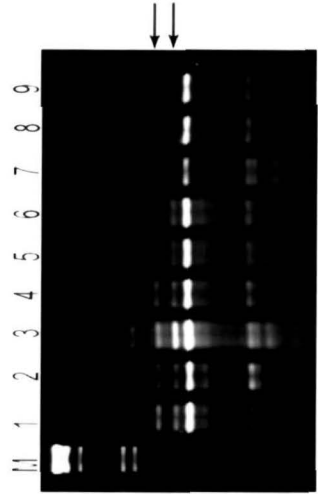
OPK-19



OPK-17



OPK-16



OPC-15

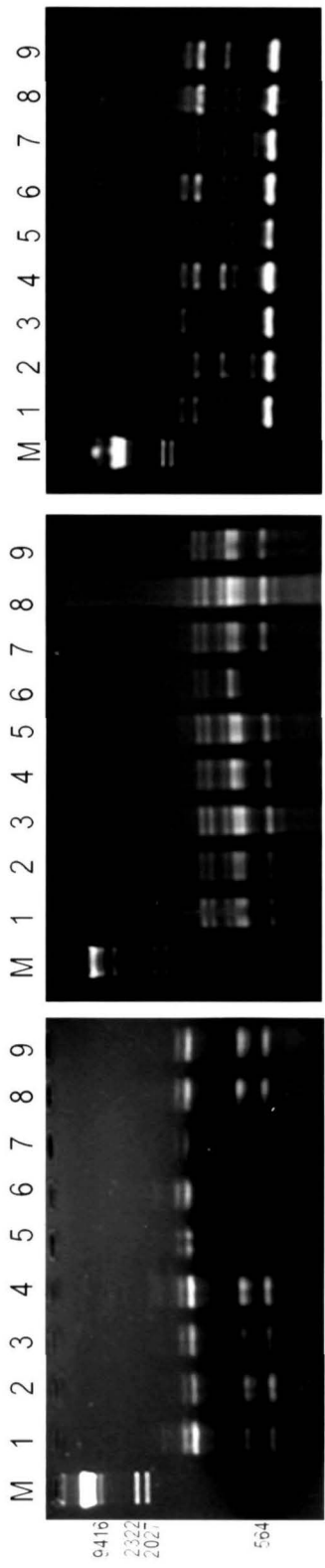
Photoplate 6.2

RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1: TM1, Lane 2: TM2, Lane 3: TF1, Lane 4: TF2, Lane 5: SM1, Lane 6: SM2,

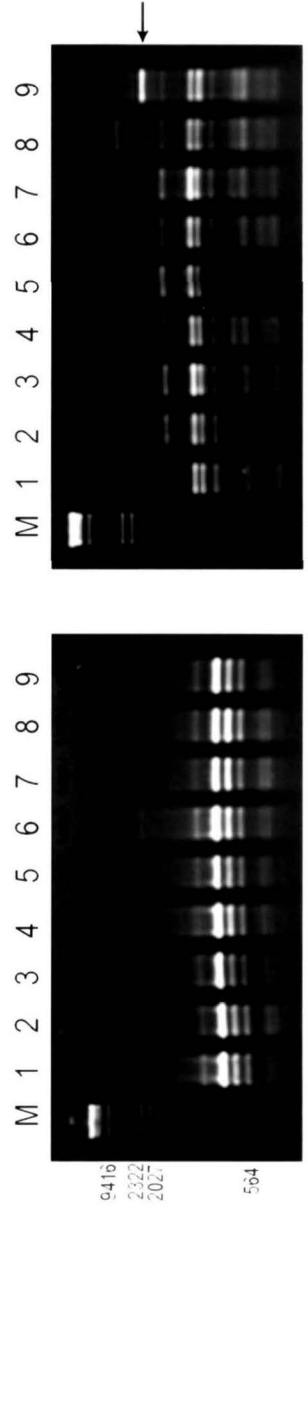
Lane 7: SF1, Lane 8: SF2, Lane 9: Bulk



OPK-18

OPH-13

OPH-20



OPK-07

OPK-04

OPK-18

OPH-20, OPH-13, OPK-18 and OPK-04 did not show any polymorphic bands (Photoplate 6.2).

With OPC-15, all samples produced 2-4 numbers of amplicons (Photoplate 6.1). Two fragments were found to be polymorphic among the different individuals. The primer, OPH-03 produced 1-5 number of bands of which one was polymorphic. OPK-04 produced 3 bands which were all monomorphic. The primer OPK-07 generated 3-4 scorable amplicons. SF2 generated an amplicon of ~ 1556 bp which was absent in the other samples. OPK-16 produced 3-4 scorable bands. A characteristic band of ~ 1444 bp was polymorphic among the different individuals. OPK-17 produced 2-4 bands of which two bands of ~ 611 bp and ~ 639 bp were polymorphic. Primer OPK-18 produced 4 amplicons which were all monomorphic. OPK-19 produced 3-4 number of bands of which one was polymorphic. Of a total of 41 bands amplified by 10 primers, only 8 fragments showed polymorphism. The total number of fragments generated by the different samples ranged from 35 to 38 (Table 6.8). The number of products generated by each primer varied from 3 to 6. The maximum percentage polymorphism (50%) was revealed by primer OPK-17 and OPC-15. The results showed that the average percentage polymorphism was around 24%.

Jaccard's similarity values between pairs of the 8 individuals ranged from 0.87 to 1.00 (Table 6.9). The lowest similarity coefficient (0.87) was found between SF1 and TM1 and, SF1 and TM2. SF2 showed a similarity coefficient of 0.88 when compared with TM1, TF1 and TF2. The similarity coefficient between SF1 and SF2 was 0.89. A similarity coefficient of 0.92 was obtained between SF1 and TF1, SF1

Table 6.8. Summary of amplified DNA fragments from different samples collected from Baghmara

Lane No.	Samples	Total No. of	Average fragments
1	TM1	38	3.8
2	TM2	38	3.8
3	TF1	38	3.8
4	TF2	38	3.8
5	SM1	38	3.8
6	SM2	38	3.8
7	SF1	35	3.5
8	SF2	37	3.7

Table 6.9. Jaccard's similarity coefficient matrix based on RAPD banding profile

	TM1	TM2	TF1	TF2	SM1	SM2	SF1	SF2
TM1	1.00							
TM2	0.95	1.00						
TF1	0.95	0.95	1.00					
TF2	0.95	0.95	1.00	1.00				
SM1	0.95	0.95	0.95	0.95	1.00			
SM2	0.95	0.95	0.95	0.95	1.00	1.00		
SF1	0.87	0.87	0.92	0.92	0.92	0.92	1.00	
SF2	0.88	0.92	0.88	0.88	0.92	0.92	0.89	1.00

and TF2, SF1 and SM1, SF1 and SM2, SF2 and SM1 and SF2 and SM2. A similarity index of 0.95 was obtained between all the tall plants except TF1 and TF2 which showed a similarity coefficient of 1.00. The short female plants also showed a similarity index of 0.95 when compared with all the tall plants. There was 100% similarity between SM1 and SM2.

Cluster analysis

A dendrogram generated by unweighted pair group method with arithmetic averages (UPGMA) algorithm revealed a major heterogeneous cluster consisting of male, female, tall and short plants together (Fig. 6.2). There was 100% similarity between TF1 and TF2 as well as between SM1 and SM2. TM1 and TM2 are also present in the same cluster with about 95% similarity between them. SF1 and SF2 are a little diverse from the rest showing about 90% similarity.

Variability among populations from different locations

For detection of variability between populations growing in three different locations i.e. Jarain, Baghmara and Lawbah, 24 individuals were selected. Out of the selected 10 primers used for the investigation, 8 primers showed polymorphism (Table 6.10). Two of the primers namely OPH-13 and OPH-20 did not show any polymorphism (Photoplate 6.3, 6.4).

Primer OPK-18 produced 3-4 amplicons (Photoplate 6.3). A characteristic band of ~ 1000 bp was present in the Jarain and the Baghmara population which was missing in the Lawbah population. With OPC-15, all samples produce 2-4 numbers of

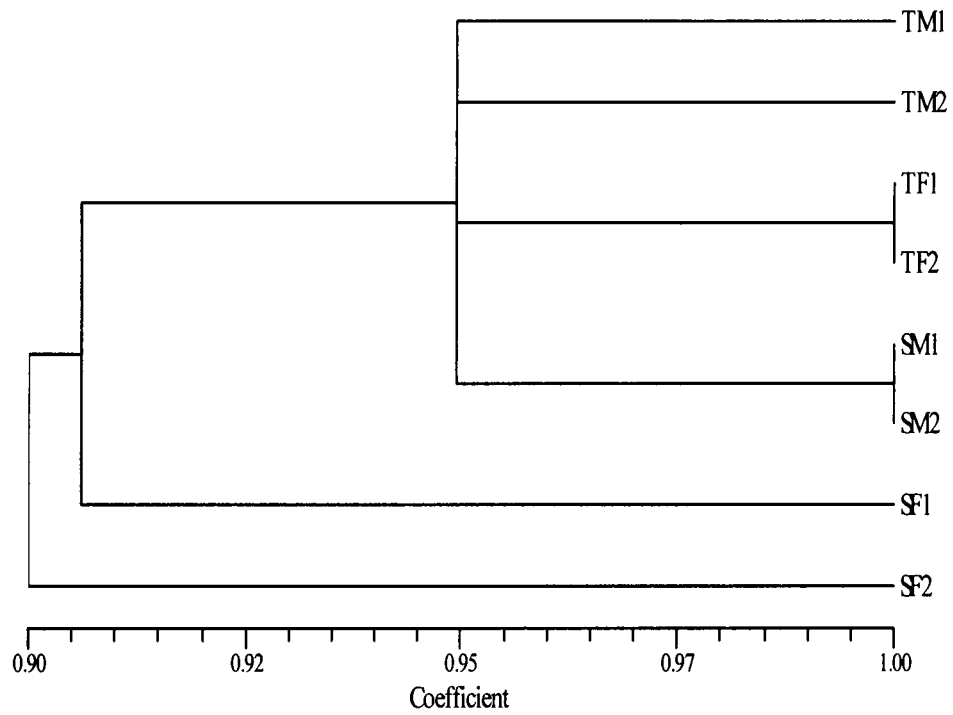


Fig 6.2. Dendrogram showing the relationship of 8 individuals from Baghmara generated using UPGMA based on Jaccard's similarity coefficient. T: Tall plants; S: Short Plants, M: Male; F: Female

Table 6.10. Polymorphism generated by the RAPD primers used in the present investigation

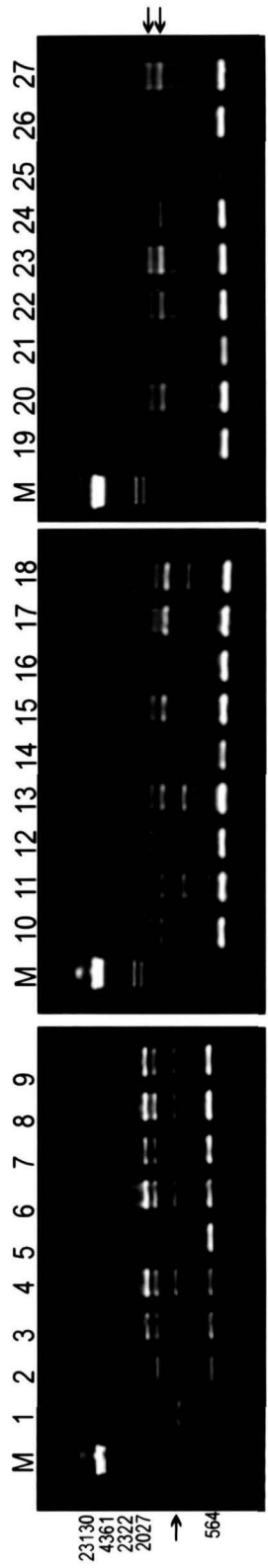
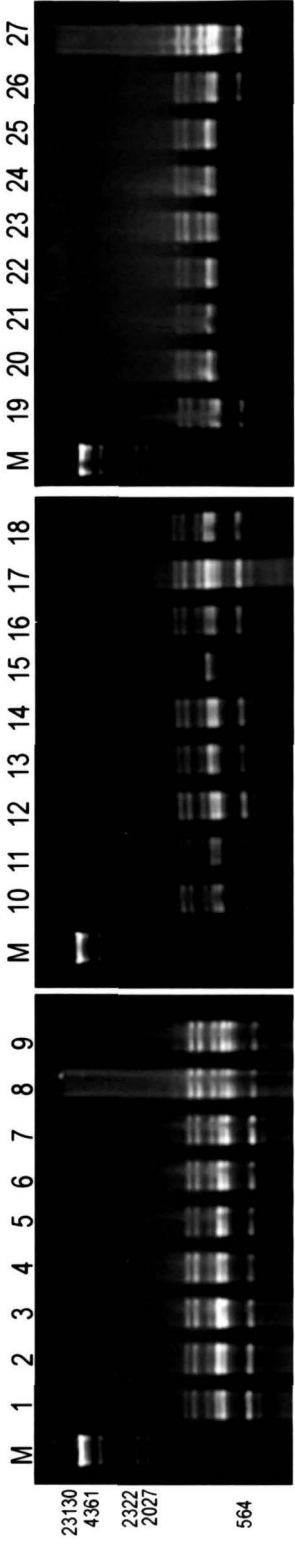
Primer	Sequence 5' to 3'	Total No. of bands	Monomorphic bands	Polymorphic bands	% Polymorphism
OPC-15	GACGGATCAG	4	2	2	50
OPH-03	AGACGTCCAG	5	1	4	80
OPH-13	GACGCCACAC	6	6	0	0
OPH-20	GGGAGACATC	3	3	0	0
OPK-04	CCGCCCAAAC	3	2	1	33.3
OPK-07	AGCGAGCAAG	4	2	2	50
OPK-16	GAGCGTCGAA	4	3	1	25
OPK-17	CCCAGCTGTG	4	2	2	50
OPK-18	CCTAGTCGAG	4	1	3	75
OPK-19	CACAGGCGGA	4	1	3	75
Total		41	23	18	Avg 43.9

Photoplate 6.3

RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1 – 8: Jarain, Lane 9: Jarain bulk, Lane 10 – 17: Baghmara, Lane 18: Baghmara bulk, Lane 19 - 26: Lawbah, Lane 27: Lawbah bulk

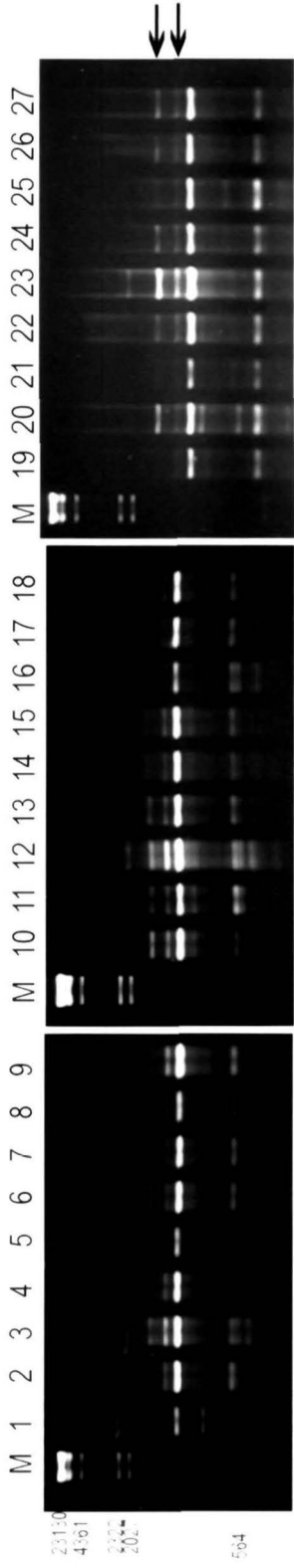


Photoplate 6.4

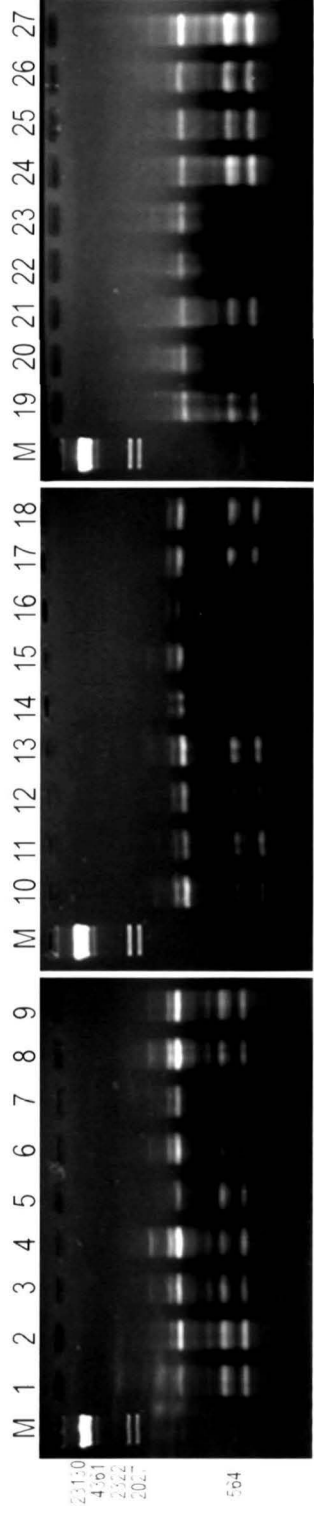
RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1 – 8: Jarain, Lane 9: Jarain bulk, Lane 10 – 17: Baghmara, Lane 18: Baghmara bulk, Lane 19 - 26: Lawbah, Lane 27: Lawbah bulk



OPC - 15



OPH-20

amplicons. Two fragments were found to be polymorphic among the different individuals (Photoplate 6.4). Primer OPH-03 produced 2-6 number of bands of which four was polymorphic (Photoplate 6.5). OPK-19 produced 1-4 number of bands of which three were polymorphic. A band of ~ 564 bp was observed in the Lawbah population but not in the Jarain and Baghmara populations. OPK-04 produced 2-3 bands of which one was polymorphic. The primer OPK-07 generated 2-4 scorable amplicons (Photoplate 6.6). When the primer OPK-07 was used, the samples J1 (Lane 1), B8 (Lane 17) and L1 (Lane 19) generated an amplicon of ~1556 bp which was absent in the other samples. However, the intensity of these amplicons varied in these samples. OPK-16 produced 3-4 scorable bands (Photoplate 6.7). A characteristic band of ~1444 bp was polymorphic among the different individuals. OPK-17 produced 2 to 4 bands of which two bands of ~ 611 bp and ~ 639 bp were polymorphic.

Of a total of 41 bands amplified by 10 primers, only 18 fragments showed polymorphism. The total number of fragments generated by the different samples ranged from 29 to 39 (Table 6.10, 6.11). The number of products generated by each primer varied from 3 to 6. The maximum percentage polymorphism (80%) was revealed by primer OPH-03. Primer OPK-18 and OPK-19 showed 75% polymorphism. The average frequency of bands was found to be 4.01bands/primer and the average percentage polymorphism was 43.9%.

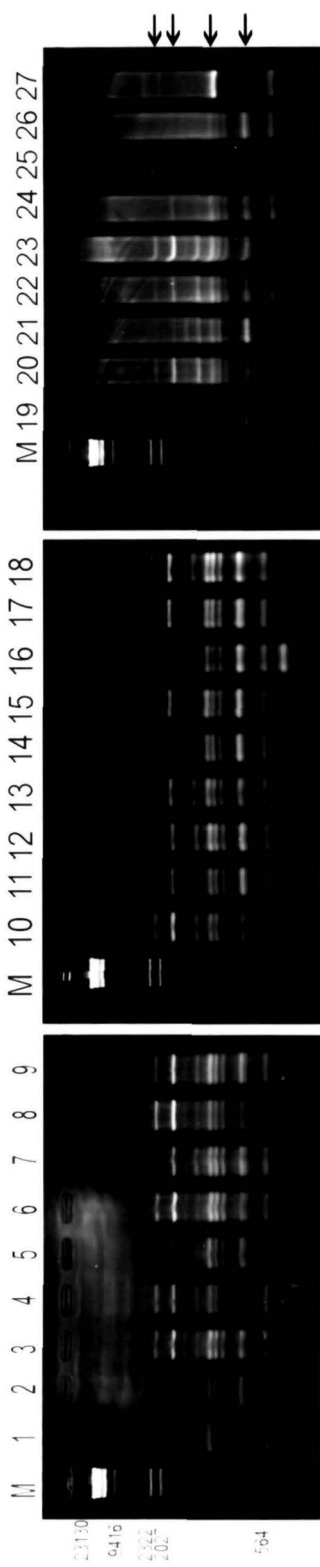
Jaccard's similarity values between pairs of the 24 individuals from different populations ranged from 0.63 to 1.00 (Table 6.12). Overall, the lowest similarity (0.63) was present between B1 and L1. Maximum similarity (1.0) was present between J4 and B5, J4 and B6, B3 and B4, B5 and B6. The minimum Jaccard's

Photoplate 6.5

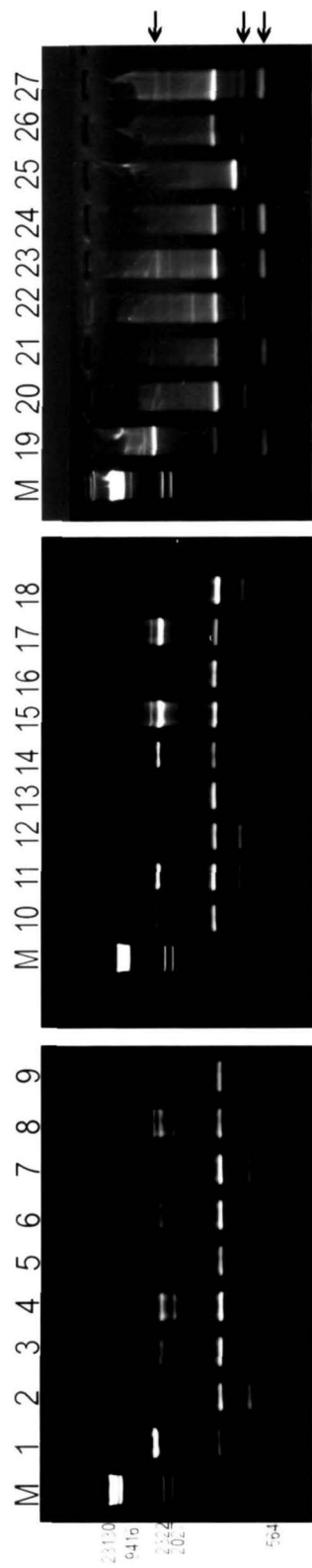
RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1 – 8: Jarain, Lane 9: Jarain bulk, Lane 10 – 17: Baghmara, Lane 18: Baghmara bulk, Lane 19 - 26: Lawbah, Lane 27: Lawbah bulk



OPH-03



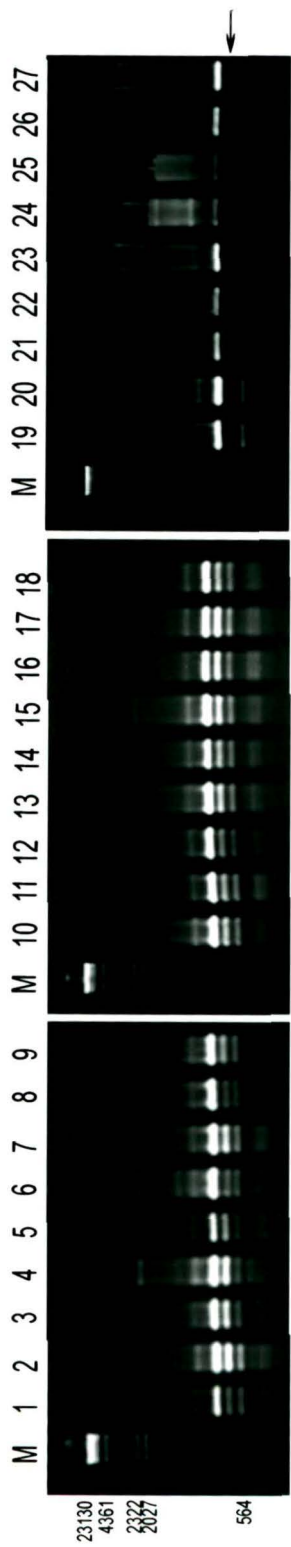
OPK - 19

Photoplate 6.6

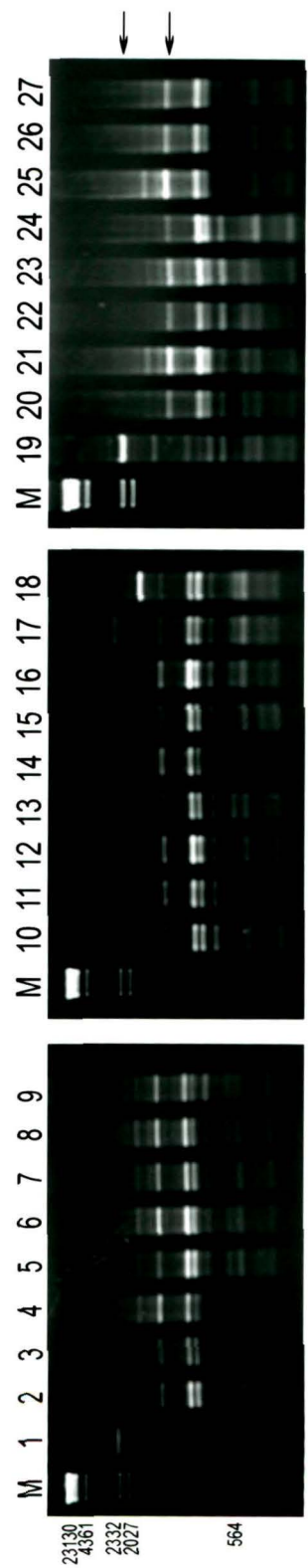
RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1 – 8: Jarain, Lane 9: Jarain bulk, Lane 10 – 17: Baghmara, Lane 18: Baghmara bulk, Lane 19 - 26: Lawbah, Lane 27: Lawbah bulk



OPK-04



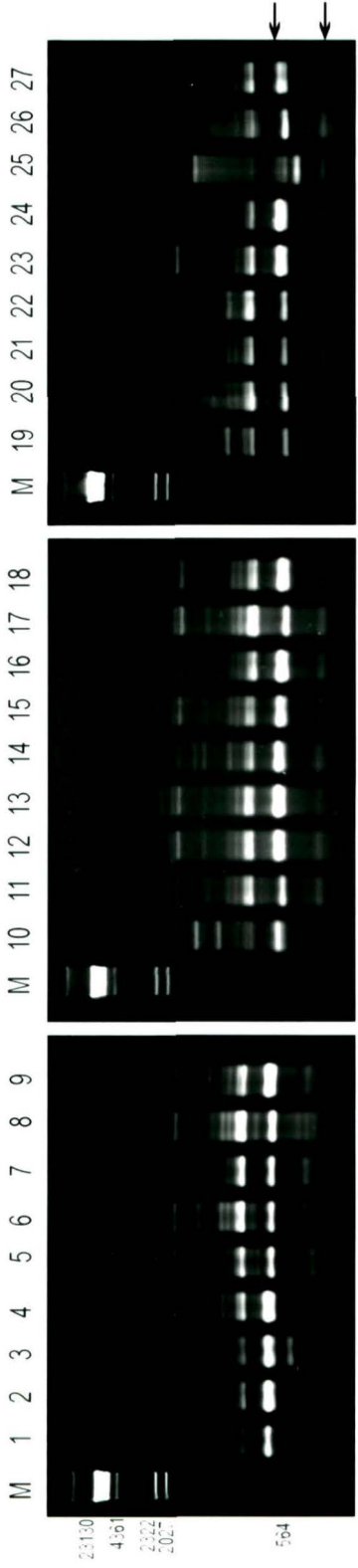
OPK-07

Photoplate 6.7

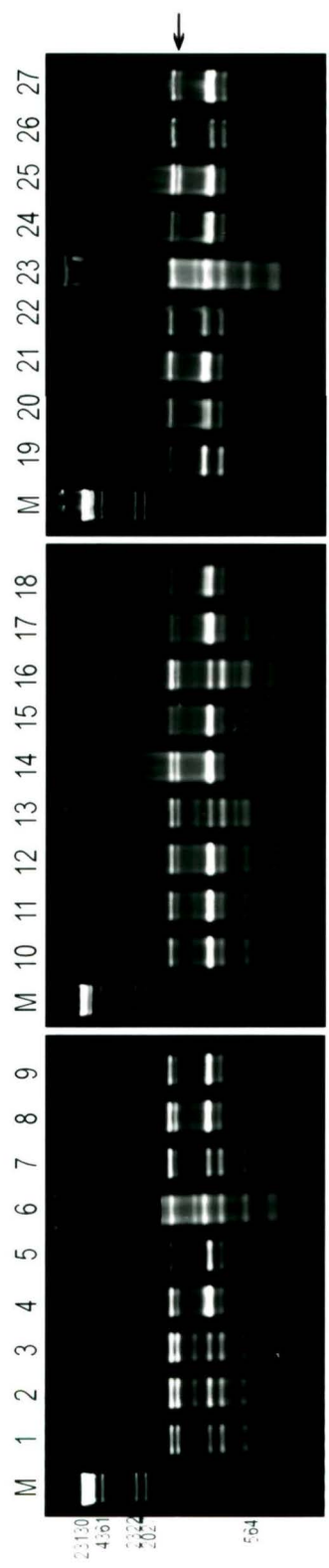
RAPD profile of *N. khasiana*

Legend M: Marker (λ DNA Hind III digest)

Lane 1 – 8: Jarain, Lane 9: Jarain bulk, Lane 10 – 17: Baghmara, Lane 18: Baghmara bulk, Lane 19 - 26: Lawbah, Lane 27: Lawbah bulk



OPK - 17



OPK - 16

Table 6.11. Number of amplified DNA fragments from different samples

Lane No.	Samples	Total No. of fragments	Average fragments per primer
1	J1	35	3.5
2	J2	37	3.7
3	J3	39	3.9
4	J4	38	3.8
5	J5	35	3.5
6	J6	36	3.6
7	J7	37	3.7
8	J8	37	3.7
10	B1	38	3.8
11	B2	38	3.8
12	B3	38	3.8
13	B4	38	3.8
14	B5	38	3.8
15	B6	38	3.8
16	B7	35	3.5
17	B8	37	3.7
19	L1	29	2.9
20	L2	34	3.4
21	L3	32	3.2
22	L4	35	3.5
23	L5	36	3.6
24	L6	35	3.5
25	L7	28	2.8
26	L8	31	3.1

Table 6.12. Jaccard's similarity coefficient matrix based on RAPD banding profile

	J1	J2	J3	J4	J5	J6	J7	J8	B1	B2	B3	B4	B5	B6	B7	B8	L1	L2	L3	L4	L5	L6	L7	L8
J1	1.00																							
J2	0.82	1.00																						
J3	0.83	0.95	1.00																					
J4	0.85	0.97	0.97	1.00																				
J5	0.80	0.95	0.90	0.92	1.00																			
J6	0.78	0.90	0.95	0.92	0.95	1.00																		
J7	0.82	0.95	0.95	0.97	0.95	0.95	1.00																	
J8	0.82	0.95	0.95	0.97	0.95	0.95	1.00	1.00																
B1	0.80	0.92	0.97	0.95	0.87	0.92	0.92	0.92	1.00															
B2	0.80	0.92	0.97	0.95	0.92	0.97	0.97	0.97	0.95	1.00														
B3	0.80	0.97	0.97	0.95	0.92	0.92	0.92	0.92	0.95	0.95	1.00													
B4	0.80	0.97	0.97	0.95	0.92	0.92	0.92	0.92	0.95	0.95	1.00	1.00												
B5	0.85	0.97	0.97	1.00	0.92	0.92	0.92	0.97	0.95	0.95	0.95	0.95	1.00											
B6	0.85	0.97	0.97	1.00	0.92	0.92	0.92	0.97	0.95	0.95	0.95	0.95	1.00	1.00										
B7	0.82	0.95	0.90	0.92	0.89	0.85	0.89	0.89	0.87	0.87	0.92	0.92	0.92	0.92	1.00									
B8	0.82	0.90	0.90	0.92	0.89	0.90	0.95	0.95	0.88	0.92	0.88	0.88	0.92	0.92	0.90	1.00								
L1	0.75	0.69	0.66	0.68	0.68	0.65	0.69	0.69	0.63	0.68	0.68	0.68	0.68	0.68	0.73	0.74	1.00							
L2	0.70	0.87	0.87	0.85	0.92	0.92	0.87	0.87	0.85	0.89	0.89	0.89	0.85	0.85	0.82	0.82	0.70	1.00						
L3	0.69	0.82	0.78	0.79	0.86	0.82	0.82	0.82	0.75	0.80	0.79	0.80	0.80	0.80	0.81	0.82	0.79	0.89	1.00					
L4	0.73	0.85	0.90	0.87	0.89	0.95	0.89	0.89	0.87	0.92	0.87	0.87	0.87	0.87	0.80	0.85	0.68	0.97	0.86	1.00				
L5	0.71	0.87	0.88	0.85	0.87	0.87	0.83	0.83	0.85	0.85	0.90	0.90	0.85	0.85	0.82	0.78	0.71	0.94	0.89	0.92	1.00			
L6	0.73	0.85	0.85	0.83	0.79	0.80	0.80	0.80	0.83	0.83	0.87	0.87	0.83	0.83	0.80	0.76	0.78	0.87	0.86	0.84	0.92	1.00		
L7	0.68	0.71	0.68	0.69	0.70	0.67	0.71	0.71	0.65	0.69	0.69	0.69	0.69	0.69	0.75	0.71	0.90	0.72	0.82	0.70	0.73	0.80	1.00	
L8	0.76	0.79	0.79	0.77	0.78	0.79	0.79	0.79	0.77	0.82	0.82	0.82	0.77	0.77	0.78	0.74	0.82	0.86	0.80	0.83	0.81	0.89	0.84	1.00

similarity values between individuals within the Jarain population was 0.76 (J1 and J5) and maximum was 1.0 (J7 and J8). Within the Baghmara population the similarity values between pairs of individuals ranged from 0.87 (B1 and B7, B2 and B7) to 1.0 (B5 and B6) while within the Lawbah population the values ranged from 0.68 (L1 and L4) to 0.97 (L2 and L4). The percent similarity within the Jarain population ranged from 76% (J1 and J5) to 100% (J7 and J4) whereas that between the Baghmara population ranged from 87% (B3, B4 and B8) to 100% (B3 and B4; B5 and B6). The percent similarity within the Lawbah population ranged from 68% (L1 and L4) to 97% (L2 and L8). Overall, the similarity matrix analysis revealed that individual B1 and L1 were most distantly related with 63% similarity.

Cluster analysis

For inter-population variability studies, the dendrogram generated separated the different individuals broadly into two clusters (Fig 6.3).

Cluster I is a heterogenous cluster containing plants from two populations i.e. Jarain and Baghmara. It consists of all eight plants from Jarain and eight plants from Baghmara.

Cluster II is a homogenous cluster containing eight plants from the Lawbah population only.

Frequencies of RAPD markers detected with different primers were calculated and this data was used to estimate the diversity index (H_o) within the three populations of *N. khasiana*. Diversity estimates for each population varied according

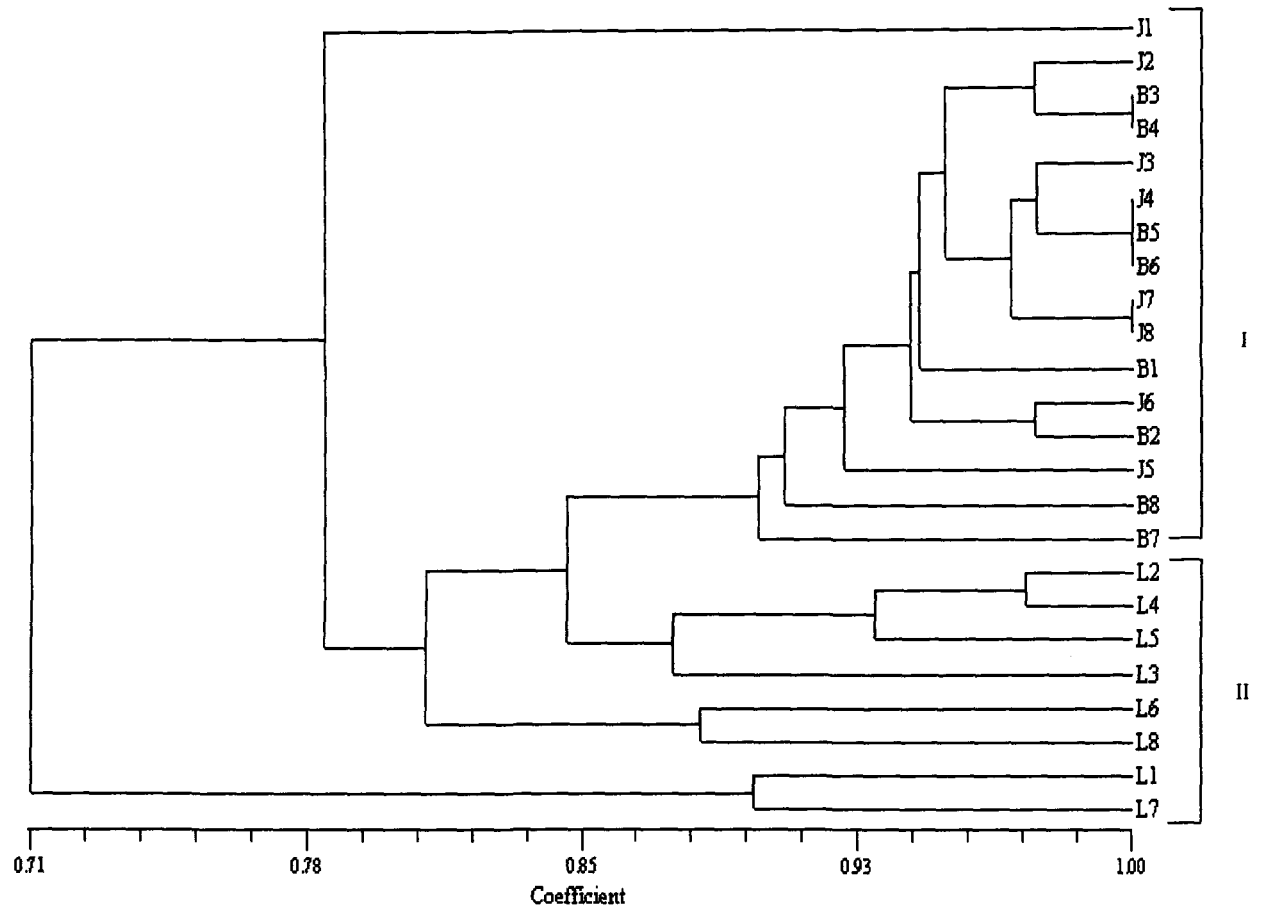


Fig. 6.3. Dendrogram showing the relationship of 24 individuals from Jarain, Baghmara and Lawbah, generated using UPGMA based on Jaccard's similarity coefficient

to primer used. Averaged over all primers, the Lawbah population exhibited the lowest variability within population whereas the Baghmara population exhibited the highest variability (Table 6.13). The three populations had Shannon index values ranging from 0.33 to 0.34. Shannon's diversity index was used to partition the diversity into within and between population components (Table 6.14). Primers OPC-15, OPH-03 and OPK-07 detected the least while OPK-04 detected the most within population variability. The average diversity present within the species, Hsp was estimated to be 0.35. Overall, the estimates of genetic diversity revealed that on an average, 96% diversity is detected within population and only 4% between populations. However, the distribution of diversity did vary between and within populations with different primers.

Discussion

The modified CTAB method described by Porebski *et al.* (1997) used for extraction of DNA ensured the uniformity of yield and purity of samples. This method was selected as it is generally employed for plants rich in polysaccharides and polyphenols.

The temperature profile, the brand of DNA polymerase, the concentration of MgCl₂, primer and template DNA can affect the reproducibility of RAPD assay (Macpherson *et al.*, 1993; Meunier and Grimont, 1993). Thus, standardization for optimum conditions to obtain consistent, reproducible RAPD pattern was necessary. In the present investigation, DNA concentration of 20 ng per 25 µl reaction volume was found to be optimum. In any RAPD analysis, concentration of genomic DNA is

Table 6.13. Estimates of genetic diversity (H_o) within three populations of *N. khasiana*

Primer	Shannon Diversity Index (H_o)		
	Jarain	Baghmara	Lawbah
OPC-15	0.26311	0.26906	0.36156
OPH-03	0.74678	0.72270	0.75918
OPK-04	0.19036	0.18426	0
OPK-07	0.21968	0.22649	0.28039
OPK-16	0.15843	0.18426	0.15573
OPK-17	0.31070	0.32296	0.33419
OPK-18	0.54173	0.55279	0.31019
OPK-19	0.33411	0.32040	0.46117
Average	0.34561	0.34787	0.33280

Table 6.14. Partitioning of genetic diversity into within and between populations for 8 primers

Primer	H_{sp}	H_{pop}	Diversity present within populations	Diversity present between populations
OPC-15	0.29511	0.29791	1.009481	0.00948
OPH-03	0.74153	0.74288	1.001829	0.00183
OPK-04	0.15635	0.12488	0.798693	0.201307
OPK-07	0.23877	0.24218	1.014288	0.01429
OPK-16	0.16828	0.16614	0.987267	0.012733
OPK-17	0.32487	0.32262	0.993075	0.006925
OPK-18	0.50417	0.46824	0.928729	0.071271
OPK-19	0.38820	0.37189	0.957999	0.042001
Average	0.35216	0.34209	0.961420	0.038580

considered to be crucial. Many workers have used different DNA concentrations ranging from 1 ng to 50 ng per 25 μ l reaction volume. Virk *et al.* (1995) used as little as 1 ng DNA for RAPD analysis of rice germplasm, Karihaloo *et al.* (1995) used 25ng DNA in 25 μ l reaction mixture when they studied RAPD variation in *Solanum melongena*, whereas Lescuyer *et al.* (1997) used 100 ng DNA per 50 μ l reaction volume for *Plasmodium falciparum*.

In the present study, out of 100 primers screened, 10 primers were used for generating polymorphism based on the clear analyzable profiles generated. Determination of minimum number of primers to be used in RAPD is important. The use of too few primers could result in an analysis that would be biased. 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). Primer base composition influences amplification strength (Williams *et al.*, 1990) and 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 in RAPD profile is independent of genome complexity (Williams *et al.*, 1990; Carlson *et al.*, 1991; Rafalski *et al.*, 1991). In RAPD reactions, the composition of the amplification product is determined by a competition between the potential priming sites available (Rafalski *et al.*, 1991). Some mismatching and annealing of template is likely to occur under the low stringent annealing conditions during thermal cycling (Williams *et al.*, 1990). Thus, where alternative priming sites occur, competition leads to a large number of initiations and a reduced number of products (Ruano *et al.*, 1991). The presence of bands of varying densities supports this.

Although low number of bands (3-6) was produced in the present study, this was advantageous as it reduced the chances of unpredictable level of homoplasy caused by co-migration of non homologous DNA fragments (McClelland and Welsh, 1994; Thorman *et al.*, 1994; Smith *et al.*, 1994). Using random primers, it is possible to survey the entire genome (Stewart and Porter, 1995). In the present study, reliable and unbiased estimates of genetic similarity could be obtained as random primers were used.

The average percentage polymorphism was found to be 24% for all the individual plants from the Baghmara population and 43.9% for all the populations taken together. This shows that *N. khasiana* has low species diversity which can be compared to that of other rare endemic species such as *Allium aaseae* (40-63%; Smith and Pham, 1996) and *Erodium paularense* (44-51%; Martin *et al.*, 1997).

The similarity estimates based on number of shared bands showed that there was variation within the individuals of the Baghmara population where there was about 87-100% similarity, although discrete variation between the tall, short, male and female plants could not be detected. Populations were also found to be diverse showing 68-100% similarity. Further, the dendrogram grouped the Lawbah population into a separate cluster suggesting a distinct genotype from the other two populations. Since Lawbah is present geographically between Baghmara and Jarain, the dissimilarity does not follow a pattern of isolation by distance, but may have resulted from different environmental conditions, such as soil type, precipitation, number of dry months within these areas, rainfall, etc. (Gillies *et al.*, 1999).

Shannon's Diversity Index was used to estimate genetic diversity of the different populations. The three populations had Shannon index values ranging from 0.33 to 0.34 with an overall diversity of 0.35 (Hsp). These values are very low when compared with other endemic and endangered plants for example 3.5 in *Rosmarinus tomentosus* (Martin and Bermejo, 2000) and 2.02 in *Changium smyrnoides* (Fu *et al.*, 2003). This could explain the inability of the species to adapt to environmental variations. Population genetic theory predicts that small, isolated populations will lose genetic diversity and become increasingly differentiated from other populations (Buza *et al.*, 2000). This suggests that the restriction into small populations following habitat loss has been accompanied by a reduction of diversity within them (Li and Xia, 2004). A reduced genetic variation lowers the adaptability of a population increasing the risk of extinction in an event of changes in habitat conditions.

Partitioning of genetic diversity within and between populations revealed that most of the genetic diversity (96%) was detected within populations. In a review of seven RAPD studies of different plant species, Hogbin and Peakall (1999) noted that variance among populations varied from 0 to 43%, the higher values being recorded in species known to undergo selfing. The relatively low diversity partitioned among populations in *N. khasiana* is, therefore, not surprising as it is a dioecious hence cross-pollinated species. It has been reported that outcrossing species tend to have higher levels of variability within populations but smaller degrees of differentiation among populations than selfing species (Hamrick and Godt, 1989; Schoen and Brown, 1991). The value is similar to those obtained in other studies of outcrossing species using RAPD; for example, 97.4% of the variation evident in *Populus tremuloides* was

present within populations (Yeh *et al.*, 1995). About 87.9% of the variation was maintained within population in *Swietenia macrophylla* when assessed using RAPDs (Gillies *et al.*, 1999). Karihaloo *et al.* (2003) reported that 94.7% of diversity in Indian mango (*Mangifera indica*) cultivars is found within regions as opposed to 5.26% among regions. Some portion of diversity (4%) was also maintained among populations in *N. khasiana*. This degree of differentiation is comparable with that observed in some other studies of outcrossing species. For example, the average among population differentiation for 25 oak species was 7% (Kremer and Petit, 1993). For *Populus tremuloides*, the among population component accounts for 2.6% of the variation (Yeh *et al.*, 1995). Since *N. khasiana* reproduces both sexually and through vegetative means, it is therefore expected that there would be larger differentiation within populations since species reproducing only sexually show larger differentiation among populations than species that reproduce both sexually and asexually (Hamrick and Godt, 1989).

The amount and partitioning of genetic variation among and within populations result from the dynamic processes of gene flow, selection, inbreeding, genetic drift and mutation (Hartl and Clark, 1994). Thus, the knowledge of the current genetic structure allows inferences about past processes. At the same time genetic variation represents the starting point for further evolutionary responses. This is of practical significance in the light of human caused habitat fragmentation, alteration or destruction. Genetic structure among and within populations also depends on the life history of a species. Origin and perpetuation of the extensive genetic variation in this

species can be attributed to obligate outcrossing due to dioceousness in the wild forms (Krishnamurthy, 1969).

The use of RAPD markers to genetically fingerprint plants which are morphologically similar or indistinguishable has been established as a reliable, efficient and very informative tool. Comparisons between molecular markers have indicated that marker type (RFLP, AFLP, and RAPD) was unimportant in separating genetic relationships among germplasm (Irwin *et al.*, 1998). RAPDs would be the marker of choice since they offer the advantage of being technically undemanding, use no radioactivity or polyacrylamide, and they are relatively cost effective as compared with the other procedures. Further, RAPD markers tend to reside in regions with many repeated sequences and therefore in non coding regions which are more susceptible to mutations. Consequently, they usually reveal more polymorphisms compared with isozymes or RFLPs which are mostly representative of conserved genome regions. DNA fingerprinting techniques such as RAPD permit the identification of taxa and the determination of phylogenetic relationships and intraspecific diversity at a molecular genetic level. The use of such techniques for germplasm characterization facilitates the conservation and utilization of plant genetic resources, permitting the identification of unique accessions or sources of genetically diverse germplasm. Assessment of genetic diversity on a large scale in natural populations, ecosystems, natural reserves or *in situ* conservation would be enhanced with quick and easy techniques such as RAPD. Biodiversity is the degree of variety in nature with three contributing components namely, habitat diversity, species diversity and genetic diversity. On the scale on which studies of biodiversity takes place, little

attention is usually paid to the measurement of genetic diversity because of the problems involved with its accurate measurement in large numbers of organisms. There is, therefore, a great demand for molecular techniques which do truly measure genetic polymorphisms and which can be applied on a large scale. RAPD may be just a technique as it could readily be used to select priority areas for conservation, to design and monitor management strategies in protected areas and to plan conservation strategies for a wide range of plant and animal genetic resources.

The threat to the genetic diversity present in wild populations of *N. khasiana* due to indiscriminate overcollection and the need for preservation of these genetic resources created an incentive for the determination of the genetic variability present within this species.

The present results indicate that RAPD can be used to establish the relationships among the different populations and even individuals within the existing populations. RAPD data from this study indicate that some variation still exists within and between the existing populations of *N. khasiana*, thus, these populations could provide materials for re-establishing other populations of this important rare and threatened species. Further more, the high genetic diversity within populations indicate that the most effective strategy for preserving genetic variation would be to conserve a large number of individuals for more diverse populations.

Information about the genetic diversity of threatened species is a prerequisite to designing suitable strategies for genetic conservation and establishing effective and efficient conservation practices (Falk and Holsinger, 1991). Declines in genetic

variation in wild populations are of great concern to conservation biologists (Hendrick, 1992). The ultimate goal of conservation is to ensure the continuous survival of populations and to maintain their evolutionary potential (Godt and Hamrick, 1995). For conservation it is necessary to protect the existing populations *in situ* in order to preserve as much genetic variation as possible, especially for those populations with higher genetic diversity. The major cause of the decline of *N. khasiana* is overexploitation for trade, forest fires, 'jhum' cultivation and damage of habitats by human activities (Bordoloi, 1977). Therefore, habitat destruction, which may lead to the elimination of species, should also be prevented.

Nepenthes khasiana Hook f. is the only species of *Nepenthes* found in India. It is endemic to the Jarain (Jaintia Hills), Lawbah (Khasi Hills) and Baghmara (Garo Hills) areas of Meghalaya, Northeast India. It is a botanical curiosity having ecological importance. The presence of the pitchers renders ornamental importance to this plant; therefore it is extensively collected for trade. The local physicians, known as the 'Kavirajas' prescribe the pitcher fluid for diabetic patients. Pitcher as well as pitcher powder is used to treat patients with cholera, frequent and painful urination, cysts, vaginal tumours and leprosy. The population of *N. khasiana* has dwindled in the last few decades due to deforestation and forest fires, excessive collection and 'jhum' cultivation. It is now threatened in its natural habitat and is listed as an endangered plant in Appendix-I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Although the seed set is very high with more than 300 seeds per capsule, germination in nature is very scant. It takes as long as 223 days for the seeds to germinate in nature.

In vitro seed germination is a concerted biotechnological approach for mass propagation and conservation of many rare and endangered plants. Seeds are preferred to vegetative material as the source of propagation material for multiplication of rare and endangered species. As the seeds are heterozygous a wider genetic base can be maintained which would hence ensure genetic diversity. Mature seeds of *N. khasiana*

obtained from the plants growing in nature were inoculated on different strengths of media viz. MS, B₅ and NN. Seed germination commenced within 30 days after culture on MS, ½ MS, B₅ and ½ B₅ media. The best germination of 70±1.8% was recorded on ¼ MS after 90 days of culture. No further increase in germination percentage was recorded after this period in any of the media tried. Physical factors such as pH, temperature and light are also critical factors that affect germination of seeds. The germination of the seeds *in vitro* was also influenced by the physical factors. Varying culture temperatures did not promote seed germination within 30 days. Maximum germination of 67±1.5% was achieved after 90 days of culture at 24°C. This increase of germination of seeds was significantly higher when compared to those incubated at both 16°C and 32°C. The seeds which were incubated at lower temperatures i.e., 0°C and 8°C did not germinate. However, at different pH ranges, the seeds started to show initial signs of germination only after 30 days of culture. After 60 days, there was no significant difference between the germination percentages at pH 3.8, 4.8, 5.8 and 6.8 with germination percentage ranging between 31% and 37%. At higher pH of the medium (i.e., 7.8), only 14% of the seeds germinated in this period. Observations taken after 90 days revealed a substantially good number of germinated seeds in all different pH with best germination percentage (68±2.2%) at pH 5.8. The seeds incubated under light intensity of 200µmolesm⁻²s⁻¹ started to germinate within 30 days of culture. However, best germination of 68±3.0% was recorded at 150µmolesm⁻²s⁻¹ after 90 days of culture. Lower light intensities inhibited the germination of seeds. The seeds kept at different photoperiods showed varying germination responses. After 90 days of culture, only 1% and 2% germination was recorded in darkness and 6h photoperiod respectively. The maximum overall germination was found to be 70%

under 18h photoperiod. There was no further increase of germination percentage after 90 days.

Plant growth regulators are essential in all physiological and developmental processes occurring during the growth of the plants. Effects of auxins (IAA, NAA and 2,4-D), cytokinins (KN, BAP) and GA₃ on seed germination of *N. khasiana* were examined. Seeds cultured in medium containing auxins singly did not show any signs of germination within 30 days of culture. However, after 60 days, maximum germination of 53% was recorded in ¼ MS medium supplemented with 0.5 mg l⁻¹ NAA which was higher than the control (36%). A maximum of only 34% of the seeds germinated in medium containing 0.5 mg l⁻¹ 2,4-D. Overall, higher concentrations of auxins in the medium were found to be inhibitory for seed germination. With the increase in auxin concentration there was a relative decrease in germination percentage. On the other hand, seeds cultured in medium containing cytokinins germinated within 30 days of culture and the percentage of germination was better as compared to that on auxin containing medium. GA₃ containing medium revealed only 3% to 17% of germinating seeds within 30 days of culture. Observations made after 60 days showed that a higher number of seeds germinated on the medium containing different concentrations of BAP and GA₃ as compared to the one with KN. Addition of auxins at different concentrations in the medium neither increased nor induced early seed germination. However, the rate of seed germination increased significantly with the addition of cytokinins and GA₃ in the medium. Of the two cytokinins, BAP was more effective than KN in accelerating germination rate. Although the exogenous supply of GA₃ induced earlier seed germination, it did not help in enhancing the germination percentage.

The interactions of growth regulators incorporated in the medium showed varying results. When KN was combined with NAA or IAA in the medium, seeds started germinating within 30 days of culture. The medium containing 5.0 mg l⁻¹ each of KN and NAA revealed a maximum germination of 68% after 90 days. With BAP and NAA in combination, only 5% seeds germinated in medium containing 5.0 mg l⁻¹ BAP and different concentrations of NAA within 30 days of culture. Although germination started late, the combination of BAP and NAA in the medium enhanced overall seed germination. The best germination of 68% was recorded in the medium containing 5.0 mg l⁻¹ each of BAP and NAA after 90 days of culture. The seeds cultured in medium having BAP and IAA in combination germinated within 30 days of culture. Maximum germination percentage (70%) was recorded in medium containing 0.5 mg l⁻¹ BAP and 2.5 mg l⁻¹ IAA. GA₃ in combination with either auxins or cytokinins inhibited early initiation of seed germination. Records of seed germination after 90 days of culture in medium containing GA₃ and IAA showed a minimum of 11% (10.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ IAA) and a maximum of 57% (5.0 mg l⁻¹ GA₃ + 0.5 mg l⁻¹ IAA). GA₃ and NAA in different combinations in the medium also had an inhibitory effect on germination wherein a maximum of 62% of seeds germinated in medium supplemented with 5.0 mg l⁻¹ GA₃ + 2.5 mg l⁻¹ NAA after 90 days of culture. A maximum of 63% of the seeds germinated after 90 days of culture in the medium incorporated with GA₃ and KN. Incorporation of GA₃ and BAP did not help in increasing the germination. A maximum of 60% germination was recorded in medium supplemented with 5.0 mg l⁻¹ GA₃ + 5.0 mg l⁻¹ BAP after 90 days of culture. Overall, the presence of different growth regulators in combination did not promote seed germination of *N. khasiana*. In all the cases, the overall germination

percentages were lower than the control. However, cytokinins in combination with IAA were beneficial for inducing earlier seed germination.

One hundred and twenty days old healthy *in vitro* grown seedlings of 3-4 cm in size with well developed roots were used for hardening. Different substrata were tested to establish the *in vitro* raised plantlets. Out of the various potting mixtures tried, soil from the natural habitat was found to be the best substratum for the survival and healthy growth of the seedlings with 97% survival. The best seedling growth (14.3 cm) and pitcher size (5.5 cm) were also obtained in this substratum. The seedlings transferred to the compost containing pebbles and powdered charcoal also resulted in 94% survival. Powdered charcoal alone and in combination with soil gave encouraging results with about 93% and 94% survival of the seedlings respectively. The highest number of pitchers (5.0) was recorded in the seedlings hardened in the mixture of soil and pebbles after 180 days of transfer. In 4-5 weeks, the seedlings were hardened and established. The plants have been maintained in the glasshouse for over two years. Hundred percent of the plants transferred to the natural habitat survived. The percentage nitrogen content of the soil collected from the natural habitats of *N. khasiana* was found to be inversely proportional to the density of the plants occurring in nature.

DNA fingerprinting using RAPD markers was carried out to analyze the genetic variability in *N. khasiana*. For analysis of variability within individuals of a single population, 10 primers were used for studying the RAPD banding patterns between tall (15-20 m) and short (10-15 cm), and male and female plants from Baghmara. Of these, 6 primers detected polymorphism. Of a total of 41 bands amplified by 10 primers, only 8 fragments were polymorphic. The total number of

fragments generated by the different samples ranged from 35 to 38. The number of products generated by each primer varied from 3 to 6. The average percentage polymorphism was 24%. Jaccard's similarity values between pairs of the 8 individuals ranged from 0.87 to 1.00. A dendrogram generated by unweighted pair group method with arithmetic averages (UPGMA) algorithm revealed a major heterogenous cluster consisting of male, female, tall and short plants together.

For detection of variability between populations growing in three different locations i.e., Jarain, Baghmara and Lawbah, 24 individuals were selected. Out of the selected 10 primers used for the investigation, 8 primers detected polymorphism. Of a total of 41 bands amplified by 10 primers, only 18 fragments were polymorphic. The total number of fragments generated by the different samples ranged from 29 to 39. The number of products generated by each primer varied from 3 to 6. The average frequency of bands was found to be 4.01bands/primer and the average percentage polymorphism was 43.9%. Jaccard's similarity values between pairs of the 24 individuals from different populations ranged from 0.63 to 1.00. The dendrogram generated, separated the different individuals broadly into two clusters: Cluster I, a heterogenous cluster comprising plants from two populations i.e., Jarain and Baghmara consisted of all plants from the two locations. Cluster II was a homogenous cluster containing plants from the Lawbah population only. Frequencies of RAPD markers detected with different primers were calculated and this data was used to estimate the diversity index (H_o) within the three populations of *N. khasiana*. Diversity estimates for each population varied according to primer used. Averaged over all primers, the Lawbah population exhibited the lowest variability within population whereas the Baghmara population exhibited the highest variability. The

three populations had Shannon index values ranging from 0.33 to 0.34. Shannon's diversity index was used to partition the diversity into within and between population components. The average diversity present within the species, Hsp was estimated to be 0.35. Overall, the estimates of genetic diversity revealed that on an average, 96% diversity was detected within population and only 4% between populations. This could be due to the reason that outcrossing species (*Nepenthes* being dioceous) tend to have higher levels of variability within populations but smaller degrees of variability among populations. The present results indicate that RAPD can be used to establish the relationships among the different populations and even individuals within the existing populations of *N. khasiana*. RAPD data from this study indicated that some variation still exists within and between the existing populations of *N. khasiana*, thus, these populations could provide materials for re-establishing this important rare and threatened species. Further more, the high genetic variability within populations indicate that the most effective strategy for preserving genetic variation would be to conserve a large number of individuals for more diverse populations.

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