Chapter 1

General Introduction

1.1 Insectivorous plants

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

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

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

The name *Nepenthes* was formally published as a generic name in 1753 in Linnaeus's famous *Species Plantarum*, which established botanical nomenclature as it exists today. "Nepente" literally means "without grief" and, in Greek mythology, is a drug that quells all sorrows with forgetfulness. In Homer's *Odyssey*, "Nepenthes pharmakon" is given to Helen by an Egyptian queen. Linnaeus (1737) explained:
"If this is not Helen's *Nepenthes*, it certainly will be for all botanists. What botanist would not be filled with admiration if, after a long journey, he should find this wonderful plant. In his astonishment past ills would be forgotten when beholding this admirable work of the Creator!" [translated from Latin by H. J. Veitch, 1897]

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

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

1.2.1. Distribution

The plant species has a very localized distribution. It is endemic to Meghalaya and is found growing from West Khasi Hills to East Khasi Hills, Jaintia Hills, East to West and South Garo Hills from 1000 to 1500 m altitude (Mao and Kharbuli 2002). It occurs in the Jarain area of Jaintia Hills and the Baghmara, Bandari, Chokpot area of Garo Hills, and few more localities, such as Nongstoin, Mukthapur, Bhagmara, Lawbah and Sonapahar in Meghalaya (Joseph and Joseph 1986). It is believed that the species represents ancient endemic remnants of older flora which usually occur in land masses of geological antiquity (Paleoendemics) (Bramwell 1972).
Fig. 1.1. (a) *Nepenthes khasiana* in its natural habitat, and (b) well developed pitchers
1.2.2. Habit

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

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

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

1.2.5. Conservation strategies

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

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

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

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

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

a. Micropropagation of *N. khasiana* using nodal segments.

b. Cytological evaluation of the germplasm and the regenerated plantlets to determine variability, if any.

c. Use of appropriate PCR-based molecular markers to define the genetic variations observed.