Chapter 4

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

4.1 Introduction

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

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

4.2.1. Plant material and regeneration

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

4.2.2. Cytological preparations for mitotic complements

The root tips of about 0.5-1.0 cm long were excised from both *in vivo* (mother plant) and *in vitro*-raised plantlets of the three successive regenerations. The root tips were pretreated with 0.002 M 8-hydroxyquinoline solution for 3 h at room temperature before being fixed in Carnoy’s fluid consisting of propanol and propanoic acid in the ratio of 3:1 for 24 h at room temperature. The root tips were transferred to 70% (v/v) ethanol and stored in a refrigerator till utilized for squash preparations. For making squash preparation of chromosomes, the fixed root tips were thoroughly washed with distilled water 3-4 times and hydrolyzed in 5 N HCl for 1 h at room temperature. The hydrolyzed root tips, after washing thoroughly, were subsequently transferred to leuco-basic fuschin solution and were kept for 45 min at room temperature under dark conditions. The stained root tips were squashed in a drop of 1% propiono-carmine under the cover glass. The cells were flattened by taping followed by removal of excess stain with Whatman filter paper pieces.

4.2.4. Microphotography

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

4.2.5. Magnification

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

4.3 Results

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

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

In the plantlets raised in the first regeneration, a total of 30 cells were analyzed out of which 76.66% cells showed somatic chromosome number as \(2n=80\) while the remaining 23.33% cells showed chromosome number of \(2n=76, 78\) (Fig.4.3).
Fig. 4.1. (a-b) Chromosome complements in *N. khasiana*, 2n=80 (Bar 10 μm)
**Table 4.1.** Analyses of root tip cells observed at metaphase in the mother plant (control) and the regenerated plantlets of *N. khasiana*

<table>
<thead>
<tr>
<th>Regenerations</th>
<th>No. of cells analyzed</th>
<th>Cells with normal chromosome</th>
<th>Cells with deviant chromosome</th>
<th>Deviant chromosome no.</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
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<tr>
<td>Control</td>
<td>30</td>
<td>30</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>30</td>
<td>23</td>
<td>76.66</td>
<td>7</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>30</td>
<td>20</td>
<td>66.66</td>
<td>10</td>
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<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>30</td>
<td>18</td>
<td>60.00</td>
<td>12</td>
</tr>
</tbody>
</table>
Fig. 4.2. (a-d) Chromosome complements in the mother plant of *N. khasiana*, 2n= 80 (Bar 10 μm)
Out of 30 cells analyzed in the plantlets of the second regeneration, 66.66% cell showed normal somatic chromosome number of 2n=80 while the remaining 33.33% cells were observed to possess deviant chromosome numbers of 2n=70, 76, 78, 84, 86 (Fig. 4.4).

In the plantlets of the third regeneration, 60% cells showed normal somatic chromosome number of 2n=80 and deviant chromosome complements of 2n=70, 76, 84, 86 were recorded in 40% cells (Fig. 4.5).
Fig. 4.4. (a-d) Chromosome complements in cells of PIIA, PIIB, PIIC, PIID and PIIE of *N. khasiana*. (Bar 10 μm). PIIA, PIIB, PIIC, PIID and PIIE represent plantlets A, B, C, D and E of the second regeneration.
Fig. 4.5. (a-d) Chromosome complements in cells of PIIA, PIIIB, PIIC, PIID and PIIE of *N. khasiana*. (Bar 10 µm). PIIA, PIIIB, PIIC, PIID and PIIE represent plantlets A, B, C, D and E of the second regeneration.
4.4 Discussion

Documentation of chromosome numbers in the genus *Nepenthes* was probably first carried out by Heubl and Wistuba (1997) where they have reported a chromosome number of 2n=80 in about 15 species of *Nepenthes* as mentioned earlier in the present chapter. Members of the family Nepenthaceae are known for remarkable uniformity in chromosome numbers of 2n=80 without any indication of numerical variations. The present studies are in agreement with these reports. However, such observations need to be substantiated with informations on more number of species representing the entire genetic spectrum of the genus. It was also observed that chromosomes lack a single localized centromere in *N. khasiana*. The chromosomes of most eukaryotes have ‘localized centromere’ which presents as a primary constriction. However, ‘non-localized centromere’ or ‘diffuse centromere’, which does not show any constriction or localized centromere position on chromosome, are known in some plants such as *Luzula* (Castro et al. 1949), and members of the family Cyperaceae (Hakansson 1958). In *Drosera* which is very closely related taxa with the genus *Nepenthes*, distinct primary constrictions or localized centromere has not been observed supporting the diffuse centromere hypothesis (Kondo et al. 1976; Kondo and Segawa 1988; Sheik et al. 1995). Similarly, in *N. khasiana*, the somatic chromosomes were observed to be very small in size and clear chromosomal gap between sister chromatids was not seen. In other words, the centromeres might have been probably diffused along the entire length of chromosome suggesting the holocentric nature of the chromosomes. Since the diffused type possesses centromere function dispersed along the whole chromosome length, in theory all
fragments of this type of chromosomes are stably transmitted after cell division. On the contrary, Kondo and Lavarack (1984) observed distinct primary constrictions in the larger chromosome of *Nepenthes*. Also, Junichi et al. (2011) reported that two larger chromosomes of *Drosera arcturi* showed primary constrictions and *D. regia* had localized-centromeric position or well-differentiated primary constrictions in most metaphase chromosomes.

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

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

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

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

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

In the present investigation, it was observed that the micropropagated plantlets of
_N. khasiana_ which is characterized by higher ploidy (probably at 8x level), produced
cells with deviant chromosome numbers _in vitro_. Genetic variations among plants
regenerated are always higher among polyploids and plants with high chromosome
numbers than those with low ploidy and low chromosome number species (Watson _et al._
1992; Skirvin _et al._ 1994). Plants regenerated from mesophyll protoplast of tetraploid
British cultivar ‘Maris Bard’ and ‘Fortyfold’ and Dutch cultivar ‘Bintje’ of potato
revealed extensive variations in chromosome number reflecting the polyploid nature of
potato (2n=2x=48), and its concomitant higher tolerance to chromosomal changes.
Numerical and structural variations in chromosome of tissue culture-raised plants are
strong evidences for possible change in genetic composition of an organism (Kunitake _et al._
in both number and structure of chromosomes in plants of four different wheat (_Triticum
aestivum_ 2n = 6x = 42) cultivars regenerated via somatic embryogenesis.

The frequency of somaclonal variations increases as the number of subcultures
and their duration increases, especially in cell suspensions and callus cultures (Reuveni
and Israeli 1990; Rodrigues _et al._ 1998; Bairu _et al._ 2006). Moreover, the rapid
multiplication of a tissue or long-term cultures may also affect genetic stability and thus
lead to somaclonal variations (Israeli _et al._ 1995). Hartmann _et al._ (1989) showed that the
long period in culture increased the number of somaclonal variants in wheat regenerants.
Rodrigues et al. (1998) observed 1.3% somaclonal variants in the fifth subculture during micropropagation of *Musa* which was found to be increased to 3.8% in the twelveth subculture. Similarly, Bairu et al. (2006) observed an increase in the rate of occurrence of variants with progressive sub-culturing of micropropagated plants of *Musa* spp. Zhenxun and Hongxian (1997) also reported that aneuploidy and mixoploidy were predominantly observed in banana regenerants cultured for long durations. A statistical model has been proposed for predicting the theoretical mutation rate with the number of multiplication cycles as the primary parameter and two main conclusions were derived from the model that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and, variable off-type percentages can be expected after a given number of multiplication cycles (Cote et al. 2001). However, the model had limited applications because of the complexity of biological systems. Etienne and Bertrand (2003) described the effects of age of embryogenic cell suspensions on frequency and phenotype of variants of *Coffea arabica*, where somaclonal variation was increased to 25% in plants produced from 12-month-old cell suspensions in plants as compared to 1.3% in plants produced from 3-month-old cell suspensions. Therefore, it is not surprising that subsequent increase of genetic variations in the plantlets of the second and the third regenerations of *N. khasiana* was observed in the present study. However, the plantlets of the three consecutive generations were morphologically very similar. This may be attributed to the ability of the normal cells to overcome the incompetent abnormal cells with decreased potential resulting in the normal phenotypic characters. Similarly, Browers and Orton (1982) observed that aberrant chromosome numbers occurred at an
appreciably high frequency in morphologically indistinguishable plants of celery derived through somatic embryogenesis. Although abnormal cells with deviant chromosome numbers are ubiquitous in tissue culture, regeneration acts as a sieve mostly permitting the growth of stable normal cells (D’Amato 1977). Moreover, chromosomal abnormalities such as loss or addition of few chromosomes arising from tissues of polyploid or hybrid origin can be tolerated (Heinz and Mee 1971; Sree Ramulu 1987; Jelenic et al. 2001).

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