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 composites 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

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

± 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 1/3\text{rd} 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 1/3\text{rd} 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 1/3\text{rd} of cup), soil (pebbles to 1/3\text{rd} 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

<table>
<thead>
<tr>
<th>Site</th>
<th>Sample</th>
<th>% N</th>
<th>Average Density (individuals / sqm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shillong</td>
<td>S</td>
<td>1.46</td>
<td>0</td>
</tr>
<tr>
<td>Lawbah</td>
<td>L1</td>
<td>ND</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>1.09</td>
<td>2.0</td>
</tr>
<tr>
<td>Jarain</td>
<td>J1</td>
<td>0.71</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>J2</td>
<td>1.07</td>
<td>1.5</td>
</tr>
</tbody>
</table>
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 hardest 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 the 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 dessication 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 (Donnely 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.