REPRODUCTIVE BIOLOGY OF ENDEMIC AND ENDANGERED INSECTIVOROUS INDIAN SPECIES *NEPENTHES KHASIANA* Hk.f.

BY

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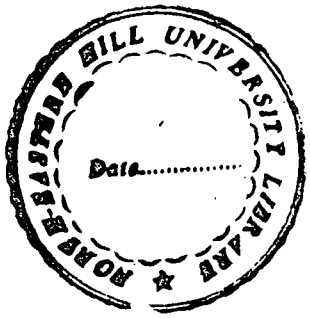

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Dedicated

to

My dear Father

NORTH - EASTERN HILL UNIVERSITY

SHILLONG
JUNE, 2004

DECLARATION

I, *Ningombam Rashi Devi*, declare that the subject matter of this thesis entitled "Reproductive biology of endemic and endangered insectivorous Indian species *Nepenthes khasiana* Hk.f." is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the North-Eastern Hill University, Shillong for the award of the degree of Doctor of Philosophy in Botany.

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CONTENTS

CHAPTER - 1

Introduction

Plants which catch and digest arthropods and other small animals are termed carnivorous plants. They possess specialized morphological, anatomical, and physiological properties. The earliest allusion to the possibility of carnivory was made about 200 years ago (Llyod, 1942; Heslop-Harrison, 1976). In 1875, Darwin published his work "Insectivorous Plants". Important contribution to the knowledge of anatomical characters has been made in particular by Goebel (1891-1893) and Fenner (1904).

The term "pitcher Plant'' is used for a member of different carnivorous plants with pitcher-like leaves. *Nepenthes* are the tropical pitcher plants. In 1976, Heslop-Harrison classified the pitcher plant (*Nepenthes*) under Group-I in which the glandular faces are immersed in the pitcher fluid.

The unigeneric family Nepenthaceae consists of approximately 74 species (Willis, 1966; Kaul, 1982). In India, the only representative of the genus Nepenthes, *Nepenthes khasiana* Hk.f. is an endemic and endangered insectivorous plant (Hooker, 1886; Jain and Sastri, 1980; Joseph and Mani, 1982), growing in Khasi, Jaintia and Garo hills of

Meghalaya state of North Eastern India. *Nepenthes khasiana* Hk.f. is a dioecious plant. The population of this species is confined in certain regions like Jarain, Sutnga, Jowai, Magheshkola and Lawbah (Haridasan and Rao, 1985; Rogers and Gupta, 1989; Choudhury, 2000). There has not been much study with reference to biotypes and varieties among these populations in sub-species level at various demographically isolated provinces. *Nepenthes khasiana* Hk.f. has a high degree of taxonomic uniqueness and critically endangered species which requires high degree of priority than the less endangered ones. The small populations of each isolated regions of this species facing several threats for its existence and its habitat because of the combined effects of shifting cultivation (Jhumming), timber extraction, mining and quarrying activities of coal and selective removal of this species because of its biological curiosities. Today the pitcher plants are restricted in its occurrence in small pockets and have become endangered by invasive weeds. Thus, the weeds prevent the seed germination and growth of other plants like *Nepenthes khasiana* Hk.f.

'Nepenthe' is derived from the Greek word means 'not and grief'. It was said to cause forgetfulness of sorrows and misfortunes. The potential medicinal uses of *Nepenthes khasiana* still remain unknown. However, the local tribal people have been using the liquid of unopened

pitcher as remedy for stomach pain and cough. Doctors in Noumea (New Caledonia) suggest their patients to take Nepenthes pills to fight stress. The small white pills are manufactured as homoeopathic treatment by a laboratory called Boiron in France (Laurent Legendre, 1999). Many of the secondary products of secretary glands may have varied types of biological activities including insecticidal, pesticidal and role in chemical ecology which lead to better understanding of plant protection mechanisms to crops. This can apparently be done with the study of biochemistry and anatomy of secretory glands.

Conservation over the long term will require management to reduce the risks, including ex-situ population which could support and interact demographically and genetically with wild population. The ultimate goal of ex-situ conservation is to provide support for the survival of species in their natural habitat. It is not alternative, but complementary to, conservation of biotypes in Botanic Gardens.

It is possible to produce enough plants in Botanic gardens and educate the local tribal people to grow it in their garden as a cottage industry i.e. tribal could grow different biotypes and sell them co-operatively at reasonable prices to satisfy all the demands without jeopardizing the limited population in the wild, because each pitcher

plant is said to bring in $800 (Đ500) in USA (Harold Koopowitz and Hilary Kaye, 1990).

The Eastern Himalayan region and North-East India are important repositories of genetic variability of various crops, horticultural and wild plants. Shifting cultivation and invasion of cleared lands by weeds have resulted in a considerable damage to natural ecosystems and rendered the region a 'hot spot' location with reference to the loss of biological diversity (Swaminathan, 1991).

Numerous studies have been published on foliar structure and development (Hooker, 1886; Fenner, 1904; Macfarlane, 1908; Kuhl, 1933; Roth, 1953; Schmid-Hollinger, 1970, 1979), glandular structure and function of the pitchers (Lloyd, 1942; Luttge, 1971; Heslop-Harrison, 1976; Adams and Smith, 1977; Fahn, 1979) and on animal inhabitants and prey in the pitchers (Beaver, 1979; Wirth and Beaver, 1979; Erber, 1979). By contrast, what little is known about the flower and fruits comes from a few studies of cultivated specimens, especially hybrids (Stern, 1917; Daumann, 1930; Kuhl, 1933). Species are usually defined according to their external morphology and anatomical characters, therefore, the study of morphology with anatomy will be helpful to identify the biotypes and sub-species in different regions. Captive breeding and viable population can be maintained over the long

period by understanding the reproductive biology which offers space for supporting population of this endangered and endemic taxon.

Nepenthes species are embryologically little known. The earlier work on the embryology of the family Nepenthaceae was reviewed by Davis (1966). Recently, Lan and Prakash (1973) studied the life history of *Nepenthes gracilis*. In 1982, Kaul observed the floral and fruit morphology of *N. lowii* and *N. villosa* growing in montane of Borneo. Subramanyam et al. (1985) illustrated the reproduction of *N. khasiana*. However, their observations are mainly based upon the one time collection of pickled specimens without observing the life cycle of *N. khasiana* periodically in their natural habitats. However, Johri et al. (1992) emphasized that the Reproductive biology of *Nepenthes khasiana* Hk.f. has not been studied thoroughly. Therefore, the present work has been undertaken with particular emphasis on the following objectives:

- To study the reproductive biology of *Nepenthes khasiana* Hk.f. this includes the different developmental aspects of male and female reproductive organs.
- Pollen germination, fertilization, embryogenesis and endosperm formation.
- Pollen and seed viability.

CHAPTER - 2

Review of Literature

A typical anther is tetrasporangiate with two microsporangia in each lobe (Bhandari, 1984; Johri et al., 1992). Bisporangiate anthers are found in some taxa, as in Adoxaceae, Circeasteraceae, Epacridaceae, Philydraceae, Restinaceae, Malvaceae (Bhandari and Nanda, 1968; Kircher, 1986; Johri et al., 1992) and in the insectivorous families like Nepenthaceae, Droseraceae, and Sarracenaceae (Watson and Dallwitz, 1992; Venugopal and Rashi Devi, 2003). Davis (1966) reported the co-occurrence of bi- and tetrasporangiate anthers in four monocotyledon and twelve dicotyledon families.

In the androecium of the family Nepenthaceae, the filaments are connate to form an androphore on the top of which anthers are arranged in a definite pattern (Hooker, 1886; Watson and Dallwitz, 1992; Kaul, 1982). The number of anthers varies from flower to flower on the same inflorescence (Kaul, 1982).

Shreve (1906), Nicholas (1908) and Livingston (1950) have given a detailed account of embryology of *Sarracenia purpurea*. The anther wall comprises persistent epidermis, fibrous endothecium, two or three-ephemeral middle layers and secretory tapetum of binucleate cells in

Sarracenia purpurea (Shreve, 1906; Johri et al., 1992). In Droseraceae also, the anther wall comprises the epidermis, fibrous endothecium, an ephemeral middle layer, and secretory tapetum.

As for the behavior of tapetum, two types are known in angiosperms: one glandular and the other amoeboid type (Maheshwari, 1950). In the glandular type the tapetum remains as a discrete layer until the pollen is mature, but in the amoeboid type the tapetum loses cell walls and forms plasmodial masses and gradually disintegrates as the pollen develops. It has long been supposed that the tapetum serves for the nutrition of developing microspores and contributes to the formation of the exine (Maheshwari, 1950). It is presumed that precursors of sporopollenin i.e. the main constituent of the mature microspore exine would pass from the tapetum to the microspores (Echlin, 1971). Secretory tapetum of binucleate cells is also occurred in Droseraceae, Aristolochiaceae and Cytinaceae. In *Adoxa,* the tapetum is amoeboid with binucleate cells (Johri et al., 1992). In *Drosera indica* and *D. peltata* the tapetal cells are pseudoplasmodial type (Venkatasubban, 1950).

In *Philydrum lanuginosum* same condition occurred (Kapil and Walia, 1965), but 2-4 middle layers and irregular biseriate secretory tapetum. In *Styphelia longiflora* the tapetal cells are uninucleate. The

dual origin of the tapetum has been observed in *Tarenna asiatica* (Periasamy and Parmeswaran, 1965). Towards the outer side the tapetum is derived from the primary parietal layer, where as on the inner side it develops from the cells of the connective. According to Periasamy and Swamy (1966), such a development occurs in all the angiosperms. In *Annona squamosa* (Periasamy and Kandaswamy, 1981), tapetum is trimorphic in origin.

The microspore mother cell undergoes meiotic divisions. Cytokinesis is simultaneous in Sarraceniaceae, Droseraceae, Cytinaceae etc. Simultaneous one is the characteristic of the dicotyledons and the successive of the monocotyledons. The occurrence of both types in different genera of the same family is also reported like in Aristolochiaceae, meiotic division is simultaneous e.g. *Aristolochia europaeum*, *A. siphon* (Tackholm and Soderberg, 1918), *A. bracteata* (Johri and Bhatnagar, 1955) and *Bragantia wallichi* (Nair and Narayana, 1961), or successive e.g. A. clematitis (Samuelsson, 1914), *A. elegans* (Johri and Bhatnagar, 1955) and *A. fimbriata* (Tackholm and Soderberg, 1918). Both types of cytokinesis is reported in *Catharanthus purillus* (Bhasin, 1971), *Conyza stricta* (Sharma and Murty, 1978) and *Vernonia cinerea* (Dakshini and Dadlani, 1978). This condition is rare.

The arrangement of microspores in a tetrad may be tetrahedral, isobilateral, linear, T-shaped and decussate. All five types are occurred in *Aristolochia elegans* and *Sparganium errectum.* The variation in tetrad formation depends more or less on the shape of microspore mother cells and the orientation of spindles during meiosis II (Chennaveeraiah and Mahabale, 1962) as well as the differential expansion of pollen and the position of germinal aperture (Knox, 1984).

The tetrad microspores are interconnected and surrounded by a thick callose wall. In *Allium tuberosum* and *Cyclamen persicum* (Bhandari et al., 1981), the callose is deposited within the primary wall around each microspore mother cell. The primary wall assists the callose in preventing the entry of macro molecules and isolating the microspores (Heslop-Harrison and Mackenzie, 1967; Bhandari et al., 1981). Due to callase activity, the callose wall around the tetrad is digested and the microspore are released in the anther locule except in some families where they remain in permanent tetrads as in Ericaceae, Juncaceae, Monimiaceae, Phylidraceae, Winteraceae, Droceraceae (Johri et al., 1992) and Nepenthaceae (Kaul, 1982; Endress, 1994; Venugopal and Rashi Devi, 2003).

The pollen grains are shed at the two celled stage in majority of the angiosperms e.g. Sarraceniacaee, Droceraceae, Aristolochiaceae etc.

(Johri et al., 1992) and three celled stage in several species e.g. *Nepenthes lowii* and *N. villosa* (Kaul, 1982) and some members of Adoxaceae (Johri et al., 1992).

The development of male gametophyte starts soon after the uninucleate pollen grains are produced. The development of the male gametophyte is remarkably uniform in the angiosperms. In most tropical plants the nucleus begins to divide almost immediately but in plants belonging to the colder region there is often a resting stage lasting from a few days to several weeks. Vacuolation occurs in the microspore which is the first cell of the gametophyte generation and its nucleus takes up a wall ward position. Such a displacement also occurs without vacuole formation e.g. *Juncus* and Cyperaceae (Wulff, 1939), *Haworthia* (Geittler, 1955).

Prior to the first mitosis in the pollen grain there is an increase in the amount of DNA in microspore nucleus (Raghavan, 1997). The first mitotic division is always unequal separating a larger vegetative cell from the smaller generative cell (Steffen, 1963; Hagemann, 1981). The reason for this unequal division is not well understood. The second mitotic division which is the division of the generative cell may take place either in the pollen grain or in the pollen tube i.e. either before or after the dehiscence of anther. This is the two-celled stage of pollen

grain at which shedding occurs in the 75% of angiosperms (Cresti et al., 1980). In pollen grains which are liberated at the three- celled stage, the generative cell divides when the pollen grains are still within the anther locule and the two male gametes or 'male germ units' are formed. It is occurred in several species, nearly 25% of angiosperms (Cresti et al., 1980; Raghavan, 1997).

The generative cell cytoplasm is highly reduced, but it contains limited number of cell organelles - mitochondria, ribosomes, endoplasmic reticulum, microtubules and dictiosomes. Generative cells of most of the species lack plastids, but some species have plastids e.g. *Oenothera hookeri.* Generative nucleus has higher amount of DNA and it undergoes some structural changes during the maturation of pollen (Knox, 1984).

Russel and Cass (1981) and Russel (1984) reported that the two sperm cells of *Plumbago zeylanica* are dimorphic in nature; one of the sperm cell is bigger while the other one is relatively smaller. The two sperm cells are differing in structure as well.

The cytoplasm of two- or three-celled pollen grains is rich in carbohydrates, lipids, proteins and amino acids (Baker and Baker, 1979; Johri et al., 1992). It also contains enzymes mostly hydrolytic for pollen germination and tube elongation. The two- and three- celled pollen

grains exhibits physiological differences in their behavior (Johri and Shivanna, 1977). The two celled pollen grains remain viable for a longer time and show a high percentage of germination in vitro, as compared to the three celled pollen grains. The morphology of the pollen grain is usually considered on the basis of its shape, size, thickness and ornamentation of exine (Knox, 1984; Johri et al., 1992). The pollen grain and its pollen tube is essential partner in fertilization and seed setting. It is also essential to have an understanding of the structure and function of pollen in order to produce new types of crop plants through hybridization.

In order for pollination to be successful, pollen must be transferred between plants of the same species. Plants typically rely on one of two methods of pollination: cross-pollination or self-pollination, but some species are capable of both. Cross-pollination is considered necessary for good seeds in *Sarracenia purpurea*. Various species of *Sarracenia* growing in the same locality very readily cross-pollinate and form viable hybrids if their flowers develop at about the same time (Macfarlane, 1917; John, 1975).

Most carnivorous plants are entomophilous, yet they sometimes capture insects including pollinators (Juniper et al., 1989; Makoto Kato,

1993). In the Droseraceae and Lentibulariaceae, small but conspicuous flowers are pollinated by various nectar-feeding insects. *Nepenthes* flowers have been thought to be pollinated by insects as the flowers secrete nectar and the pollen is dispersed in tetrads (Kaul, 1982; Juniper et al., 1989). Makoto Kato (1993) studied the floral biology and insect visits to flowers of *Nepenthes gracilis* in a montane forest in Sumatra to discover the pollination system of *Nepenthes* and reported that *Nepenthes* flowers which are characterized by dioecious condition, nectariferous tepals, secreted nectar of low sugar concentration at night, and were visited by nocturnal pyralid moths and a few diurnal calliphorid flies, whereas dish-type flowers usually produce thick nectar and have been regarded as fly pollinated (Faegri and Van der Pijl, 1979). In a hot sunny tropical habitat, nectar secreted on exposed tepals immediately evaporated. *Nepenthes* flowers without nectar in daytime were rarely visited by insects. Insect visits to the flowers, however, have seldom been observed in natural habitats in the tropics at least during the day (Holttum, 1954). Male flowers have a higher nectar production rate but lower concentration of nectar than female flowers, where as in *Cucurbita pepo*, the female flower produce more nectar than male flower (Nepi and Pacini, 1993). The pattern of nectar production of tepals is regarded as attracting nocturnal flying insects and avoiding ants, while

the pitchers attract ants by nectar secreted on the pitcher rim. In *Sarracenea psittacina* and *S. purpurea* species, a tall scape (relative to their leaf height) positions the flower well above the trapping space occupied by the pitcher openings.

The dioecious ant plant *Macaranga hullettii* (Euphobiaceae) in South east Asia, intraspecific pollen transfer by thrips species, *Neoheegeria* sp. (Thysanoptera) was proved by pollen loads of thrips taken from receptive pistillate inflorescences (Ute Moog et al., 2002).

Cucurbita pepo carries male and female flowers on the same plant, and is pollinated by nectar-collecting bees. Both types of flower are opened for only 6 hour; male flowers open and close half an hour earlier than female flowers. Female receptivity has two aspects, that of the stigma lasting 4 days, and that of the ovules lasting 2 days (Nepi and Pacini, 1993).

Anderson and Hill (2002) studied the reproductive biology of *Hamamelis virginiana* (Hamamelidaceae) and reported that the homogamous, self incompatible flowers emit a faint odor, bear nectar with sucrose ratios typical of bee- and fly-pollinated flowers, and produce abundant sticky pollen. Bees and flies are likely pollinators. Inspite of too many flowers, this plant yield remarkably low fruit set (Test of > 40,000 flowers showed natural fruit set to be < 1%) in which

the flowering time, breeding system, and clumped distribution of plants are responsible for it. Because all other species of *Hamamelis* flower from late winter to early summer where as *H. virginiana* flowers from late September to late November. Male flowers of some *Nepenthes* species are known to give off a foetid smell that might be an insect attractant (Juniper et al., 1989), while both sexes of the *N. gracilis* flowers did not (Makoto Kato, 1993). Small moths are sometimes attracted by floral odors (Thien et al., 1985).

Pollen grains must be among the shortest-lived independent bodies in nature, for there are few which can remain alive for more than a few days after they have been shed, while some can live for only a few hours (Echlin, 1968; Sporne, 1974).

Gabriele et al., (1999) reported in *Metrosideros excelsa* that pollen is highly viable (93.65%), and stigma receptivity extends for at least 9 days, as indicated by peroxidase activity, pollen germination, pollen tube length 24h after pollination, and seed set.

In *Cucurbita pepo* (Nepi and Pacini, 1993), pollen viability determined by fluorescein diacetate (flurochromatic reaction) decreases by 20% during anthesis and more rapidly after the flower closes. This decrease is due to dehydration of the grain, especially around the pore where the intine is exposed. However, in *Tectona grandis* Linn.f.

(Suwan and John, 1997) pollen released at 11.00h has the highest viability (92.2%) but is no longer viable 3 days (84h) after anthesis. In vitro pollen tube growth is fast (140 μmh^{-1}) and increases significantly within the first 8h.

In vitro pollen germination is one of the most convenient and reliable methods used to test the viability of fresh or stored pollen. The media used for in vitro germination of pollen of different species ranges from simple sucrose/boric acid media (Linskens, 1967) to complex media containing polyethylene glycol (Zhang and Croes, 1982; Shivanna et al., 1997) and various amino acids (Read et al., 1993). A medium has been developed that has been widely used and found to be suitable for more than 86 plant species (Brewbaker and Kwack, 1964).

Gelling of the medium is not beneficial for *Pinus kesia* (Katiyar, 1991) and *P. densiflora* (Tanaka, 1955) pollen because incorporation of bacto-agar in 0-03% sucrose +5ppm of boric acid medium reduced both germination and tube growth. But, gelling of the medium improves pollen germination and pollen tube growth in *Pinus patula* (Kapoor and Dobriyal, 1980). Chauhan and Katiyar (1996) worked out the requirements for optimal pollen germination and pollen tube elongation in *Schima wallichi.* Even at the optimal concentrations (10% sucrose + 50 ppm boron + 400 ppm calcium + 300 ppm magnesium + 100 ppm

potassium + 0.8% agar at pH 7.0) only 36.03 percent pollen germinated. In *Schima khasiana* the medium consists of 15% sucrose + 50 ppm boron + 300 ppm calcium + 300 ppm magnesium + 150 ppm potassium + 0.8% agar at pH 7.3 (Goswami, 2002). Jayaprakash and Sarla (2001) developed an improved medium for germination of *Cajanus cajan* (L:) Millsp. pollen in vitro which consists of 37.5% sucrose + 15% polyethylene glycol 4000 + 250 mgl^{-1} boric acid + 300 mgl^{-1} calcium nitrate + 100 mgl^{-1} potassium nitrate + 200 mgl^{-1} magnesium sulphate + 1% agar + .-amino caproic acid (0, 100, 250, 500, 750 or 1000 mgl^{-1}). This is the first time .-amino caproic acid (EACA) has been used as a constituent of pollen germination medium. The media containing 750 or 1000 mgl^{-1} of EACA gave >91% pollen germination. But, in a liquid medium consisting of 40% sucrose, 250 mgl^{-1} boric acid and 200 mgl^{-1} calcium nitrate only 48% pollen germinated (James et al., 1987).

Understanding of various factors influencing pollen germination and tube growth are a prerequisite for the success of hybridization programme (Vasil 1964; Mercy et al., 1978). Some of the factors which influence pollen germination and tube growth in plants are carbohydrate (sugar), boron, calcium, enzymes, plant hormones, magnesium, potassium, agar, pH and certain physical factors such as temperature and the requirements are species specific (Johri and Vasil, 1961). The

germination of pollen grains commonly occurs on the stigma. The stigmatic exudates provide a suitable medium for pollen germination. It has recently been shown that lipids present in the stigma are involved in directional pollen tube growth (Arts et al., 1998; Jayaprakash and Sarla, 2001). High levels of boron in the stigmatic and stylar tissues promotes the absorption of sugars, increase oxygen uptake by pollen and also play a role in the synthesis of pectic materials; thus stimulating pollen germination and pollen tube growth (Pfahler, 1968; Stanley, 1971).

Pollen grains require an optimum concentration of sugar solution for germination (Mukherjee and Das, 1964) because sugars regulate osmotic pressure and serve as the respiratory substrate (Johri and Vasil, 1961). Compared to other several sugars, sucrose is a better source for pollen germination and pollen tube growth in most of the plant species investigated (Tupy, 1966). Effect of various oligosaccharides on the in vitro growth of *Camellia japonica* pollen tube was investigated. It was found that amongst sucrose, raffinose, metezitose, cellobiose, turnose and isomaltose, the first four promoted pollen tube growth (Nakamura and Suzuki, 1985). The sucrose concentration required for optimal germination varies from species to species. Pine pollen though does not require exogenous supply of carbohydrate to germinate (Hellimers and Machlis, 1956) exogenous supply of sucrose (0.03-0.1%) enhances

pollen germination and tube elongation in *Pinus kesiya* (Katiyar, 1989). In *Cajanus cajan* (L:) Mill sp. the optimal sugar requirement for pollen germination is 37.5%. In *Medicago sativa* pollen 20% sucrose is most suitable for optimum germination while 10% sucrose induces tube growth (Gupta, 1979). Effect of sucrose, glucose and fructose on *Corylus avellata* pollen germinability was investigated by Kim et al. (1985). They found that 10 to 25% sucrose was most effective while glucose permitted good germination at lower concentrations.

Boron is also essential for pollen germination and tube growth. The optimal boron requirement for pollen germination and tube elongation differs in different plant species: Graminae 150 ppm; Cucurbitaceae 200 ppm; Leguminosae 100-150 ppm (Vasil, 1964). Boric is a better source of boron than borax for pollen germination and tube elongation (Vasil, 1964). Stanley and Linskens (1974) stated that plants requiring high boron for in-vitro pollen germination are rich in endogenous boron. Boron influences water relationship thus preventing pollen tube bursting and enzymatic steps in the biosynthesis of carbohydrates and growth hormones and regulators. The effect of boron on pollen germination and pollen tube elongation is many fold: (i) it forms sugar borate complexes which help to increase absorption, translocation and metabolism of sugars (Stanley and Loewus, 1964; Vasil,

1964), (ii) it is involved in the synthesis of pectin materials required for the wall of actively growing pollen tubes (Stanley and Loewus, 1964), (iii) it stimulates chemotropic activity of Ca^{++} (Mascarenhas and Machlis, 1964) and (iv) it increases O_2 uptake by pollen (O'kelley, 1957).

Pollen grains in larger populations during culture germinated well than when lesser number of pollen grains was cultured. Brewbaker and Kwack, 1963 termed this phenomenon as population or mutual stimulation effect which is subsequently identified this factor to be calcium ion. In *Tradescantia virginiana* calcium is involved in vesicle fusion as well as the plasticity of the tip wall components of the pollen tube (Picton and Steer, 1983). The Ca^{++} enhances pollen germination in *Zea mays*, *Triticum aestivum*, *Sorghum vulgaris*, *Gossypium arboretum*, *G. hirsutum* and *Hibbiscus cannabinus*.

Magnesium, Potassium and Sodium are the supporting ions which enhance the stimulatory effect of Ca (Brewbaker and Kwack, 1963). However, pollen of many plant species does not require (exogenous) supply of Mg or K for pollen germination e.g. *Pinus kesiya*.

Pollen grains germinate in a wide range of pH but best results are obtained at pH 5.5-6.5 (Johri and Vasil, 1961). They stated that pH may not directly affect pollen germination but whatever effect is produced

may be entirely due to the cations and anions present in the medium in the form of buffer salts.

Pollen germination can be substantially altered by the temperature and length of storage. The response to storage is associated with pollen source (Pfahler, 1973). Pinus pollen grains stored at 4°C remain viable for 2 ½ years without any decline germination capacity (Nygaard, 1969; Katiyar, 1991). Temperature is an important factor in pollen germination and pollen tube growth. Pollen of most plants show optimum germination and tube growth between 20-30°C (Johri and Vasil, 1961). In *Theobroma cacao* pollen Ravindran, 1977 observed no germination below 10°C and above 40°C. Gawel and Robacker (1986) reported that in *Gossypium hirsutum* pollen optimum temperature for tube elongation is 30°C. Vasil and Bose (1959) observed marked swelling or bursting of the tips of the pollen tubes at higher temperature.

Irradiation-induced tube elongation is reported in *Nicotiana* (Swaminathan and Murty, 1959; Michie J and Bohm L (1989), Douglas fir *Pseudotsuga menziesii* (El-Lakany and Sziklai, 1970; Livingston and Stettler, 1973; Vonder Donk et al., 1978), *Pinus sylvestris* (Zelles and Ernst, 1973; Zelles and Fendrik, 1973; Zelles and Seibold, 1976; Zelles et al., 1979), *Pinus* (Seibold et al., 1979), *P. wallichiana* and *P. patula* (Katiyar and Chauhan, 1987a, b).

In irradiated pollen of *Nepenthes alata* and *N. longsdorffi* the generative nucleus undergoes normal division to give rise two gamete nuclei (Grnt et al., 1980). Sharma (1977) pointed out that in *Amaryllis vittata* pollen, during tube growth the polysaccharides already present in the pollen are utilized by the elongating tube. Chhabra and Malik (1977) stated that in the early phase of tube initiation protein and RNA present in pollen are also used. It is also well established that toxic substances and calcium antagonists influence germination and inhibit the growth of the pollen tube (Gentile et al., 1973; Gentile et al., 1978; Pfahler 1981; Picton and Steer 1985; DuBay and Murdy 1983; Scholz et al., 1985; Michie and Bohm, 1989). Randolf and Robert (1986) reported that the pollen germinability is influenced by flower age, drying time, and pollen source while Pfahler (1967) observed that storage environment and date of collection are also crucial factor.

The ovule, or the megasporangium, is attached to the placenta of the ovary by a stalk, the funicle. It is a mass of potentially fertile tissue called nucellus, enveloped by one or two integuments (Bouman, 1974; Johri et al., 1992). The ovule may have one or two, internal and external integuments and in some species without integument (Sporne, 1969; Dahlgren 1980). The number of integuments is constant for most

families (Maheshwari, 1950) designated as uni- (e.g. Orchidaceae, Sarraceniaceae, *Meliosma* sp.) and bitegmic (e.g. Magnoliaceae, Euphorbiaceae, Nepenthaceae, Droceraceae, Aristolochiaceae etc.).

In the majority of families with bitegmic ovules, the inner integument overgrows the outer and forms the micropyle. The number of integuments indicates the evolutionary status of the family. The bitegmic condition occurs usually in dicotyledonous polypetalous families, where as the unitegmic condition is associated with most of the sympetalous families (Bouman, 1984; Johri et al., 1992).

In the yellow passion fruit (Passifloraceae), there are two integuments: the outer integument which covers the inner integument, until they meet each other and form the micropyle (Margarete et al., 2002). Also, in *Herminium monorchis* (Orchidaceae), two integuments are formed, but only the internal integument forms the micropyle (Frederickson, 1990). Watson and Dallwitz (1992) reported that outer integument contribute to the micropyle in Nepenthaceae. But, Kaul (1982) in *Nepenthes lowii* and *N. villosa* and Subramanyam et al. (1985) in *N. khasiana* observed that the long micropyle is formed by the inner integument alone.

The inner integument originates, in most cases, from the dermal layer and the external integument derives from the dermal and hypodermal levels (Johri et al., 1992; Margarete et al., 2002).

In angiosperms, the inner integument is always dermal except in *Codiaum variegatum* and *Euphorbia milii* (Bor and Bouman, 1974) and *E. geniculata* (Bor and Kapil, 1976). The outer integument is generally subdermal, but its dermal origin has been observed in Dichapetalaceae (Boesewinkel and Bouman, 1980; Boesewinkel and Geenen, 1980; Boesewinkel and Been, 1979).

The ortho-, ana-, hemiana-, Campylo- and amphitropous forms of ovules which are classified according to the position of the ovule in relation to hilum are considered as basic forms (Johri et al., 1992). The ovule is anatropous in Sarraceniaceae, Droseraceae and Nepenthaceae (Kaul, 1982; Johri et al., 1992; Watson and Dallwitz, 1992; Subramanyam et al., 1995).

The hypodermal archesporium is usually one-celled in Sarraceniaceae, Nepenthaceae and Aristolochiaceae etc. (Shreve, 1906; Nair and Narayana, 1961; Subramanyam et al., 1985), but multicellular archesporium develops in some families like Casuarinaceae (Swamy, 1948), Rosaceae, Sterculiaceae Compositae, Umbeliferae and Liliaceae (Bouman, 1984). In Droseraceae, there are one to three archesporial cells

(Narasimhachar, 1949). There may present more than one archesporial cell in some species like *Potentilla nivea* and *Passiflora edulis* (Eriksen and Frederickson, 2000; Margarete et al., 2002).

In tenuinucellate ovules e.g., Sarraceniaceae, Hydnoraceae (Johri et al.,1992), the archesporial cell functions directly as megaspore mother cell whereas in crassinucellar ovules e.g., *Drosera burmanni* (Narasimhachar, 1949; Patankar, 1956), Nepenthaceae (Stern, 1917; Watson and Dallwitz, 1992; Subramanyam et al., 1995), Phylidraceae, Aristolochiaceae, and *Passiflora* sp. (Margarete et al., 2002) etc., the archesporial cell cuts off a parietal cell. But, in *Drosera rotundifolia* (Pace, 1912), D. indica (Narasimhachar, 1951) and *D. peltata* (Venkatasubban, 1950), the archesporial cell directly functions as the megaspore mother cell.

Occurrence of callose during megasporogenesis was first noticed in the ovules of *Orchis maculata* by Rodkiewicz and Gorska- Brylass (1967), subsequently by Kuran (1972) and Kapil and Tiwari (1978). Callose invariably develops in species with Polygonum, Oenothera and Allium types of embryo sac but is absent in tetrasporic forms (Rodkiewicz, 1970; Kapil and Tiwari, 1977). Its deposition is closely related with the isolation of cellular connection between the meiocyte and the surrounding nucellar cells (Israel and Sagawa, 1965). In species

with polygonum type of ontogeny callose gradually decreases at the chalazal end of the megasporocyte.

The megaspore mother cell undergoes meiotic divisions in which the heterotypic division results in the formation of a dyad. The homotypic division occurs in each dyad cell and a linear e.g., Sarraceniaceae, *Drosera* species. Nepenthaceae (Johri et al., 1992; Subramanyam et al., 1995) and *Passiflora* species (Margarete et al., 2002) or T- shaped tetrad e.g., *Aldrovanda vesiculosa* (Johri et al., 1992). In Aristolochiaceae and Sarraceniaceae, linear or T-shaped tetrad is produced (Johri et. al., 1992). The chalazal one undergoes three mitotic divisions, usually resulting in a cell with eight nuclei which is the polygonum type of embryo sac formation, as described for about 70% of the angiosperm species (Maheshwari, 1950; Reiser and Fischer, 1993) e.g., Sarraceniaceae (Johri et al., 1992), Nepenthaceae (Davis, 1966; Watson and Dallwitz, 1992; Subramanyam, 1995), Droseracea, Aristolochiaceae, Cytinaceae (Johri et al., 1992) and *Passiflora* species etc. (Margarete et al., 2002). Allium type of embryo sac development is reported in *Prosopanche bertoniensis* (Chodat, 1916) and tetrasporic Adoxa type in *Hydnora Africana* (Dastur, 1992).

The number of megaspore nuclei take part in the formation of embryo sac may vary, due to variations in meiosis, cytokinesis, period

and number of mitotic divisions (Maheshwari, 1950; Reiser and Fischer, 1993; Cameron and Prakash, 1994). Megagametogenesis is highly coordinated with the sporophytic tissue of the ovule (Grossniklaus and Schneitz, 1998).

The female gametophyte (megagametophyte) is formed within the ovule, because inside the ovule the process of megasporocyte differentiation, production of a functional megaspore (megasporogenesis), formation of the embryo sac (megagemetogenesis), and embryogenesis occur (Reiser and Fischer, 1993).

Maheshwari (1937, 1941, 1946, 1947, 1948), Johri (1963a, b) and Willemse and Went (1984) have reviewed different types of embryo sacs in angiosperms. Depending on the number of megaspore nuclei taking part in its formation, the embryo sac is designated as Mono-, Bi- and Tetrasporic and several such types.

Swamy and Krishnamurthy (1975) have simplified the typology of embryo sac development by focusing attention on the exclusive features of the megagametophyte. The attainment of haploidy, the establishment of polarity, an elaboration phase to the formation of the egg apparatus, and a mature organization are recognizable in all types. The micropylar pole of the embryo sac is very regular in structure, whereas the chalazal pole shows a great variability. Of casual nature are factors like the wall

formation during meiosis, the number of mitotic divisions after formation of the megaspore, cell-wall formation after mitotic divisions and the fusion of nuclei. With the time of establishment of polarity as the guiding factor, four types of embryo sacs are distinguished. The Polygonum type as representative for the 'Supra- Homeotypic' category, the Allium type representative of the 'Homeotypic' category I, and the Penaea type and Fritillaria type as representatives of the 'Homeotypic' category II.

The process leading to the mature embryo sac may differ, but its organization, in most angiosperms, follows the same pattern. At the time of fertilization, the embryo sac presents the tricellular egg apparatus, with the egg cell and two synergids, three antipodal and two nuclei in the centre cell, fused or not. These cells take part in the fertilization, embryogenesis, and nutrition of the embryo sac and embryo (Maheshwari, 1950; Johri and Bhatnagar, 1973; Kapil and Bhatnagar, 1981; Reiser and Fischer, 1993; Chamberlin et al., 1994).

The development of more than one type of embryo sac in a family, or genus, or even in a species, is not uncommon. Kapil and Prakash (1966) recorded five different types (Polygonum, Endymion, Drusa, Penaea and Adoxa) of development in *Delosperma cooperi* (Aizoaceae). Dharamadhaj and Prakash (1978) observed twin embryo sacs, one

monosporic and the other bisporic, in the same nucellus of an ovule of *Capsicum annum* var. *acuminatum*.

An endothelium develops at the two-nucleate embryo sac (Johri et al., 1992) in Sarraceniaceae. Narasimhachar (1951) observed false endothelium development around the embryo sac in *Drosera indica*.

The accumulation of starch grains in the embryo sac is a common feature in several families, such as Acanthaceae, Aizoaceae, Bruniaceae, Cactaceae, Crassulaceae, Frankeniaceae, Portulaceae and Tiliaceae (Johri et al., 1992).

The antipodal cells are located on the opposite side of the egg cell, they are usually three, frequently varying in size (Maheshwari, 1950; Cameron and Prakash, 1994) and number, as it occurs with the number of nuclei of these cells, as recorded in the Compositae family. Narasimhachar (1951); Johri et al., (1992) reported that the antipodals are conspicuous in *Drosera peltata* and *D. burmanni*. In *Passiflora edulis*, the antipodals are positioned at the chalazal end of the gametophyte, and had a triangular shape, their nuclei are small, as compared to other nuclei in the gametophyte (Margarete et al., 2002).

The polar nuclei fuse before fertilization in Nepenthaceae (Watson and Dallwitz, 1992; Subramanyam et al., 1995). But, in Sarraceniaceae, the polar nuclei fuse at the time of fertilization.

The fertilization process of plants is governed by different kinds of cell-cell interactions. In higher plants, these interactions are required both for recognition of the pollen grain by the female reproductive system and to direct the growth of the pollen tube inside the ovary. Two distinct types of Principles, mechanical and chemotropic, have been suggested to account for the directed growth of the pollen tube toward its target, the embryo sac. The guidance of the pollen tube depends on the architecture and chemical properties of the female reproductive tissues, where as, the ovule provides a signal for the target-directed growth of the pollen tube (Hulskamp et al., 1995; Raghavan, 2000).

In particular, the mechanisms that enable only one pollen tube to interact with each female gametophyte, there by preventing polyspermy, are not understood. This is might be due to the attractive and repulsive interactions between female and male gametophytes (because of the emission of attractants by the female gametophyte and repulsion between pollen tubes) (Shimizu and Okada, 2000).

In flowering plants, two gametes from a single pollen fuse with two female gametes, the egg and central cell to form the embryo and

endosperm, respectively. This is known as double fertilization. Faure (2003) concluded that both male gametes have the capacity to fuse with the egg cell in maize by investigating double fertilization in maize: the two male gametes from a pollen grain have the ability to fuse with egg cell.

Lord and Russell (2002) studied the mechanisms of pollination and fertilization in plants. In flowering plants, pollen grains germinate to form pollen tubes that transport male gametes (sperm cells) to the egg cell in the embryo sac during sexual reproduction. Pollen tube biology is complex, presenting parallels with axon guidance and moving cell systems in animals. Pollen tube cells elongate on an active extra cellular matrix in the style, ultimately guided by stylar and embryo sac signals. A well documented recognition system occurs between pollen grains and the stigma in sporophytic self- incompatibility, where both receptor kinases in the stigma and their peptide ligands from pollen are now known. Complex mechanisms act to precisely target the sperm cells into the embryo sac. These events initiate double fertilization in which the two sperm cells from one pollen tube fuse to produce distinctly different products: one with the egg to produce the zygote and embryo and the other with the central cell to produce the endosperm.

When patterns of cell cycle activity in sperm of *Arabidopsis* and other flowering plants are compared, developmental analysis reveals that heterochronic alterations (Changes in the relative timing of ontogenetic events) in cell cycle activity are a central cause of the diversification of patterns of gametogenesis in higher plants. The pattern of prefertilization S phase activity in the sperm of a flowering plant stands in marked contrast to all other non- plant eukaryotes (from ciliates to yeast to sea Urchins to mammals) where sperm remain in G1 during development, prior to the initiation of gametic fusion.

After pollination, one of the synergid shows signs of degeneration (Jensen et al., 1983; Johri et al., 1992). The pollen tubes penetrate the embryo sac, and enter the degenerated synergid through the filiform apparatus and liberated the two male gametes through a pore.

Double fertilization in angiosperms (Steffen 1963b; Korobova 1974; Battaglia 1981; Belyaeva 1981; Van Went and Willemse 1984) follows pollination. In this process one of the male gametes fuses with the egg cell (syngamy) while the other male gamete fuses with the secondary nucleus or the two polar nuclei (triple fusion).

Maheshwari (1950) and Johri and Ambegaokar (1984) discussed the role of persistent pollen tubes and emphasized that these are merely

the remnants of a 'dead' structure which are not at all concerned in absorbing and transmitting the nutrients.

In Droseraceae, triple fusion precedes syngamy (Johri et al., 1992). The process of syngamy results in the formation of zygote. Usually, a transverse division occurs in the zygote, and separates a spherical terminal cell 'ca' from the basal cell 'cb' (Soueges, 1913; Crete, 1963; Sivaramkrishna, 1978).

The term proembryo is applied from the two-celled condition till the initiation of organs in the embryonal mass. The development of proembryo up to an octant stage and the formation of two types of octant are similar in dicotyledons and monocotyledons (Swamy, 1962; Guignard and Mestre, 1969; Guignard 1975; Johri et al., 1992).

The derivatives of the two-celled proembyro, 'ca' (apical cell) and 'cb' (basal cell), lead to six types of embryogeny (Soueges, 1934; Johansen 1945; Maheshwari, 1950; Crete, 1963; Johri et al., 1992): Onagrad/ Crucifer (includes *Myosurus*, *Juncus*, *Capsella*, *Veronica*, *Mentha*, *Trifolium*, *Ruta* and *Lilium*); Asterad (includes *Muscari*, *Geum*, *Polysonum*, *Urtica*, *Lamium*, *Oxalis* etc.); Solanad (includes *Hyoscyamus*, *Nicotiana*, *Papaver*, *Sherardia* and *Linum*); Chenopodiad (includes *Chenopodial*, *Polemonium* and *Myosotisvariations*); Caryophyllad (includes *Sagina*, *Vacaria*, *Corydalis*, *Sedum*, *Medicago*,

Drosera, *Sagittaria* etc. and Piperad type (includes Piperaceae, Loranthaceae etc.).

In Caryophyllad type, the embryo proper is derived from the daughter cells of 'ca' only; in addition, the tier 'ca' also contributes to the formation of suspensor. However, as in the Crucifer, Asterad, Solanad, Chenopodiad type, the daughter cells of 'cb' may or may not take part in the formation of the proper embryo.

In Droseraceae, a transverse division in the zygote results in an apical, and a basal cell. The apical cell develops in to the embryo proper, and also contributes to the formation of the suspensor. The basal cell may or may not divide, and add to the suspensor in *Drosera burmanni* and *D. indica*. The embryogeny may be of the Caryophyllad type. In *Dionaea muscipula* the terminal cell of the two-celled proembryo divides vertically, indicating the Asterad or Onagrad type of development (Johri et al., 1992).

In Sarraceniaceae, a transverse division of zygote occurs after the formation of about 150 endosperm cells. The terminal cell of a uniseriate row of five to seven cells of the proembryo develops into the embryo proper. The suspensor is strongly curved (Johri et al., 1992). Watson and Dallwitz (1992) also reported Caryophyllad type of embryogeny in the Sarracaniaceae family.

The Solanad type of embryo development is found in *Hydnora africana* (Davis, 1966) and Aristolochiaceae (Johri et al., 1992; Subramanyam et al., 1995). But it needs confirmation.

Swamy and Lakshmanan (1962) reported that in the dicotyledonous embryo, the two opposite cells of a terminal quadrant give rise to two cotyledons where as in monocots, the number of sectors of a quadrant involved in the formation of a single cotyledon varies.

The mature embryo is minute and linear in Droseraceae, Sarraceniaceae and Nepenthaceae (Kaul, 1982; Johri et al., 1992; Watson and Dallwitz, 1992; Subramanyam et al., 1995).

Endosperm, a product of double fertilization, develops only in angiosperms (Johri et al., 1992). The nutritive role of endosperm has long been recognized. It nurses the embryo from the proembryo stage till it becomes self -dependent and regulates the pattern of development (Krishnamurthy, 1988). The endosperm contains the growth regulators, and coconut milk. In *Drosera* it contains plenty of starchy food reserves (Johri et al., 1992) and in Sarraceniaceae, the endosperm stores aleurone grains (Shreve 1906; Johri et al., 1992).

The Nuclear type of the endosperm development is observed in Droseraceae and free nuclei aggregate in the chalazal region which

functions as a haustorium. Centripetal wall formation is initiated in the micropylar region, after the formation of 32-64 free nuclei. Ultimately, the entire endosperm becomes cellular (Johri et al., 1992).

The endosperm remains free - nuclear through out in *Floerkea proserpinacoides* (Maheshwari and Johri, 1956) and in *Oxyspora* (Subramanyam, 1951). However, in the majority of plants it does become cellular.

In cellular type, the division of the primary endosperm nucleus is followed by wall formation e.g., Sarraceniaceae (Shreve, 1906), Aristolochiaceae, Cytinaceae (Johri et al., 1992).

As the Nuclear type of endosperm has been observed in a larger number of taxa than the other two types, it may be considered to be primitive (Johri et al., 1992).

The formation of different types of a haustorium has been reported in several angiosperms like Anacardiaceae, Cucurbitaceae, Euphorbiaceae, Leguminaseae and Proteaceae (Walia and Kapil, 1965; Johri et al., 1992).

Williams and Friedman (2002) reported diploid biparental endosperm in *Nuphar polysepalum*, a basal angiosperm and showed that diploid endosperms are common among early angiosperm lineages and may represent the ancestral condition among flowering plants.

Nepenthes are found in diverse habitats from Madagascar and India to northern Australia, New Caledonia, and the Phillipines. The areas with the most species in the most diverse habitats are Borneo, Sumatra, and Malaysia. They are found on beaches, in hot steamy jungles and cold wind-blown ridge tops. Different species grow in sand, acid bogs, or alkaline volcanic soils. Some species like intense sun while others are at home in dense shade (ICPS seed bank, 2002).

Most of the temperate zone species are found in bogs. Bogs typically have very acid soils that may be low in certain minerals which are required by plants for normal growth. The theory has thus been advanced that these insectivorous bog plants use their prey as supplemental sources of minerals.

Pitcher plant seeds mature in July through September in the Southeastern U.S. habitats, depending on species and localities. In the warmer region, if the seeds are shed before the fall sets in, the germination takes place in a month or so, and tiny seedlings will emerge in that year, although the germination is often delayed until the following spring in many localities. After twin cotyledons, a seedling produces tiny juvenile leaves which are already hollow, pitcher leaves (Shreve, 1906).

Moisture, temperature, oxygen and light are considered essential factors for seed germination. The New Zealand woody species *Beilschmiedia tawa*, *Dysoxylum spectabile*, *Griselinia lucida*, and *Weinmannia racemosa* germinated rapidly with a high degree of success when kept moist and in maximum available daylight. In the dark, germination rate was slower but the success was similar for *Beilschmiedia* and *Griselinia*. *Dysoxylum* seeds died in the dark, possibly because they were too wet. Germination of *Weinmannia* seeds were inhibited in the dark but they germinated well when put in the light (Burrows, 1999a). *Alseuomia purilla, Geniostoma rupestre, Myrtus bullata, Aristotelia serrata, Coprosma robusta, Cordyline australis*, *C. banksii*, *Myrtus obcordata, Schefflera digitata* and *Solanum aviculare* seeds germinated well in the light. *Cordylene* seeds showed 100% germination success in the dark. In a relatively well lit, moist treatment seeds from freshly collected fruits of the New Zealand woody sps. *Alseuosmia macrophylla*, *A. purilla*, *Cordyline banksii*, *Geniostoma rupestre*, *Myrtus bullata* and *Solanum avivulare* germinated in autumn-winter, or winter-spring. Germination of a few *A. macrophylla* seeds was delayed until spring of the 2nd year. Success was high (97-100%) for all species except *Solanum* (72-82%). Germination in dark was nil for *A. macrophylla* but for the other species ranged from 82% (*Solanum)* to

100% (*Cordyline*); *A. macrophylla* seeds germinated when brought into the light (96% success) (Burrows, 1996).

Simon Moore (1994) studied the effects of low temperature on seed germination of New Zealand species of *Pittosporum* and reported that imbibed seeds of *P. eugenioides*, *P. obcordatum* and *P. tenuifolium* showed almost no germination and marked a loss of viability after 3-4 months moist storage in light at 21°C. In contrast, 8 weeks or more of low temperature stratification resulted in almost complete germination of seeds.

Miriam et al. (1994) studied the effects of increasing stratification times as well as the effects of light and dark on germination in *Pernettya mucronata* (L.F) Gaudich ex. G. Don. The percentage germination at 60days was significantly higher in the light (45-6%) than in the dark (29.4%). The percentage germination at 60 days was also significantly higher after a 300 h chilling pretreatment (40.4%) compared with that of unstratified seeds (22%).

Sarracenia leucophylla, *S. flava*, *P. vulgaris*, *Drosera intermedia* seeds need a stratification period for good germination percentage except *N. ventricosa.* Seed will start to germinate after four or five weeks (Mark Pogany, 1999). Germination season is generally during April (spring season).

High percentage of seed germination (98.4%) is reported in *Metrosideros excelsa* (Gabriele Schmidt-Adam et al., 1999*)*, *Melicytus ramiflorus* (87%) (Herron and Clemens, 2001), high seed viability for up to 24 years in eleven species of *Carmichaelia* (Ingrid Gruner and Heenan, 2001). This species showed no specific light requirements for seed germination.

Schutz. et al. (2002) the role of dormancy, temperature and light in the regulation of seed germination of four annual Asteraceae, *Millotia myosotidifolia*, *podotheca gnaphalioides*, *P. chrysantha*, *Ursinia anthemoides*. Seeds of all species were strongly dormant at maturity. Germination percentages were highest at average temperature (25°C). Most of the species germinate better in darkness than in light. Simon Moore (1994) studied the effects of low temperature on seed germination of New Zealand species of *Pittosporum* and reported that imbibed seeds of *P. eugenioides*, *P. obcordatum* and *P. tenuifolium* showed almost no germination and marked a loss of viability after 3-4 months moist storage in light at 21°C. In contrast, 8 weeks or more of low temperature stratification resulted in almost complete germination of seeds.

Erythronium japonicum (Liliaceae) seeds germinated at 15°/5°C or 10°C (light 12h/ dark 12h alternating temperature.), starting on day

135, but if kept at 25°C or 30°C they did not (Tetsuya Kondo et al., 2002).

Nezar et al. (2003) studied the effect of maturity stage on germination and dormancy of fresh and air- dried seeds of bitter vetch (*Vicia ervilia* L.) and concluded that maximum and minimum dormancy was achieved for air-dried and dry-prechilled seeds harvested at the brown pod stage.

Much of seed viability depends upon storage conditions. The ideal storage condition for seeds is somewhere cool and dry. A capped jar in the refrigerator serves the purpose (Sherry Rindels, 1995). *Nepenthes* seeds will last upto 12-14 months provided the seeds are treated correctly. The seed of *N. fusca* (that collected in Sabah) showed at least 90% germination after 14 months (Phill Mann, 1998). Seeds of species such as *N. bicalcarata* and *N. ampullaris* seem to have a very short life of a matter of weeks. When considering the climate where they occur, it is possible that the seed never actually 'dries' and there must be a considerable percentage of moisture all the time. Seed collected should be 'dried' to avoid moulds taking over and then placed them into the small envelopes and into a plastic bag. These then should be stored in the 'butter' section of the refrigerator (Phill Mann, 1998).

In vitro germination of *Nepenthes* seeds usually takes 11 days to 3 months. Some seeds still germinate after 6-7 months. It may be mainly depends on how long we can keep the cultures going before they dry.

Sterilization would play a large part in seed germination, as factors such as to much bleach (or too long), insufficient rinse after sterilization would definitely have adverse reaction in seed germination (Phill Mann, 1998).

CHAPTER - 3

Materials and methods

Development in the staminate and pistillate flowers

Male and female flowers of *Nepenthes khasiana* Hook.f. at various stages were collected from Jowai, Jaintia Hills, Meghalaya state, India (92.10° N - 25.25° E; Plate-3). For the light microscopy the materials were fixed in a phosphate buffered solution of 2-3% glutaraldehyde, dehydrated in propanol and embedded in glycol methacrylate (Technovit 7100). To ensure proper fixation the tepals were removed before fixing the androecium and gynoecium. A rotary microtome was used to produce 7-10 μm thick sections that were stained with safranin-fastgreen and erythrosine. Callose was stained with decolourized aniline blue (Shivanna and Rangaswamy, 1993). Proteins were stained with mercuric bromophenol blue (Mazia, Brewer and Alfert, 1953) and nucleic acids with Azure blue (Heslop-Harrison, 1979). Photomicrographs were taken by using Nikon E600 and Leitz fluorescence microscopes. External surfaces of the androecium and gynoecium were studied morphologically by using Scanning electron microscope (SEM). The following methods were employed for SEM studies:

1. Flowers were collected, androecium as well as gynoecial parts were dissected longitudinally with razor blades, fixed in 2-3% glutareldehude prepared in 0.1M phosphate buffer, pH 7.2 at 4°C for 8 hours, thoroughly washed in 0.1M phosphate buffer and postfixed in 1% OsO_4 for 2 hours.

2. The materials were then dehydrated in increasing concentration of acetone.

3. Dehydrated materials were critical point dried in a Jeol JCPD-5 critical point dryer, 3-methyl butyl acetate solution as the exchange liquid.

4. Dried materials were sputter coated with gold in an Eiko ion coater and examined with a Joel 35 SEM at 15 KV.

Pollen Viability

Male inflorescences bearing flower buds about to anthesis were brought to the laboratory from the collection site in the month of July. The cut ends of the inflorescence peduncles were immersed in water. On anthesis of flowers, pollens from the freshly opened flowers were immediately stained with fluorescein diacetate for viability.

Pollen viability of fresh pollen grains was tested by using Fluorochromatic Reaction (FCR) Test (Heslop-Harrison, 1970; Shivanna and Rangaswamy, 1993).

1. Stock solution of Fluorescien diacetate solution (FDA) was prepared in acetone (2mg/ml). It can be stored in the refrigerator for months.

2. 10% sucrose solution was prepared to prevent bursting of pollen grains. 300 mg/l of calcium nitrate was added into the sucrose solution to improve the response of pollen systems.

3. To 2-5ml of sucrose solution in a small glass vial drops of stock solution of FDA were added until the resulting mixture shows persistent turbidity.

The mixture was used within 30 min. from preparation; otherwise most of the FDA would precipitate.

4. A drop of sucrose-FDA mixture was taken on a slide.

5. Sufficient amount of fresh pollen grains were suspended in the preparation.

6. The preparation is incubated in a humidity chamber (>90%RH) for 5-10 min.

7. At the end of the incubation period, a coverglass was lowered and observed the preparation under the fluorescence microscope with HPWB (High Performance Wide Band) filter.

8. Pollen grains that fluoresce brightly (which gave a bright, yellowish green) were scored as viable. And those that didn't fluoresce were non viable. For calculating pollen viability, total number of viable and non viable pollen grains was counted from 20 microscopic fields. Five replicates were maintained.

Pollen germination and Pollen tube growth

Freshly collected pollen grains just after anthesis were inoculated for germination test. The basal medium for germinating pollen in vitro was followed after Brewbaker and Kwack (1963). The optimal requirements of pollen germination and pollen tube elongation were worked out by altering one factor at a time. The Brewbaker and Kwack's medium was modified by incorporating the optimal requirements of

Nepenthes khasiana pollen and was used for the study of pollen germination and pollen tube growth in *Nepenthes khasiana*. Effect of light on germination and tube elongation were investigated by incubating the pollens in dark and white light. The light intensity was 530-750 lux. Pollen were incubated for 10 hours in the germinating medium and then fixed by putting a drop of FAA (formalin: glacial acetic acid: 50% ethanol 1:1:10) on the incubated pollen. Per treatment 10 slides were maintained. Data on pollen germination were recorded by scoring 10 microscopic fields chosen randomly per slide. All the 10 slides were scored for data recording. Thus, altogether 100 observations were made for an inflorescence. Pollen germination was determined by taking mean of all the 100 readings. Pollen tube elongation was measured in 100 pollen tubes per slide, from ten different microscopic fields. The mean length of the 1000 tubes so measured gave the tube growth. The data were analysed statistically by one - way ANOVA.

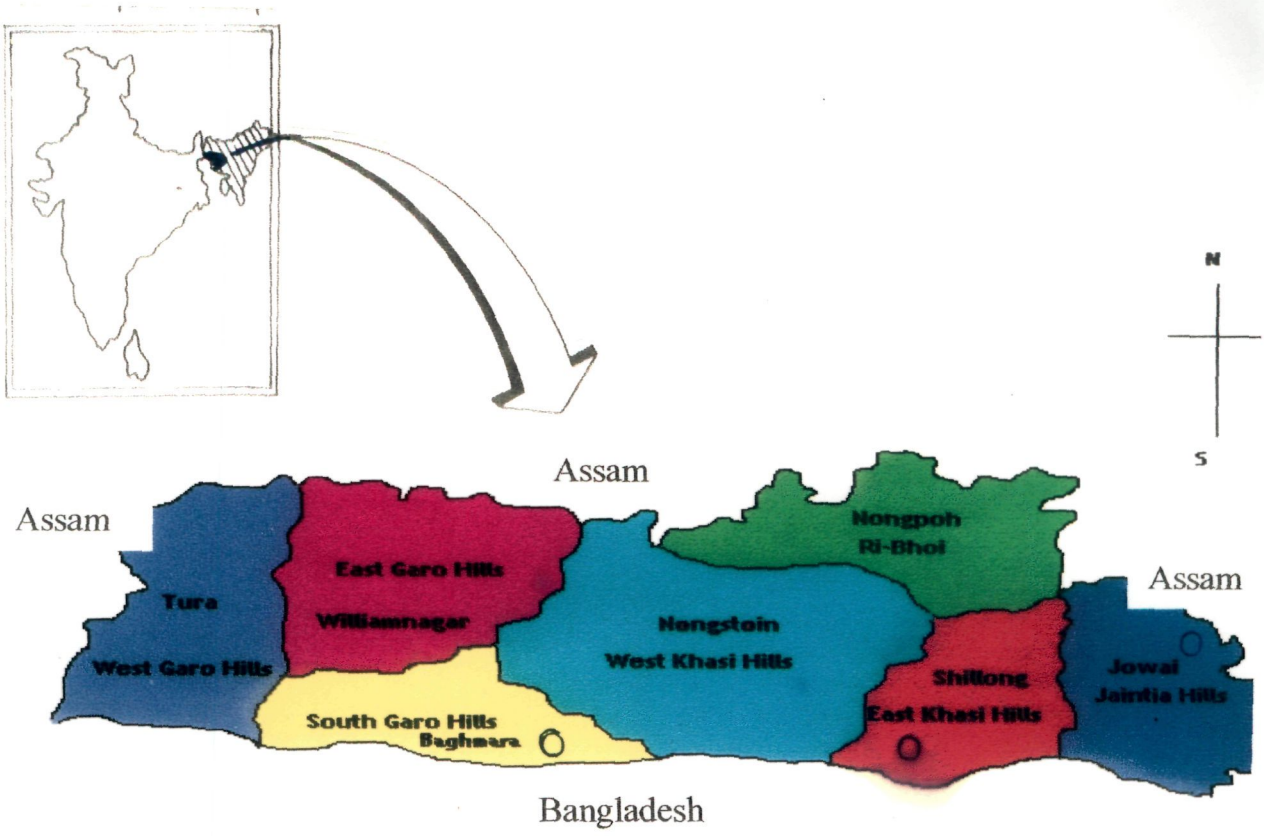
Seed Viability

Seed germination test was performed for seed viability. Sterilized seeds of *Nepenthes khasiana* were germinated in petriplates on soaked (for 2-4 hours to moisten it evenly and to remove water soluble toxic substances) Whatman filter paper No.1, sterilized sand moistened with

glass double distilled water in BOD incubator at 25° C in light, dark and 16h dark 8h light conditions. The intensity of the light was 1000-1250 lux from white lamps.

Sterilized seeds were also germinated in petriplates on Whatman filter paper No.1, sterilized sand moistened with glass double distilled water in BOD incubator at 20°C, 25°C, 28°C temperatures and in continuous light, dark and 16h dark 8h light conditions to find the effect of temperature and light conditions on seed germination. Subsequent moistening is done with glass double distilled water. Emergence of radicle, plumule above sand was considered as germination. Data were recorded regularly till the germination was over. Data were collected on days to commence the germination, days taken to complete the germination and total germination percent. The data were analysed with the help of student's 't' test.

Plate – 3



○ ***Distrbution of Nepenthes khasiana Hk.f. in Meghalaya***

CHAPTER - 4

Male Flower

4.1 *Introduction*

The unigeneric family Nepenthaceae consists of approximately 74 species (Willis, 1966). In India, the only representative of the genus *Nepenthes*, *Nepenthes khasiana* Hk.f. is an endemic and endangered insectivorous plant (Hooker, 1886; Jain and Sastri, 1980; Joseph and Mani, 1982). In the androecium, the filaments are connate to form an androphore on the top of which 25-30 bisporangiate anthers are arranged in a definite pattern. The reproductive biology has not been thoroughly investigated in *N. khasiana* (Johri, Ambegaokar and Srivastava, 1992). Davis (1966) reviewed the embryological characters of the family Nepenthaceae. The life history of *N. gracilis* was studied by Lan and Prakash (1973) and that of *N. khasiana* by Subramanyam, Narayana and Sundari (1985). Kaul (1982) also described the structure of the stamens on *Nepenthes lowii* and *Nepenthes villosa*, but the detailed study of development of anther and microsporogenesis have not been studied so far. Therefore, the present investigation aims to study the structure and development of the androecium and microsporogenesis in *Nepenthes khasiana* Hk.f.

4.2 *The structure of male flower*

Nepenthes khasiana Hk.f. is a dioecious plant. Flowers small, conspicuous having four tepals. Flowers are in racemose inflorescence. Racemes lateral and terminal, puberulous, up to 65 cm long, in each inflorescence an average of 100-150 flower buds and 4-8 flowers per inflorescence open at a time (Plate-4.2a, b). Flowers tetramerous, 0.5 - 1.0 cm. in diameter, greenish-red or greenish brown in colour, pedicels 0.8- 1.0 cm., ebracteate, fragrant, regular, cyclic, hypogynous disc absent (Plate-4.2.1a). Tepals free, regular, oblong, 3mm-4.5mm across, 4mm-6mm in length and imbricate. Floral nectaries (epithelium nectaries) distributed on the upper surface of four tepals which secrete nectar (Plate-4.2.1b). The staminate and pistillate inflorescences are similar, except for somewhat stouter peduncles and pedicels in the pistillate. Staminate flower buds globose, pistillate flower buds ovoid - oblong.

In the male flower, stamens are 25-30, coherent. In stamens, filaments united into a column to form an androphore and crowned by the usually connate anthers. Maximum height of an androphore is 4-5mm (Plate-4.2.1c). Anthers bisporangiate dehiscing via longitudinal slits, extrorse. Anther is around 3-4mm in diameter, pollen shed in aggregates of tetrads (Plate-4.2.1d). Pollen grains numerous and 16.6 μm in diameter, three-celled.

4.3 *Development of the Stamen*

In the male flowers, the filaments of the anthers are connate to form an androphore. The anther heads are sessile in the bud but as the flower opens the androphore elongates and the anthers are inclined upward about 45°. The number of anthers varies from 25 to 30 from flower to flower on the same inflorescence. The bisporangiate anthers are disposed in a regular pattern (see Plates-4.2.1a, c; 4.3a). On initiation the androphore primordium comprises a mass of undifferentiated meristematic cells. It grows in height by intercalary meristematic activity. After reaching 10 μm in length the vasculature differentiates in acropetal direction. Simultaneously on the top of the androphore 25 to 30 staminal primordia develop, which also consist of meristematic cells. Soon each staminal primordium becomes bilobed condition, which is the region where sporangia with two thecae develop later (Plate-4.3b). The maximum height of an androphore is 4-5 mm, which reaches within 24 hrs.

4.3.1 *Archesporium and anther wall formation*

A plate of four to five hypodermal cells differentiates into an archesporium at each of the two corners. Each archesporial cell is radially elongated, densely cytoplasmic with a large nucleus and

nucleolus (Plate-4.3c). The number of archesporial cells increases by additional anticlinal divisions from the already differentiated archesporial initials. Although the entire archesporial initials divide periclinally, the time of division varies in different archesporial cells. The cells derived from the first periclinal division are all of equal sized daughter cells without a clear difference between them. The inner row of cells is much larger than the outer ones. The former becomes the primary sporogenous cells and later the primary parietal cells.

The primary parietal cells undergo periclinal divisions to form four to five layers, of which the outermost functions as an endothecium with distinct annual thickenings, the innermost, the tapetum, and remaining two to three as middle layers. The anthers dehisce extrorsely by vertical slits whose walls have flared, exposing the great yellow mass of tetrads that is held above the tepals. Each pollen grain is three celled when shed. The pollen grains in a tetrad adhere firmly (Plate-4.3d).

4.3.2 *Tapetum*

The outer peripheral portion of tapetum is formed from the primary parietal layers. The tapetal cells that abutting at the connective side arise from the cells of the connective tissues, which are much larger in size and sometimes bi- or tri- layered. Thus, the tapetum has a dual

origin, from (1) the derivatives of the parietal cells (parietal tapetum) and (2) the connective cells (connective tapetum). Both the parietal tapetum and connective tapetum are of secretory type (Plate-4.3d). The cells become binucleate during prophase-I of meiotic division in the microspore mother cells, whereas the connective tapetal cells become two to three nucleate condition. The parietal tapetum becomes dark and loses its contents very earlier than the connective tapetum when the microspores are in tetrads (see Plate-4.3d). The parietal tapetum disintegrates a little later when microspores attain their maximum size after disappearance of the callose sheath.

The cells of the endothecium remain rectangular and develop thickening in the inner tangential and two lateral walls at the stage when the microspores become vacuolated and enlarged to the maximum size. The characteristic fibrous thickenings develops when the pollen are formed. The mature anther wall comprises the epidermis and endothecium.

4.4 *Meiosis and cytokinesis*

Pollen grains are arranged in a tetrahedral and decussate arrangement, so that there are two types of cytokinesis in *N. khasiana*. In the first type, meiosis is not uniform in all the microspore mother cells.

The microspore mother cells are held together by the distinct callose wall (Plate-4.4a), which dissolves after the completion of cytokinesis and before the onset of pollen wall deposition. After the first meiotic division of the nucleus, no plate formation is formed and the nucleus spindle disappears. Centripetal furrowing of the cytoplasm begins when four daughter nuclei are in telophase-II and is completed by centripetal furrowing, which is the characteristic feature of simultaneous division result into tetrahedral pollen grains (Plate-4.4b). The second type is heterotypic, in which a cleavage furrow starts after meiosis I, but its further development ceases during meiosis II. It resumes growth at the end of meiosis II (Plate-4.4c). Simultaneously, additional furrows develop centripetally from the periphery. So the four microspores are arranged in a decussate manner (Plate-4.4d). During the initial stages of microspore enlargement the cytoplasm does not increase rapidly enough to fill the entire lumen of the microspore wall. Cytoplasmic vacuolation increases and when the microspores enlarge to their maximum size, the cytoplasm forms only a thin layer around the nucleus with radiating strands. Subsequently the cytoplasm increases quickly and the nucleus shifts its position to the distal pole where it divides to form the vegetative and generative cells.

4.5 *Discussion*

The bisporangiate anther has been reported only in few families (Bhandari, 1984). The insectivorous families Nepenthaceae, Droseraceae and Sarracenaceae show a tetrasporangiate condition (Watson and Dallwitz, 1992). But, an interesting feature in *N. khasiana* is that it shows a bisporangiate condition. Davis (1966) reported the co-occurrence of bi- and tetrasporangiate anthers in four monocotyledon and twelve dicotyledon families. Such a co-occurrence feature is absent in *N. khasiana* Hk.f. The number of sporangia ranges from 25-30 on each androphore in *Nepenthes khasiana* Hk.f. The same condition has been reported in *Nepenthes lowii* (Kaul, 1982). The plate of archesporial cells, hypodermal in origin, has been reported in many plants (Maheshwari, 1950; Periasamy and Swamy, 1959; Periasamy and Kandasamy, 1981; Swamy and Krishnamurthy, 1990). The anther development is dicot type (Davis, 1966; Poddubnaya, 1976; Subramanyam et al., 1985). The origin of the tapetum is dual in origin; a part of it is derived from the primary parietal cell, which is a single layer whereas another part, which is abutting the androphore, is derived from the connective tissue of the androphore, which is multilayered, multinucleate and the nuclei are hypertrophid. These tapetal cells are much larger in size than the parietal tapetum (Periasamy and Swamy,

1966). However, the dual origin of tapetum has not been reported in *Nepenthes lowii* and *Nepenthes villosa* (Kaul, 1982). Subramanyam et al. (1985) also did not mention this fact. The secretory type of tapetum also confirms the observation of Subramanyam et al. (1985). The connective tapetum degenerates earlier than the parietal tapetum. The further differentiation of the androphore and its elongation take place only when the pollen grains are in tetrads. The same phenomenon has also been observed in *Nepenthes lowii* and *Nepenthes villosa* (Kaul, 1982). But the activity of an intercalary meristem, which is responsible for the elongation of androphore, has not been observed in the above-mentioned species.

As the microspore mother cells enter into reduction division, there is a distinct callose wall around each microspore mother cell. The endothecium acquires wall thickenings at the end of meiosis II, simultaneously the callose wall around the tetrads starts to disappear.

In general, the type of cell division in the microspore mother cell (meiocyte) can be classified as successive or simultaneous. *N. khasiana* exhibits simultaneous type of cytokinesis, but there are two variations within it. 1. Simultaneous centripetal constriction furrows at the end of meiosis II. 2. It is intermediate where in the first furrowing that commences after the first nuclear division is not completed until the

second nuclear division. As a result the completion of cytokinesis may be simultaneous although the initiation of the division furrow is successive. A similar condition was reported in *Magnolia* (Farr, 1918; Stoudt, 1960) and in *Annona* (Juliano, 1935).

In view of the unique morphological flux exhibited within the relatively few members of insectivorous families of the order Sarraceniales studied so far with regard to various aspects of the microsporangium, a study of more members of this group may bring to light other features that may be of value in the understanding of the morphology and evolution of the angiosperms as a whole.

Plate - 4.2

Nepenthes khasiana Hk.f.

Male Inflorescence

An Enlarged View of Male Inflorescence

Plate - 4.2.1

a. An enlarged view of a male flower. x 10.

b. An enlarged view of a single tepal showing the nectariferous glands embedded on it . x 50

C and D. SEM of androecium and pollen grains; in C the filaments connate to form androphore. C- x 50;
D- x 1000.

Plate - 4.2.1

Nepenthes khasiana Hk.f.

A Male Flower

Tepal with Nectar glands

Scanning Electron photographs of Androecium and Pollen grains

Plate - 4.3

a: L. S. of young male flower bud, showing the bisporangiate condition of anther. ba = bi - sporangiate anther; pmc = Pollen mother cells. x 1200.

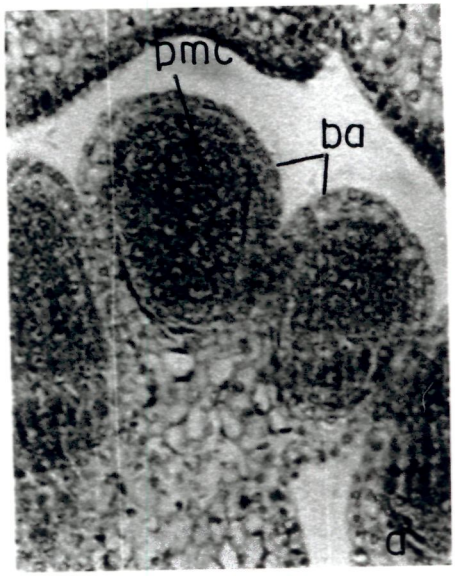
b: L.S. young male flower bud showing bilobed staminal primordia (sp). x 1700.

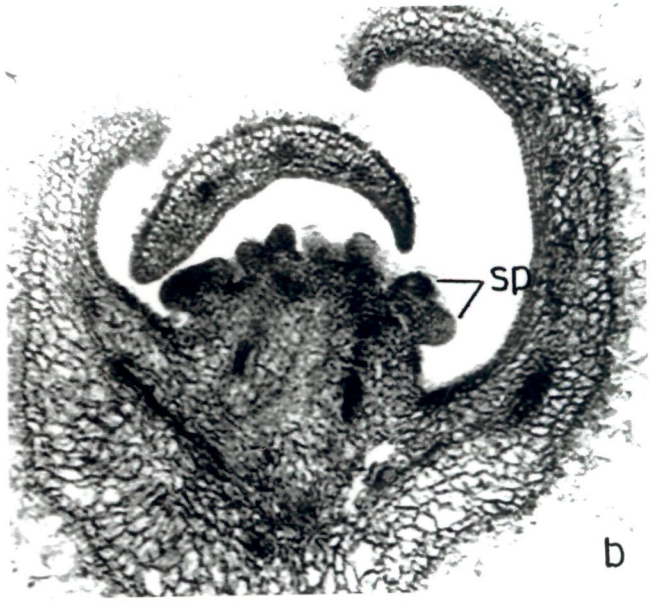
c: T. S. of anther lobe depicting tapetum, connective tapetum (ct); parietal tapetum(pt) and tetrahedral pollen grain (tp). x 600.

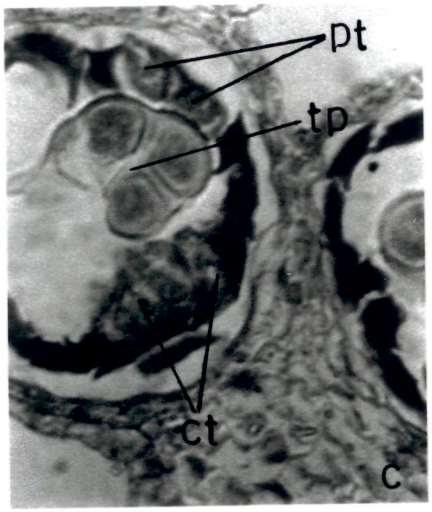
d: An enlarged view of an anther primordial lobe showing a plate of archesporial initials (ai). x 3500.

Plate - 4.3

Nepenthes khasiana Hk.f.







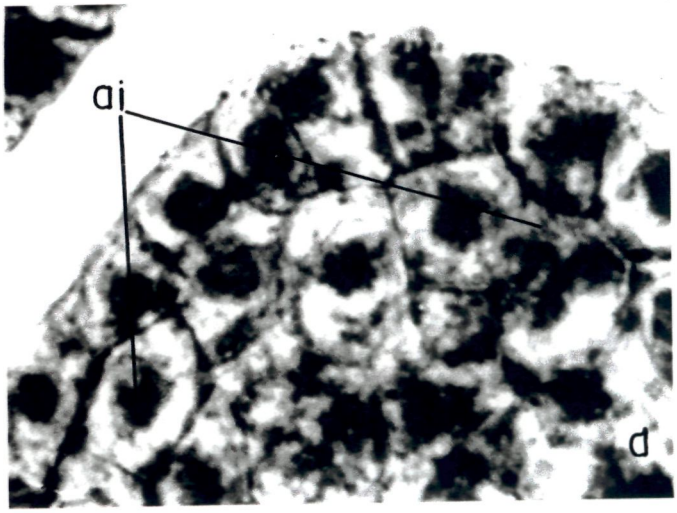


Plate - 4.4

a: T.S. of anther lobe stained with aniline blue showing the fluorescence of callose deposition. x 800.

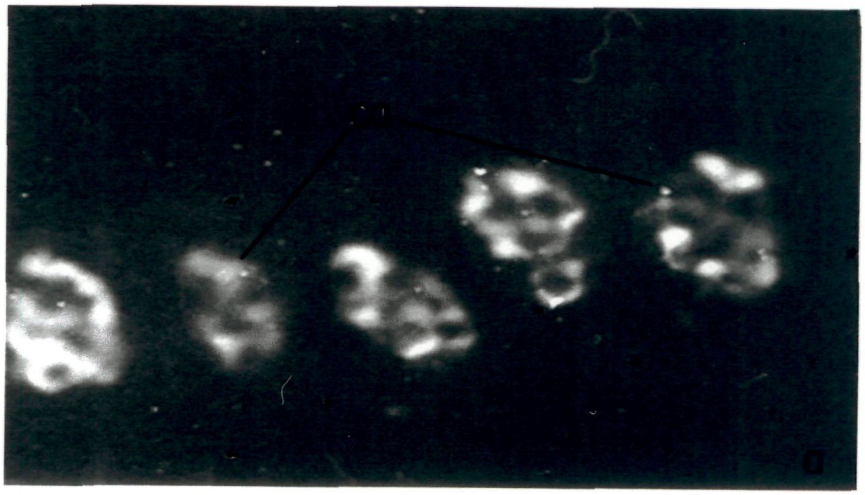
b: Simultaneous division of telophase stage of Meiosis II. x 3000.

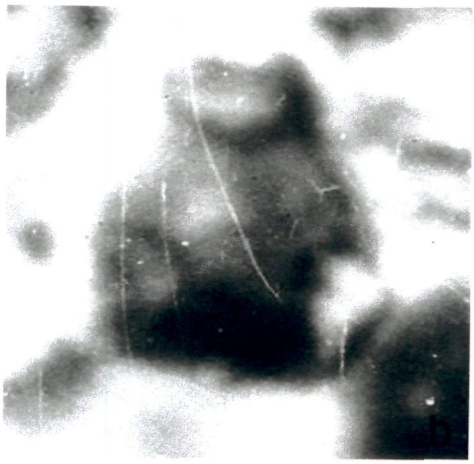
C: Showing heterotypic division of anaphase stage of meiosis II. x 3000.

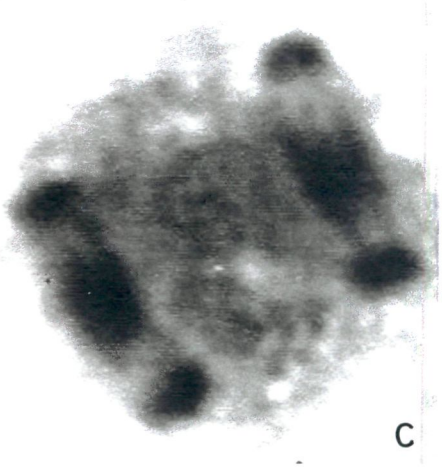
d and e: Scanning electron micrographs of pollen grains depicting the tetrahedral and decussate arrangement of pollen grains. x 4500 - 5650.

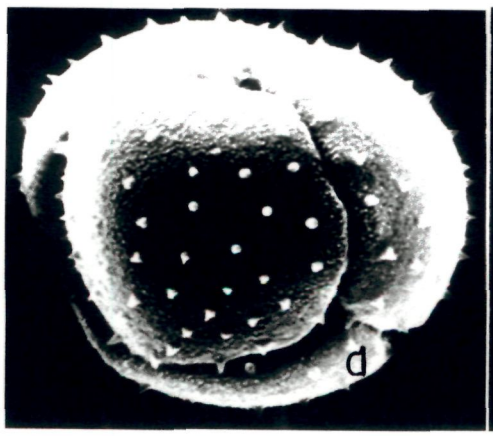
Plate - 4.4

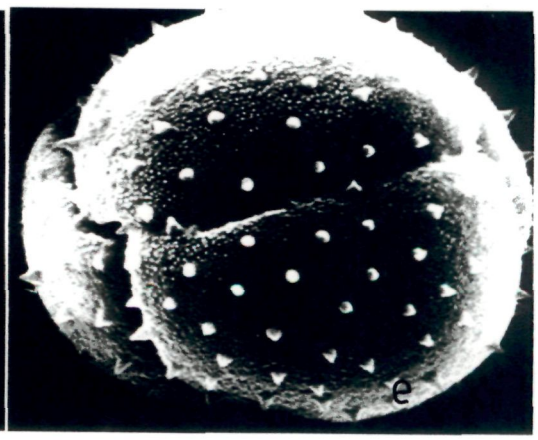
Nepenthes khasiana Hk.f.











CHAPTER - 5

Pollen viability and Pollen germination

5.1 *Introduction*

The pollen grains are extremely reduced, three or bi-celled, haploid male plant of flowering plants, have a number of specialized functions to perform. The primary functions are the production of two sperm cells and their transport within the pollen tube that travels through the style, seeks out the ovule, grows into the embryo sac, and delivers the baggage of sperms, where they participate in double fertilization. Most of our knowledge about the pollen germination is based on in vitro studies. At the time of anther dehiscence, the pollen grains of most flowering plants are bicellular: they contain a vegetative cell and a generative cell. Nearly 75 % of flowering plants studied shed pollen in bicellular stage, and 25% released pollen as tricellular grains following sperm production (Brewbaker, 1967; Raghavan, 2000). Mascarenhas (1992, 1993) studied the molecular events that take place during pollen tube growth and differentiation. The floral and fruit morphology of two species of *Nepenthes* was studied by Kaul (1982) in which he described the structure of pollen grains. Venugopal and Rashi Devi (2003) described the development of anther and microsporogenesis of

N. khasiana Hk.f. But, the pollen viability, pollen germination and tube elongation were not studied in detail in. *N. khasiana* Hk.f.

Understanding of various factors influencing pollen germination and tube growth are a prerequisite for the success of hybridization program (Vasil, 1964; Mercy et al., 1978). Viable pollen will help in giving successful result for plant breeders in breeding programme which requires huge expenditure of time as well as money. Some of the factors which influence pollen germination and tube growth in plants are carbohydrate (sugar), boron, calcium, enzymes, plant hormones, magnesium, potassium, agar, pH and certain physical factors such as temperature and light. The requirements are species specific (Johri and Vasil, 1961). So far, no pollen viability and pollen germination and tube elongation studies on *Nepenthes khasiana* are known to have been published. Since, pollen grains are the microspores which give rise to male gametes and which takes important role in fertilization, the objective of this chapter was to study the pollen viability and germination and tube elongation in *Nepenthes khasiana* Hk.f. This investigation could provide information to explore important aspects of plant reproduction.

5.2 *Results*

The percentage of viable pollen fluoresce brightly and non viable one didn't fluoresce is shown in Plate-5.2. *Nepenthes khasiana* Hk.f. shows high pollen viability percentage. The Fluorochromatic Reaction (FCR) test revealed that 89.62 % of pollen grains were viable (Plate-5.2.1; Table-5.2.4). Pollen viability should be considered different from germination, for instance, self or cross incompatible pollen, though viable may fail to germinate due to lack of certain essential factors (Goswami, 2002). It is however necessary that viable pollen should give high percentage of germination for guessing the effective fertilization (Semalty and Sharma 1996).

Significant differences in germination percentage were found among pollen grains inoculated in different media. According to Mulcahy and Mulcahy (1983), the bicellular pollen generally germinates readily in culture, but *N. khasiana* pollen began germination after keeping 10 hours in the culture medium, because the pollens of *N. khasaina* are tricellular.

Table-5.2 summarized data on the effect of temperature (15, 20, 30, 35°C), sucrose (5, 10, 15, 20%), boron (H_3BO_3 - 50, 100, 150, 200 ppm), Calcium ($Ca(NO_3)_2 . 4H_2O$ - 250, 300, 350, 400, 500 ppm), magnesium ($MgSO_4 . 7H_2O$ - 100, 150, 200, 250 ppm), potassium (KNO_3

Table - 5.2: Effect of temperature and dark treatment on pollen germination and pollen tube elongation in *Nepenthes khasiana* Hk.f.

Factor	Pollen germination (%)	Pollen tube length (μm)
Temp (° C)		
15	-	
20	-	
25	38.08 ± 1.29 *	113.2 ± 3.27*
30	-	
35	-	
Sucrose (%)		
5	88.17 ± 2.46*	234.5 ± 4.18*
10	38.08 ± 1.29	113.2 ± 3.27
15	24.18 ± 1.41	116.7 ± 424
20	-	-
LSD	5.36	11.39
Boric acid (ppm)		
50	-	
100	38.08 ± 1.29	113.20 ± 3.27
150	42.64 ± 2.11*	130.50 ± 3.03*
200	00.89 ± 0.02	41.22 ± 0.88
LSD	4.14	7.61
Calcium nitrate (ppm)		
250	1.60 ± 0.13	101.20 ± 3.83
300	38.08 ± 1.29	113.20 ± 3.27
350	39.37 ± 2.33*	129.90 ± 5.27*
400	00.51 ± 0.001	51.84 ± 1.18
LSD	3.80	10.50
Magnesium sulphate (ppm)		
100	-	-
150	-	-
200	38.08 ± 1.29*	113.2 ± 3.27*
250	-	-
Potassium nitrate (ppm)		
50	-	-
100	38.08 ± 1.29*	113.2 ± 3.27*
150	-	-
200	-	-
Agar (%)		
0.4	-	
0.6	38.08 ± 1.29*	113.2 ± 3.27*
0.8	-	
1.0	-	
pH		
5.0	-	-
6.0	-	-
7.0	00.52 ± 0.003	28.95 ± 1.21
7.1	00.54 ± 0.003	39.65 ± 0.59
7.3	38.08 ± 1.29	113.20 ± 3.27
8.0	84.04 ± 2.94*	157.40 ± 4.03*
9.0	19.5 ± 2.3	92. 23 ± 2.6
LSD	4.59	7.67

± = SE, * Significantly different from other treatments (Significance tested with one-way ANOVA).

- 50, 100, 150, 200 ppm), agar (0.4, 0.6, 0.8, 1.0%) and pH (5.0, 6.0, 7.0, 7.1, 7.3, 8.0, 9.0) incubated for 10 hours in dark in an incubator maintained at 25°C except when effect of temperature on pollen germination and tube growth was studied. The pH of the medium was adjusted to 7.3 except when effect of pH on pollen germination and tube growth was studied.

Temperature is an important factor in pollen germination and pollen tube elongation. The effect of temperature under dark treatment on pollen germination and pollen tube elongation are shown in Table-5.2. Pollen germination was not at all occurred at the temperature of 15°C, 20°C, 30°C and 35°C respectively (see Table-5.2). The pronounced germination of pollen and pollen tube elongation was observed at 25°C (see Table-5.2). Similarly the concentrations of magnesium sulphate (200 ppm), potassium nitrate (100 ppm) and the percentage of agar (0.6%) induced pollen germination and tube elongation whereas the other concentrations of above mentioned chemicals and percentage of agar did not cause any effect at all (see Table-5.2).

Out of the four different concentrations of sucrose (5, 10, 15, 20%), the 20% sucrose was not favourable. On the otherhand, 5% of sucrose enhanced the pollen germination and tube elongation. 10% and

15% of sucrose showed little effect on pollen germination and tube length.

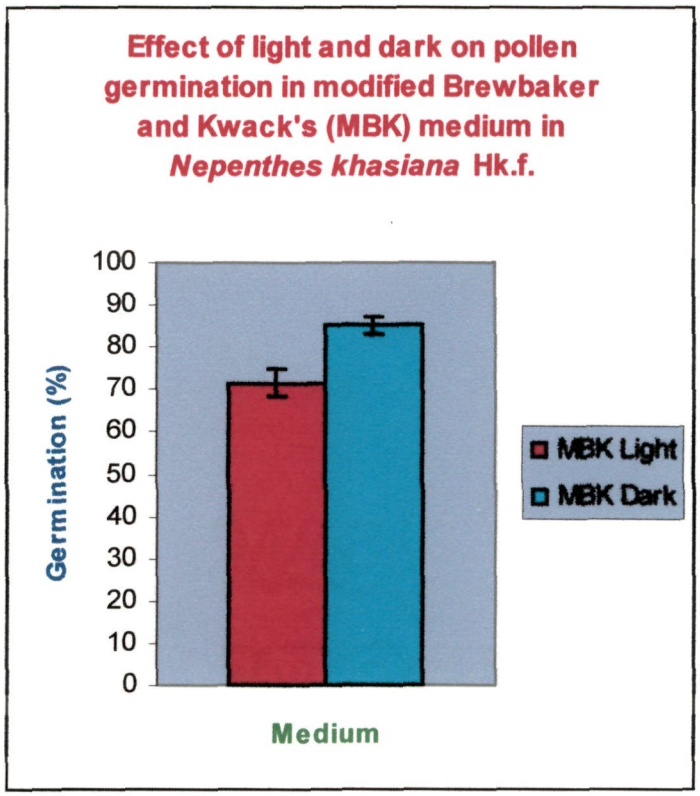
The pollen germination and tube elongation were significantly higher at 150 ppm of boron than the other concentrations. At 200 ppm of boron, the pollen germination and tube elongation were highly reduced whereas at 50 ppm, no pollen germination was found. 100 ppm of boron was favourable for pollen germination and tube elongation.

The pollen germination and tube elongation were more or less similar at 300 ppm and 350 ppm calcium but slightly higher at 350 ppm of calcium. Very less pollen germination and pollen tube elongation were observed at 250 ppm and 400 ppm of calcium respectively.

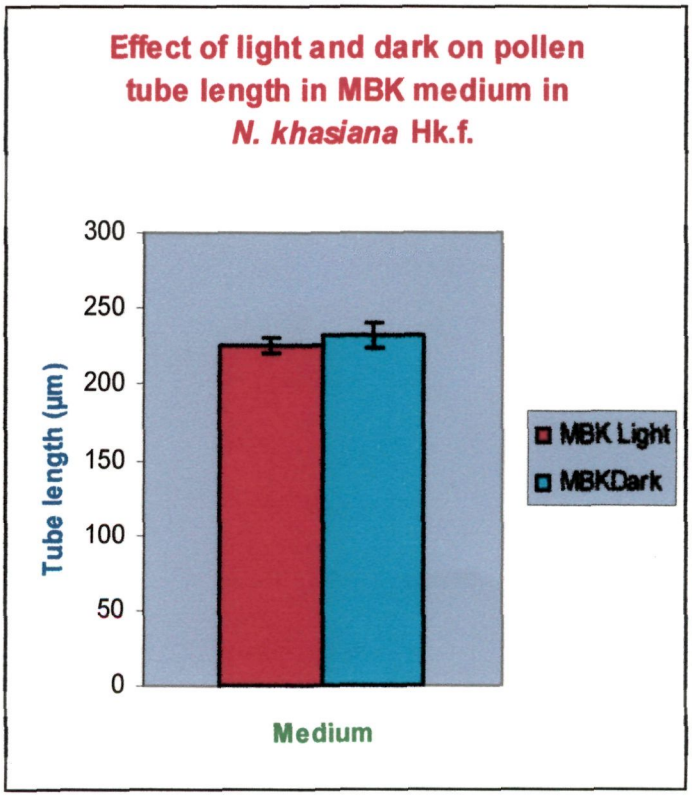
Maximum pollen germination (84.04%) and tube length (157.4 μm) were observed at pH 8.0. Less pollen germination and tube elongation were occurred at pH 9.0, 7.3, 7.1, 7.0 whereas pH 5.0 and 6.0 were not favourable for pollen germination and tube elongation.

Thus, a medium containing the above mentioned optimal concentrations (i.e. 5% sucrose, 150 ppm boron, 350 ppm calcium, 200 ppm magnesium, 100 ppm potassium and 0.6% agar) at pH 8.0, at 25°C in dark showed 85.10 percent pollen germination and 231.75 μm tube length (Plate-5.2.2a, b; Fig. 5.2a, b).

Fig. 5.2


Effect of light and dark on pollen germination in modified Brewbaker and Kwack's (MBK) medium in *Nepenthes khasiana* Hk.f.
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
MBK Light
MBK Dark
Medium

a


Effect of light and dark on pollen tube length in MBK medium in *N. khasiana* Hk.f.
Tube length (µm)
300
250
200
150
100
50
0
MBK Light
MBKDark
Medium

b

Table-5.2.1 showed data on pollen germination and tube growth at 25°C temperature with different concentrations of, sucrose, boron, calcium. magnesium, potassium and agar concentrations by incubating under 530-750 lux of light (white lamp). Subsequently pH for germination of *Nepenthes* pollen was determined by altering the pH (5.0, 6.0, 7.0, 7.1. 7.3 and 8.0) of the basal medium. Here also, pollen germination occurred at 25°C only and 5% of sucrose revealed higher pollen germination and tube elongation whereas 10% and 15% of sucrose showed less pollen germination and tube elongation. At 20% of sucrose no pollen germination was occurred.

Out of the four different concentrations of boron (50, 100, 150 and 200 ppm), 200 ppm boron inhibit the pollen germination. On the other hand, 50 ppm boron showed significantly higher pollen germination and tube elongation whereas, 100 ppm and 150 ppm of boron showed less pollen germination and tube elongation. Similarly, 350 ppm of calcium was favourable. On the other hand, less pollen germination and tube elongation were showed at 300, 250 and 400 ppm of calcium. In magnesium, potassium and agar effect, germination occurred at 200 ppm magnesium, 100 ppm of potassium, 0.6% agar only. pH 7.1 showed optimal pollen germination and tube elongation where as pollen

Table - 5.2.1: Effect of light on pollen germination and pollen tube length in *Nepenthes khasiana* Hk.f. at 25°C of temp.

Factor	Pollen germination(%)	Pollen tube length (μm)
Sucrose (%)		
5	51.08 ± 2.04*	193.00 ± 11.33*
10	22.92 ± 1.20	116.70 ± 4.24
15	2.83 ± 0.11	87.88 ± 3.97
20	-	-
LSD	4.86	21.32
Boric acid(ppm)		
50	67.69 ± 0.74*	374.2 ± 11.74*
100	22.92 ± 1.20	116.7 ± 4.24
150	4.47 ± 0.16	104.4 ± 6.79
200	-	
LSD	2.38	23.80
Calcium nitrate(ppm)		
250	2.70 ± 0.05	103.62 ± 5.65
300	22.92 ± 1.20	116.70 ± 4.24
350	30.99 ± 1.60*	129.43 ± 6.41*
400	00.44 ± 0.007	33.35 ± 2.08
LSD	2.79	13.95
Magnesium sulphate(ppm)		
100	-	-
150	-	-
200	22.92 ± 1.20*	116.7 ± 4.24*
250	-	-
Potassium nitrate(ppm)		
50	-	-
100	22.92 ± 1.20*	116.7 ± 4.24*
150	-	-
200	-	-
Agar(%)		
0.4	-	-
0.6	22.92 ± 1.20*	116.7 ± 4.24*
0.8	-	-
1.0	-	-
pH		
5.0	00.36 ± 0.002	28.84 ± 1.18
6.0	26.26 ± 1.22	74.20 ± 3.45
7.0	40.39 ± 2.01	120.76 ± 3.45
7.1	74.84 ± 2.77*	224.10 ± 4.59*
7.3	22.92 ± 4.24	116.70 ± 4.24
8.0	12.41 ± 4.24	114.33 ± 11.19
LSD	4.81	15.64

± =SE, * Significantly different from other treatments (Significance tested with one-way ANOVA).

germination and tube elongation were less at pH 7.0, 6.0, 7.3, and 8.0 respectively and least at pH 5.0.

It was observed that *N. khasiana* Hk.f. pollen showed 71.50% of pollen germination and 225.44 μm tube growth under light condition in the medium containing the above mentioned optimal concentrations (i.e. 5% sucrose, 50 ppm boron, 350 ppm calcium, 200 ppm magnesium, 100 ppm potassium and 0.6% agar) at pH 7.1 at 25°C (Plate-5.2.2c, d; Fig. 5.2a, b; Table-5.2.2). By comparing the results of the two media kept in dark and light conditions, pollen showed significantly higher germination in dark (see Plate-5.2.2a, b, c, d; Fig. 5.2a, b; Table-5.2.2). But, there is not much difference in tube growth (Fig. 5.2b; Table-5.2.2). Pollen tube length is slightly higher in dark. Therefore, the concentrations of 5% sucrose, 150 ppm boron, 350 ppm calcium, 200 ppm magnesium, 100 ppm potassium and 0.6% agar at 8.0 pH at 25°C in dark represent the requirements for optimal pollen germination and tube growth in *Nepenthes khasiana* .

The Brewbaker and Kwack's (1963) medium was modified by incorporating the optimal requirements of *Nepenthes khasiana* Hk.f. pollen enumerated above. Comparision of pollen germination and tube growth in Brewbaker and Kwack's medium (1963) and the modified medium revealed that germination and tube growth were significantly

Table - 5.2.2: Effect of light and dark on pollen germination and pollen tube length in modified Brewbaker and Kwack's (MBK) medium in *Nepenthes khasiana* Hk. f.

Medium	Pollen germination (%)	Pollen tube length (μm)
MBK Light	71.50 ± 3.27	225.44 ± 4.44
MBK Dark	85.10 ± 2.17*	231.75 ± 7.88

± = SE, * Significantly different from MBK Light medium. Significance tested with 't' test (p=0.05).

Table - 5.2.3: Comparision of pollen germination and pollen tube length in Brewbaker and Kwack's (BK) and modified Brewbaker and Kwack's (MBK) media in *N. khasiana* Hk.f.

Medium	Pollen germination (%)	Pollen tube (μm)
BK	38.08 ± 1.29	113.20 ± 3.27
MBK	85.10 ± 2.17***	231.75 ± 7.88***

*** Highly significant from BK medium at $p<0.001$ level.

Table - 5.2.4: Pollen viability, pollen germination and pollen tube length in *N. khasiana* Hk.f.

Pollen viability (%)	Pollen germination (%)	Pollen tube length (μm)
89.62 ± 1.98	85.10 ± 2.17	231.75 ± 7.88

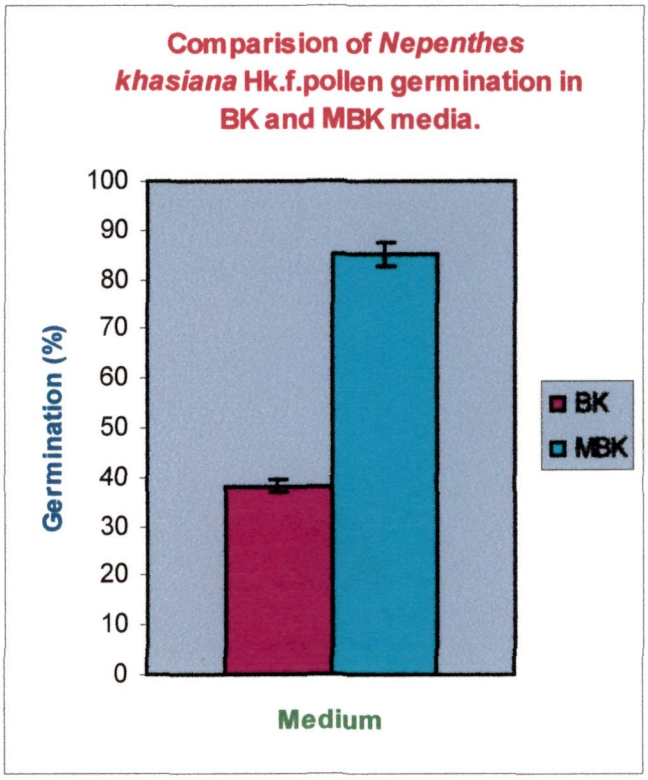
higher in the modified medium (Plate-5.2.3; Fig. 5.2.1; Table-5.2.3). In the modified medium the germination and tube length were 85.10 percent and 231.75 μm respectively (see Table-5.2.3). Obviously, therefore very high degree of pollen fertility could be reported in *Nepenthes khasiana*.

5.3 *Discussion*

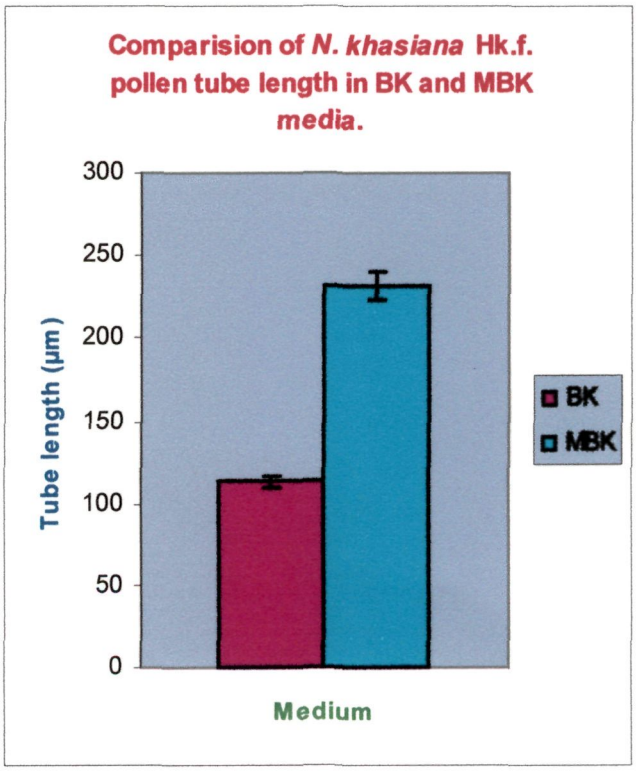
Nepenthes khasiana Hk.f. shows high pollen viability (89.62%). In all the angiosperm species the pollen viability decreases with time (Pacini et al., 1997). Binucleate pollen generally survives longer than trinucleate and germinates readily in vitro (Stanley and Linskens, 1974). Entomophilous species also generally have longer pollen viability (Pacini et al., 1997). But, in *Nepenthes khasiana* the pollens seem remain viable for only a short period. This suggests that the pollen grains are in a highly active metabolic state and, as a result, the biochemical changes probably occur over time.

Pollen germination can be substantially altered by the temperature. Pollen of most plants show optimum germination and tube growth between 20 - 30°C (Johri and Vasil, 1961). In the case of *Nepenthes khasiana*, pollen grains germinated only at 25°C. The sucrose concentration required for optimal germination varies from species to

Fig. 5.2.1


Comparision of Nepenthes khasiana Hk.f.pollen germination in BK and MBK media.
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
Medium
BK
MBK

a


Comparision of N. khasiana Hk.f. pollen tube length in BK and MBK media.
Tube length (µm)
300
250
200
150
100
50
0
Medium
BK
MBK

b

species e.g., *Solanum aviculare*, *S. laciniatum*, *S. sisymbrifolium* require 10% sucrose; *Corylus avellata* require 10 to 25% sucrose (Kim et al., 1985). In *Nepenthes khasiana*, 5% sucrose enhances pollen germination and tube elongation (see Table-5.2 and 5.2.1).

The pollen germinability is influenced by flower age, drying time and pollen source (Randolf and Robert, 1986). No pollen germination and tube growth were occurred in the stored pollens in *N. khasiana* and collected few hours after anthesis. However, in *Asclepias exaltata*, flower age has little effect on pollen germinability (Shannon and Wyatt, 1986). Fresh pollen grains collected during the anthesis gave optimal pollen germination and tube elongation in *Nepenthes khasiana*. Pollen grains must be among the shortest-lived independent bodies in nature, for there are few which can remain alive for more than a few days after they have been shed, while some can live for only a few hours (Echlin, 1968; Sporne, 1974). Pollen grains germinate in a wide range of pH but the best results are obtained at pH 5.5-6.5 (Johri and Vasil, 1961). But, in *Nepenthes khasiana* optimal germination and tube elongation were seen at pH 8.0 under dark.

Lights of different colour influenced pollen germination and tube elongation in *Schima wallichii* (Chauhan and Katiyar, 1996). Similarly, in *Nepenthes khasiana* also dark had significant effect on pollen

germination. However, there was not much difference in tube elongation as compared with the light treatment.

As such the pollen viability and germination percentage were high, which result into a high degree of pollen fertility in *N. khasiana*. Therefore, one could expect more viable seeds after fertilization. Usually, high fruit set is also reported in *Nepenthes gracilis*. (Holttam, 1954; Makoto Kato, 1993). Probably the maximum fruit set in these two species may be due to the higher percentage of pollen viability and pollen germination. In *N. khasiana* also a high rate of seed viability has been observed.

Plate – 5.2

Nepenthes khasiana Hk.f.

POLLEN VIABILITY

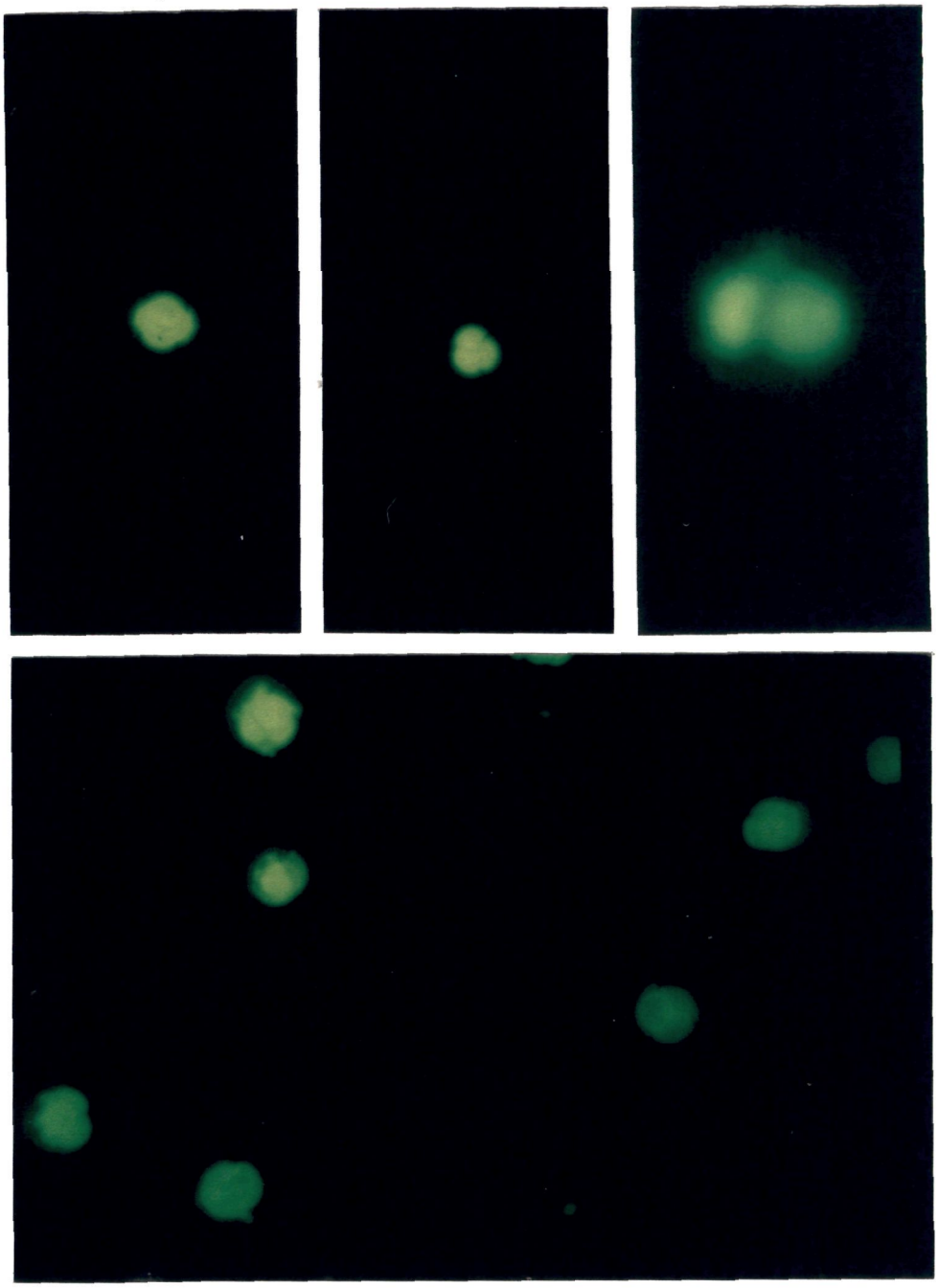


Plate - 5.2.1

All photographs portraying the pollen viability with Flurochromatic Reaction Test (FCR). Note the differential fluorescence within the tetrads. x 150 - 750.

Plate–5.2.1

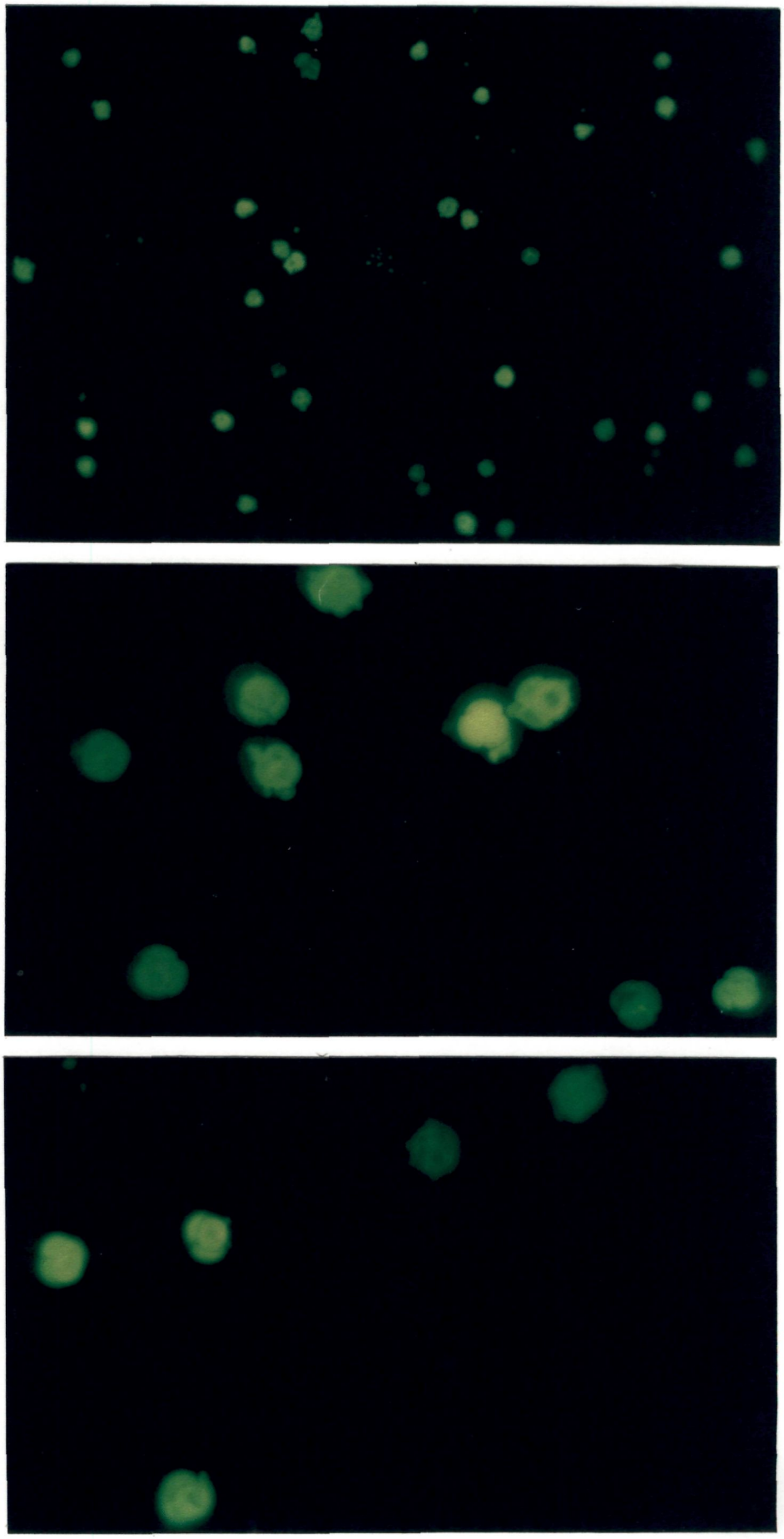


Plate - 5.2.2.

a: **Germination of pollen grains in Modified Brewbaker and Kwack's (MBK) medium in Dark. 10 x 4X**

b: **Germination of pollen grains in Modified Brewbaker and Kwack's medium in Dark. 10 x 40X.**

C: **Germination of pollen grains in Modified Brewbaker and Kwack's medium in Light. 10 x 4X.**

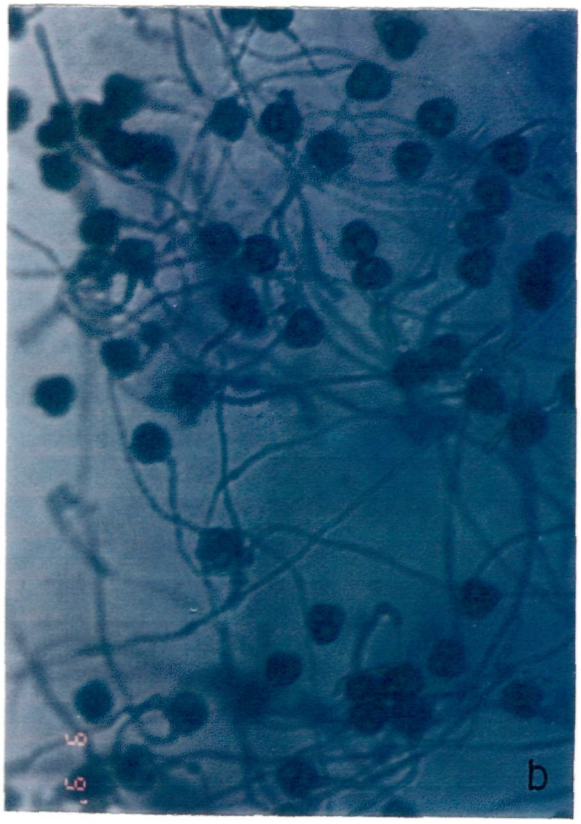
d: **Germination of pollen grains in Modified Brewbaker and Kwack's medium in Light. 10 x 40X.**

Plate – 5.2.2

Nepenthes khasiana Hk.f.

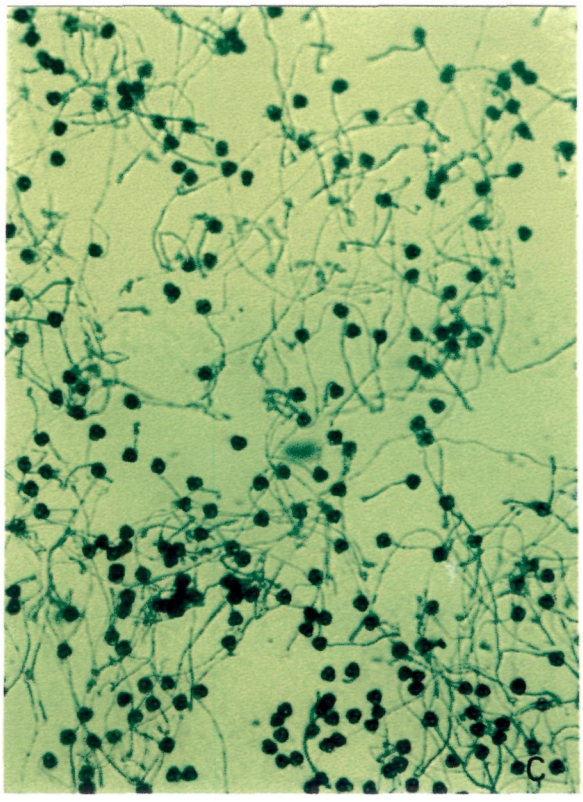
Pollen Germination in MBK Medium
(Dark)

10 x 4X

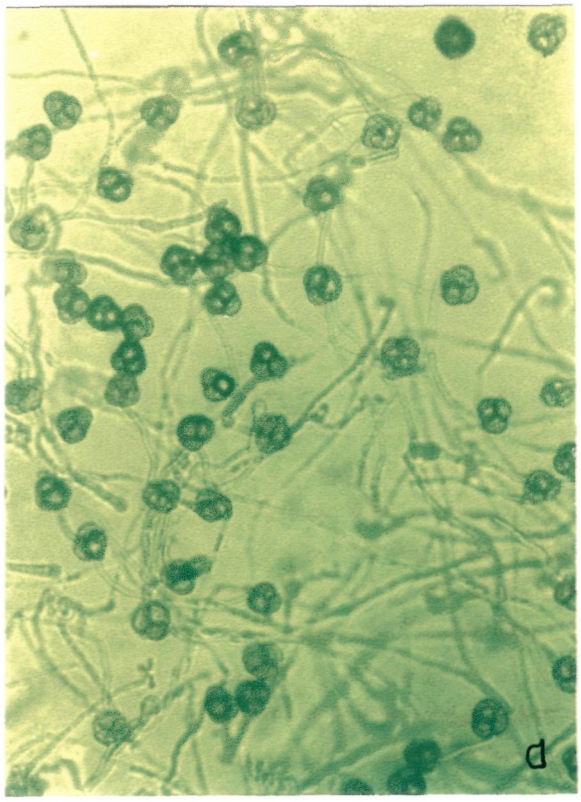


10 x 40X

(Light)



10 x 4X



10 x 40X

Plate - 5.2.3.

a: **Germination of pollen grains in Brewbaker and Kwack's (BK) medium. 10 x 4X.**

b: **Germination of pollen grains in Brewbaker and Kwack's (BK) medium. 10 x 40X.**

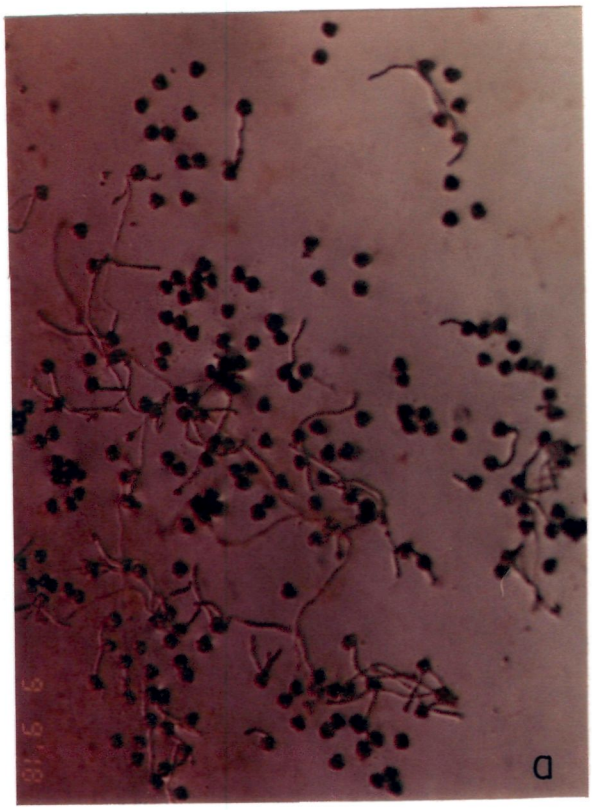
C: **Germination of pollen grains in Modified Brewbaker and Kwack's (MBK) medium. 10 x 4X.**

d: **Germination of pollen grains in Modified Brewbaker and Kwack's (MBK) medium. 10 x 40X.**

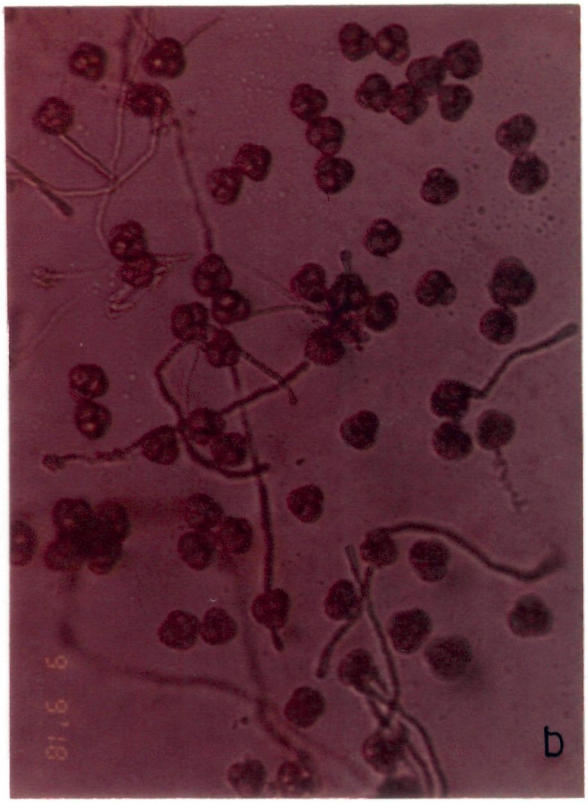
Plate–5.2.3

Nepenthes khasiana Hk.f.

Pollen Germination
in BK Medium

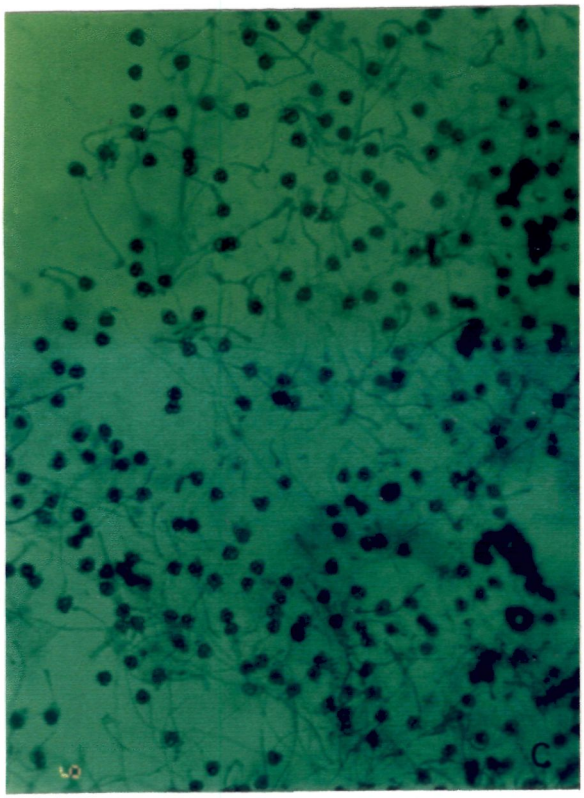


10 x 4X

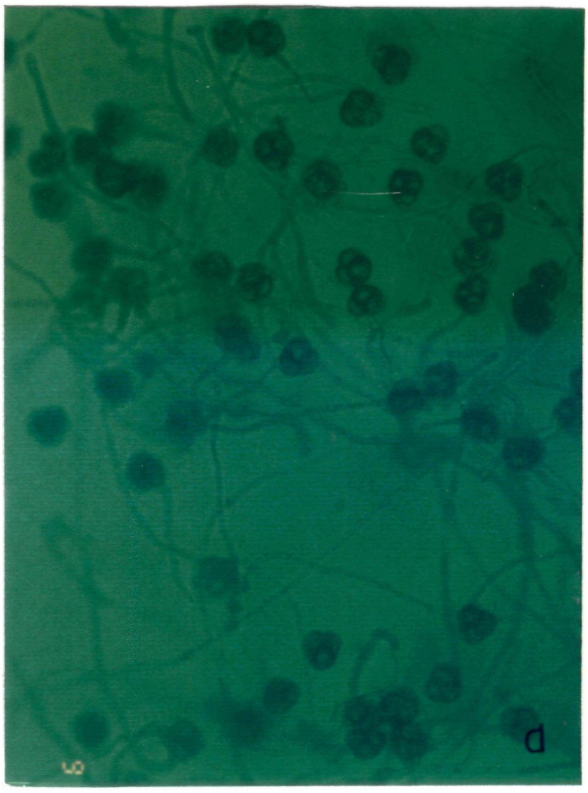


10 x 40X

in MBK Medium



10 x 4X



10 x 40X

CHAPTER - 6

Female Flower

6.1 *Introduction*

Out of approximately 72 species (Willis, 1966) in the world, the only representative of the Family Nepenthaceae in India is *Nepenthes khasiana* Hk.f. Numerous studies have been published on glandular structure and functions in the pitcher (Lloyd, 1942; Luttge, 1971; Toekes et al., 1974; Adams and Smith, 1977; Fahn, 1979; Heslop-Harrison, 1976; Frazier, 2000). As far as Reproductive Biology of the genus *Nepenthes* is concerned, only limited studies were conducted. Lan and Prakash (1993) studied the life history of *Nepenthes gracilis*. Kaul (1982) reported the floral and fruit morphology of *N. lowii* and *N. villosa* from Borneo (Indonesia). Subramaniam et al. (1985) also insisted that more detailed studies are necessary *in N. khasiana.* According to Johri et al. (1992) the reproductive biology of *N. khasiana* has not been studied thoroughly. Therefore, this chapter deals with the initiation of ovule to the embryo formation.

6.2 *The Structure of female flower*

The female flowers are on racemose inflorescences (Plate-6.2a). Female flower shape and size are similar to that of male flowers, epithelium nectaries are also present (Plate-6.2d); gynoecium tetracarpelary, syncarpous, size of the pistil 5-8 mm; ovary superior, tomentose (Plate-6.2.1a). Stigma is almost sessile, 2 mm-3 mm in diameter, discoid, four lobed, dry type, papillate (Plates-6.2c; 6.2.1a). Placentation parietal with many rows of ovules (fusing placentae lead to the appearance of axile). Ovules numerous, more than 100 per locule in longitudinal series, attached to the placenta, ascending, non- arillate, anatropous, bitegmic (Plate-6.2.1b). Fruit is non-fleshy (leathery), dehiscent, elongated capsule. Capsules 1.5-3 cm. long, ellipsoid- oblong, loculicidally four valved, valves persistent, 200-400 seeded. Seeds filiform, spindle shaped, winged, numerous, minute, 0.5-0.7 cm. long (Plate-6.2.1c). Seeds are endospermic when young, seeds with starch. Embryo well differentiated (small), axile, straight cotyledons two, linear, plano convex; radicle is short inferior.

Flowering and fruiting time is from June to December. Seed germination is phanerocotylar.

6.3 *Ovule, embryo sac formation and fertilization*

The ovule is anatropous, bitegmic and crassinucellate (see Plate-6.2.1d). The ovule is initiated on the placenta as a small dumb shaped protuberence and its histogenic organization in similar to that of tunica-corpus concept of shoot apex which grows in height be repeated cell division and subsequent cell enlargement. The Nucellus is demarked from funicullus at the time of the initiation of inner integument (Plate-6.3a). As the Nucellus is attained sub-globular shape, the female archesporial cell is clearly differentiated and distinguished in the third layer of the nucellus from the apex when the growth of the inner integument reaches almost towards the apex (Plate-6.3b). The archesporial cell is enlarged radially and it stains heterochromatically (Plate-6.3c, d). Both the chalazal end and the raphal side exhibit pronounced downward and upward growth as a result of which the mature ovule is very much elongated. The funicular vascular trace ends bluntly at the base of hypostase.

The nucellar cells are densely cytoplasmic with distinct nuclei. In the mature ovule distinct hypostase is present in which cell walls are highly thickened (see Plate-6.2e). The inner integument grows much faster than the outer integument. The deeply seated female archesporial cell functions as megaspore mother cell which directly undergoes

meiosis. The first heterotypic meiotic division results into dyad cells (Plate-6.3.1a); the second homoetypic (meiosis II) leads into two types of configuration: (1) Lenear tetrad (Plate-6.3.1b); (2) 'T' shaped tetrad (Plate-6.3.1c). The functional megaspore mother cell is micropylar in the linear configuration (Plate-6.3.1d) while in the 'T' shaped tetrad the functional megaspore is the chalazal one (Plate-6.3.2a, b). Since the female archesporial cell functions directly as megaspore mother cell the formation of parietal layer is completely absent.

The functional megaspore is radially elongated with dense cytoplasm with prominent nucleus and nucleolus (see Plate-6.3b, c, d; 6.3.1d; 6.3.2b). The megaspore nucleus of the linear tetrad undergoes three successive free nuclear divisions to form eight nucleate embryo sac normal or polygonum type (Plate-6.3.3a, b, c, d). The egg apparatus consists of two synergids and an egg cell, the synergids show vacuole at the chalazal pole and the filiform apparatus at the micropylar pole where as in the egg cell the vacuole is at the micropylar pole and the egg nucleus towards the chalazal end. The two synergids are apart from each other at the micropylar side (see Plate-6.3.3a). The filiform apparatus is sub-globose in shape. In the egg cell of the egg apparatus, the nucleus is at the micropylar end while in the chalazal end vacuole is located. The three antipodals are densely stained whereas the central cell contains two

polar nuclei. During the elaboration of embryo sac, the nucellular cells start to disintegrate. There are three antipodals which are darkly stained and show the sign of disappearance (see Plate-6.3.3a, b).

In the tetrasporic type of embryo sac formation, the archesporial initial directly functions as meiocyte. The meiocyte is comparatively larger in size and radially elongated with dense cytoplasm and prominent nucleus (Plate-6.3.4a, b). It undergoes meiosis I and II without cell plate formation (Plate-6.3.4c), as a result of which four nucleate Coenomegaspore is formed (Plate-6.3.4d). Out of four nuclei two are located at the micropyle and the remaining two at the chalazal pole. The four nucleate Coenomegaspore undergoes two successive mitotic divisions to form 16 nucleate stage. The 16 nuclei are arranged in the following manner (Plate-6.3.5).

(1). In the micropylar end 3 celled egg apparatus consisting of a single egg cell and two synergids. The egg cell shows distinct polarity in it, the nucleus and the vacuole are located chalazal to micropylar pole respectively.

(2). The antipodals consist of six cells lie together at the chalazal end of the embryo sac.

(3). In the central cell at base of the egg apparatus the remaining six nuclei fuse to form the secondary nucleus of hexaploid in nature.

The pollen tube enters into one of the synergids. After releasing of male gametes the two synergids are disappeared. The fusion of one of the male gamete with female gamete is of intermediate type (Plate-6.3.5a, b, c). The fusion product forms the zygote. The male gamete fuses with the secondary nucleus to form primary endosperm nucleus (see Plate-6.3.5d).

6.4 *Zygote*

The zygote is somewhat ovoidal in shape and the wall formation is completed at the chalazal portion of the zygote. The zygote increases in volume after fertilization. The zygotic cell exhibits a pronounced polarity of dispersion nucleus and most of the cell organelles towards the chalazal pole whereas the vacuole at the micropylar pole (Plate-6.4a, b).

6.5 *Embryogenesis*

The first division of the zygote is transverse and the micropylar cell (the basal cell, designated as 'cb') is considerably larger than the apical cell (conventionally designated as 'ca') which is directed towards the chalazal end (see Plate-6.4c). The basal cell 'cb' undergoes only one transverse division to form the two celled suspensor, while in the 'ca' subsequent few divisions take place in the transverse plane resulting in a

filament of eight cells. It should be noted that the shape of the terminal cell is a hemispherical and that of the subterminal cells, a squat cylinder (Plate-6.5a, b, c).

The increase in volume of the filamentous proembryo is initiated by the vertical division in the terminal cell. Another set of walls in the same plane, but at right angles to the previous wall formation is occurred in the daughter cell as a result of which the four derivative cells are aligned in a single tier. Similar type wall formation occurs in a few of the sub-terminal cells of the filament.

The further development of embryo from the octant stage (Plate-6.5.1a, b, c, d) following numerous cell divisions in various planes causes the proembryo to assure a globular configuration (Plates-6.5d; 6.5.2a, b). The globular embryo passes through a phase before cotyledons (pco) and epicotyle (pvt) become outwardly evident at specified loci and there is a transition from radial symmetry to bilateral symmetry. The embryo is now heart shaped which is the characteristic feature of the dicotyledons.

6.6 *Endosperm*

The endosperm is nuclear type. The two polar nuclei are distinct which are attached with each other located in the centre of the embryo

sac (Plate-6.6a, b). At the time of fertilization it moves towards the micropylar pole just below the egg apparatus. The fusion product of two polar nuclei and are of the sperm constitute the primary endosperm nucleus which is triploid in nature (3n) (Plate-6.6c, d). It remains at the place of formation i.e. micropylar pole. The three nuclei of the primary endosperm nucleus are distinct and the primary endosperm suspended in a thin film of cytoplasmic strand (Plate-6.6d). The primary endosperm nucleus undergoes several mitotic divisions as a result of which several nuclei all of which are suspended in a cytoplasmic strand around the central vacuole (Plate-6.6a, b). Initially the endosperm is nuclear later on cellularization takes place to form the cellular endosperm with a distinct chalazal haustorium (Plate-6.6.1d, e). The endosperm is very small when compare to the seed size and its role for the nutrition of embryo is very little because it is completely absorbed by the developing embryo in the early stage i.e. at the time when the embryo reaches globular stage. Further development of the embryo depends upon the store reserve products in the inner integument (tegmen).

6.7 *Discussion*

In Nepenthaceae the development of Crassinucellate ovule, initiation of ovule primordium and inner integument is similar to that of

other angiospermous families. But the initiation and derivation of inner integument is 5-6 cells below the apex of the ovular primordium. Thus, the initiation of inner integument demarcates the funiculus and the nucellus. The development of both the integuments confirms the observations of Bouman (1984). The outer integument develops later when the inner integument directly covers three fourth of the nucellar tissue in *Nepenthes khasiana*. But, the micropyle is formed by the inner integument alone.

The archesporial initial is always single and it originates from the third layer of the nucellus but not hypodermal. I did not come across any multicellular archesporium, as reported by Subramanyam et al. (1985) in *N. khasiana*. But the present study confirms the observations of Kaul (1982) and Subramanyam et al. (1985) with regard to the chalazal end of the ovule exhibits pronounced growth (see Plate-6.2.1d). Davis (1966) reported the formation of a single parietal layer in Nepenthaceae and in *Drosera burmanni* (Narasimhachar, 1949; Patanapar, 1976). Kuhl (1933) and Kaul (1982) also reported the derivation of parietal cell and megaspore mother cell from the archesporial initial. But in *N. khasiana* parietal tissue is absent. The archesporial initial directly functions as megaspore mother cell which is similar to that of Sarraceniaceae

(Shreve, 1906), *Drosera rotumdifolia*, *D. indica* and *D. peltata* (Venkatasuban, 1950; Narasimhachar, 1951).

The megasporemother cell or meiocytes undergoes meiosis I and II result into 'linear' and 'T' shaped tetrads. In the former the micropylar megaspore is functional while in the later the functional megaspore is chalazal one. The 'T' shaped tetrad has also been reported in *Aldrovanda vesiculata* (Batygina and Yakovlev, 1985). Subramanyam et al. (1985) reported linear tetrad only in the same plant *N. khasiana*.

The embryo sac formation is polygonum type, eight nucleate in *N. khasiana*. Similar feature has been reported in Sarraceniaceae and *Droseraceae* (see Johri et al., 1992).

Apart from the Normal type or Polygonum type of female gametophyte development, an interesting feature which has also been observed in *N. khasiana*, is the formation of the tetrasporic embryo sac development which has not been reported so far in the order Sarraceniales. More than one type of embryo sac formation has also been reported in members of Ranunculaceae (Vijayaraghavan, 1970). Kapil and Prakash (1966) recorded five different types (Polygonum, Endymion, Drusa, Penaea and Adoxa) of development in *Delosperma cooperi* (Aizoaceae). Dharamadhaj and Prakash (1978) observed twin embryo sacs, one monosporic and the other bisporic, in the same

nucellus of an ovule of *Capsicum annum* var. *acuminatum*. According to Swamy and Krishnamurthy (1975), Supra - Homeotypic (Polygonum type) category and Homeotypic category II (tetrasporic) have been observed in *N. khasiana*.

In majority of the angiosperm the three cells of the egg apparatus share the common wall surface, to form triangular arrangement (Willemse and Van Went, 1984; Raghavan, 2000). Therefore in *N. khasiana* the two synergids are distinct and their filiform apparatus are oval in shape at the micropylar end. The egg is slightly projecting beyond the two synergids. The egg cell and two synergids are highly vacuolated and strongly polarized cells in *N. khasiana* (see Schulz and Jensen, 1968 in *Gossypium*; Mansfield et al., 1991 in *Arabidopsis*).

As mentioned earlier, the polygonum type of embryo sac originates from the monosporic development while the tetrasporic coenomegaspore gives rise to the Drusa type of embryo sac.

Nawaschin and Guignard (1898) described independently double fertilization in angiogperm. Out of the three types of karyogamy during fertilization (Gerassimova - Navashina, 1960), intermediate type has been observed in *N. khasiana*. The zygote show distinct polarity in its distribution of nucleus and organelles at the chalazal pole. The structure of zygote in *N. khasiana* is similar to that of *Gossypium*, *Capsella*

(Jensen, 1964, 1968; Schulz and Jensen, 1968; Cocucci and Jensen, 1969). The cytoplasm, nucleus and nucleolus are deeply stained with biological dyes. This feature indicates the zygote is the site of very active metabolism which includes synthesis of m RNA and protein in *N. khasiana*. After fertilization there is distinct cell wall around the zygote. Jensen (1968) in Cotton, Mogensen and Suthan (1979) in *Nicotiana* and Mogensen (1972) in *Quercus* reported the formation of complete PAS- positive cell wall around the zygote after fertilization. The zygote of *N. khasiana* slightly enlarges in size after fertilization. This enlargement is probably due to the large vacuole occupies at the micropylar end of the zygote.

As reported by Natesh and Rau (1984) after the first division of the zygote the differential distribution of cytoplasmic organelles and the location of nucleus, the further course of embryogenesis is already foreshadowed in the zygote itself. In *N. khasiana* also in the two celled proembryo the apical cell 'ca' has most of the cytoplasm and prominent nucleus than the basal cell 'cb'.

Another interesting feature has been observed in *N. khasiana* in the course of embryogenesis in which the embryo development is Caryophyllad type irrespective of its embryo sac organization. The Caryophyllad type has been reported in Droseraceae (see Crete, 1963;

Natesh and Rau, 1984). But, Subramanyam et al. (1985) reported Solanad type of embryo in the *N khasiana.*

In *N. khasiana* the endosperm development is of the nuclear type at the beginning. The two polar nuclei are attached with each other at the time of triple fusion. The product of triple fusion result into triploid primary endosperm nucleus. The three nucleoli condition is distinct in the primary endosperm nucleus as well as its derivative nuclei. A prominent chalazal haustorium draws nutrition for the developing embryo. Though the role of endosperm for the development of embryo is limited, its supply of nutrition during the early embryo development is significant. Also, a similar type of endosperm and haustorium was described by Batygina and Yakovlev (1985) in *Aldrovanda vesiculata* and *Dionaea muscipula.* However, in Sarraceniaceae the endosperm is cellular type (Shreve, 1906). Subramanyam et al. (1985) did not report the presence of endospermic haustorium in *N. khasiana.* Kaul (1982) did not observe endosperm in *N. lowii* and *N. villosa*, because his observation was based upon the nature seeds of these two species. In *N. villosa* and *N. lowii.* The endosperm was present in the younger stage of seed development which was totally consumed by the developing embryo.

Taxonomic consideration:

Johri et al. (1992) pointed out that scanty data are available on the embryology of Nepenthaceae. Based on the present investigations the following conclusion has been arrived:

Bentham and Hooker (1862-1893) placed Nepenthaceae under Monochlamydae along with Aristolochiaceae and Cytinaceae.

Engler and Diels (1936) kept the family Nepenthaceae under the order Sarraceniales which includes Sarraceniaceae, Nepenthaceae and Droseraceae.

Dahlgren (1981) erects three order: Sarraceniales (Sarraceniaceae); Theales (Nepenthaceae); Droserals (Droseraceae). Table-6 summarizes the embryological data of the order Sarraceniales as well as its allied families. It is evident from the table that the families Nepenthaceae and Droseraceae share the maximum correlation with respect to the tetrad pollen grains, crassinucellate ovule, absence of endothelium, nuclear endosperm and Caryophyllad type of embryogeny. Therefore, the families Nepenthaceae and Droseraceae would be placed together under the separate order Nepenthales in the subclass Dilleniidae as proposed by Cronquist (1988) and the family Sarraceniaceae as the sole family in the order Sarraceniales (as per Takhtajan, 1980).

Plate - 6.2

a: A female plant with racemose inflorescences growing along with *Osbeckia* sp. (Reduced into ¼ of the natural size).

b: An enlarged view of female inflorescence showing the greenish ovaries and red coloured tepals . (Reduced into ½ of the natural size).

C: An enlarged view of female flower. Note the indumentums throughout the ovary, pedicel and lower surfaces of tepals. The stigmatic flaps are recurreved. x 10.

d: Another enlarged view of dorsal surface of a single tepal, displaying the embedded nectariferous glands. x 20.

Nepenthes khasiana Hk.f.

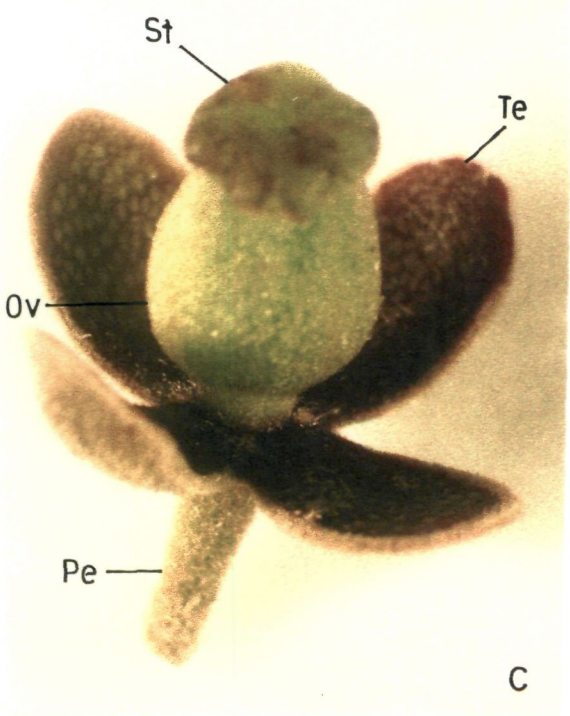
Female Inflorescence

a

An Enlarged View
Of Female Inflorescence

b

Female Flower



c

An Enlarged View of a Tepal
Showing Nectary Glands

d

Plate - 6.2.1

a, b and C: Scanning Electron micrographs of Ovary, L.S. of Ovary and a single ovule showing the type of hairs, arrangement of ovules on placenta and outer surface of the ovule respectively.

st.l - Stigmatic lobes; Pl - placenta; Ov - ovules; Mi - micropyle; Ra - Raphe and Ch - Chalaza.

a = x 3; b = x 40 and C = x 250.

d : L.S. of ovary exhibiting the bitegmic, anatropous (Ao) and crassinucellate condition. x 90.

e: Cleared ovule with lactic acid displaying the micropyle (Mi); Vasculature (Va); Hypostase (Hy) and Chalaza (Ch). x 200.

Plate – 6.2.1

Nepenthes khasiana Hk.f.

STRUCTURE OF GYNOECIUM AND OVULES

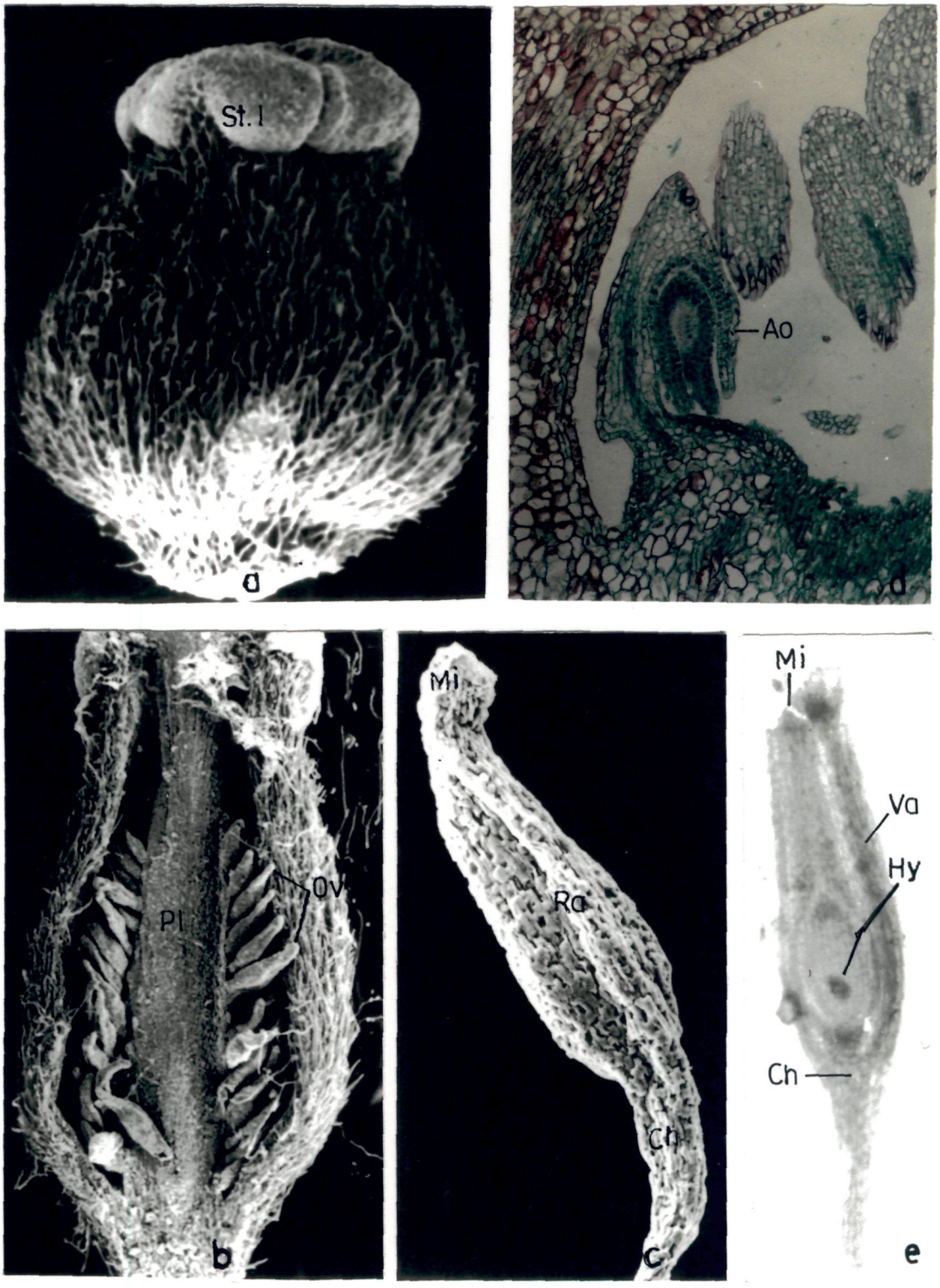
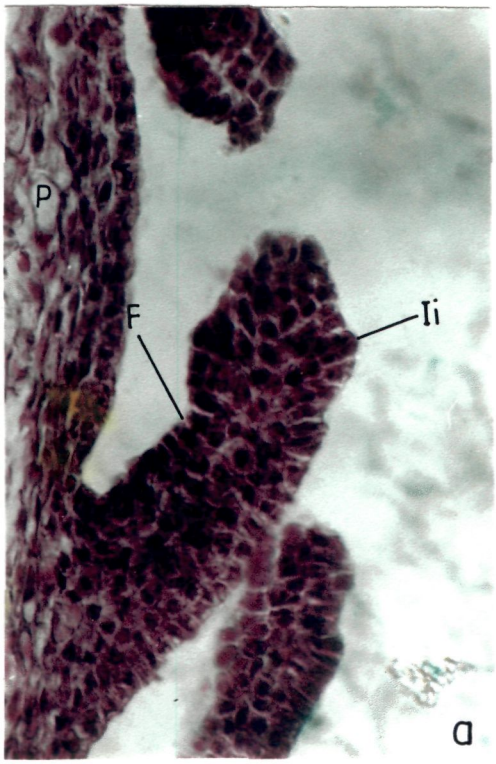


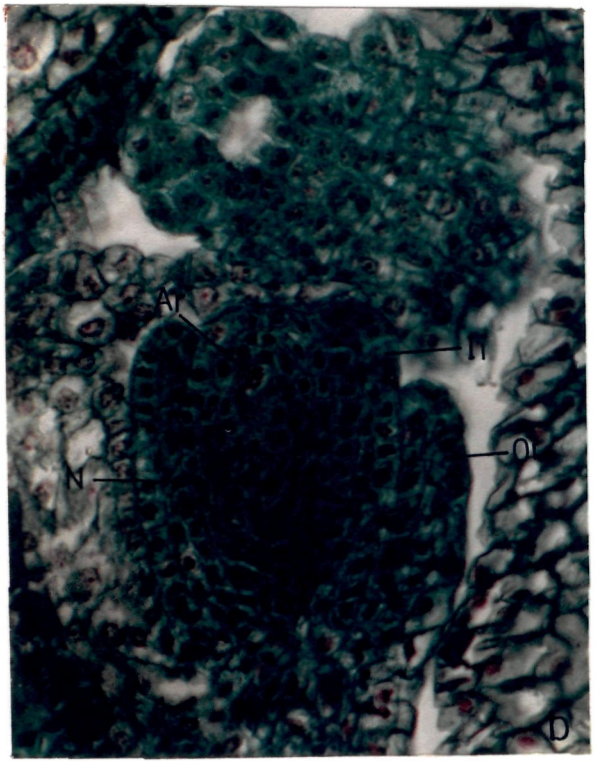
Plate – 6.3

MEGASPOROGENESIS

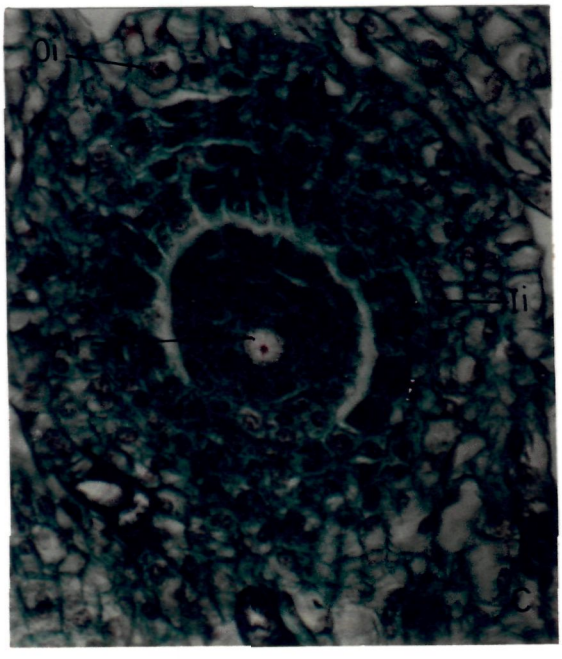
Ovule initiation



Archesporial Cell and Development of Outer Integument



T.S. of Ovule with Archesporial Cell



Enlargement of Archesporial Cell before Meiosis

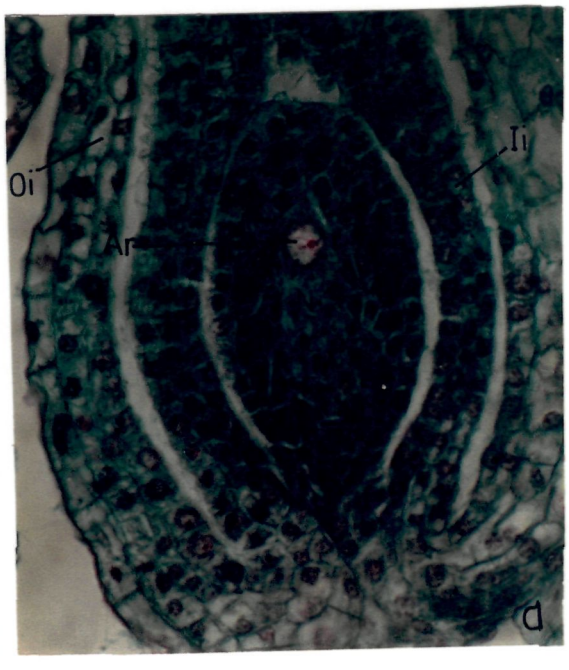


Plate - 6.3.1

L.S. of Ovule

a: Dyad stage after meiosis - I.

b: Linear tetrad configuration

C: 'T' shaped tetrad

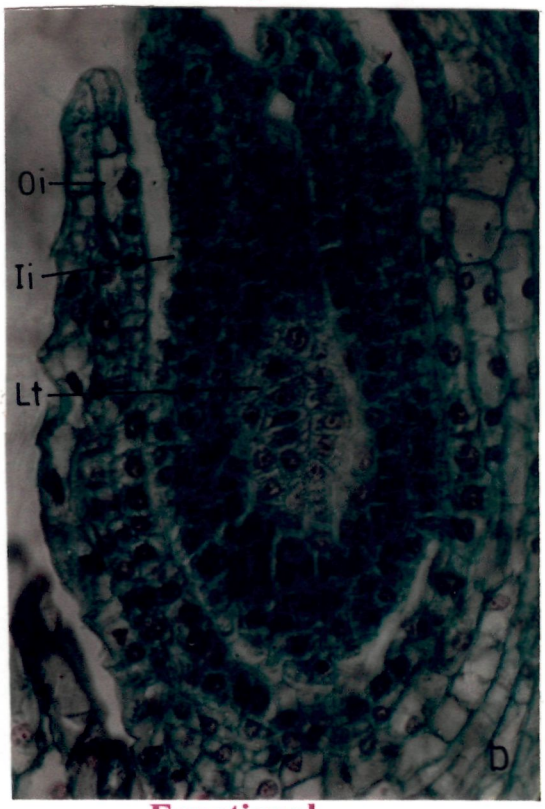
d: In linear tetrad, the functional megaspore is micropylar one.

All are x 515.

Plate – 6.3.1

Meiosis I
Dyad Stage

Meiosis II
Tetrad Stage (Linear)



'T' Shaped
Tetrad

Functional
Micropylar Megaspore

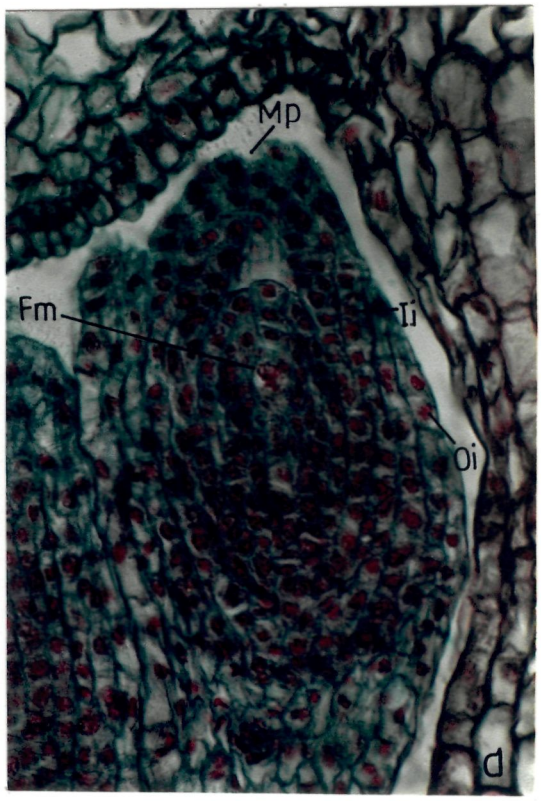


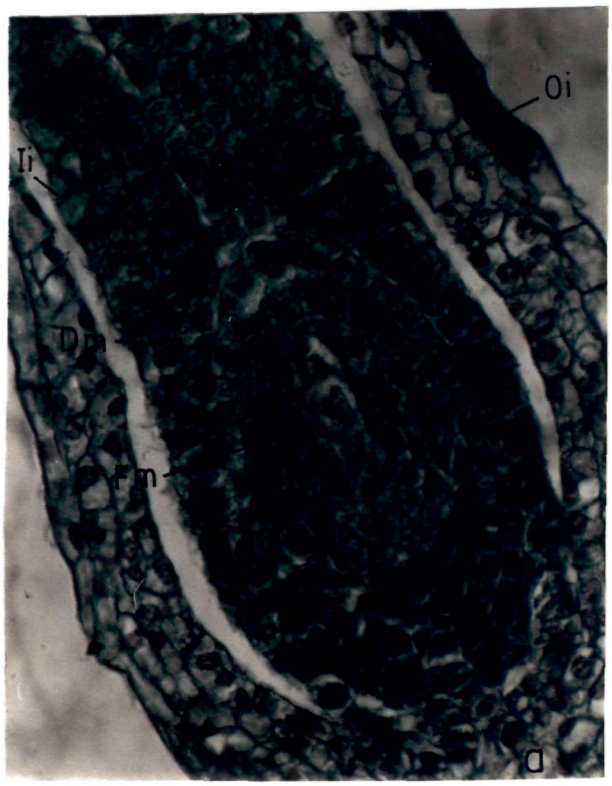
Plate - 6.3.2

a: **In 'T'shaped tetrad the functional megaspore (Fm) is Chalazal one. Note the three micropylar non-functional megaspores are at degenerating stage. x 600.**

b: **T. S. of ovule showing the functional megaspore at metaphase stage . x 600.**

Plate–6.3.2

'T' Shaped Tetrad with Functional Chalazal Megaspore and Three Degenerating Megaspores



Functional Chalazal Megaspore (Metaphase Stage)

Plate - 6.3.3

a: L.S. of ovule showing the Polygonum type of embryosac organization. Note the synergids (Sy) and filliform apparatus (Fa). x 1200.

b: L.S. of ovule with the Polygonum type of embryosac showing the Egg cell (E) and out of two polar nuclei one is seen. x 1400.

C: T.S. of ovule displaying the outer (Oi) and inner (Ii) integuments; embryo sac with egg cell (E) and degenerating stage of the nucellus (Dn). x 425.

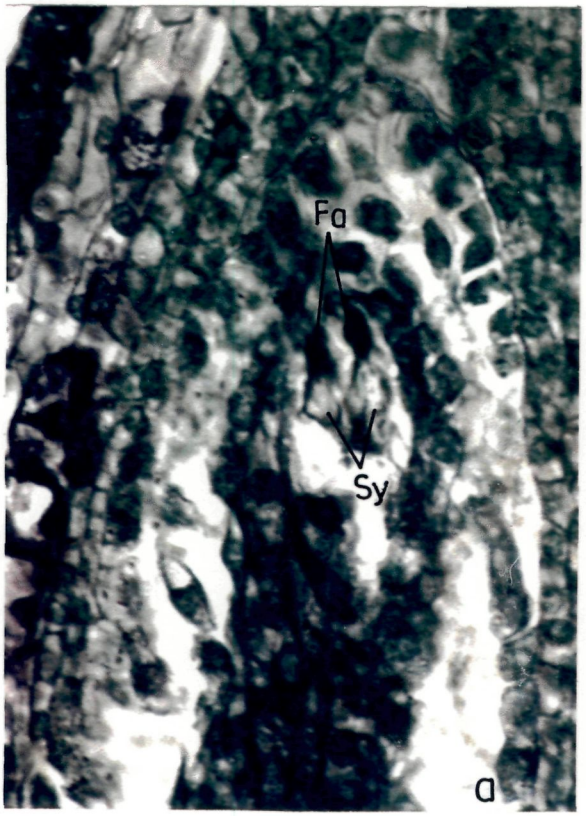
d: T.S. of ovule showing the two polar nuclei (Pn). x 450.

Plate–6.3.3

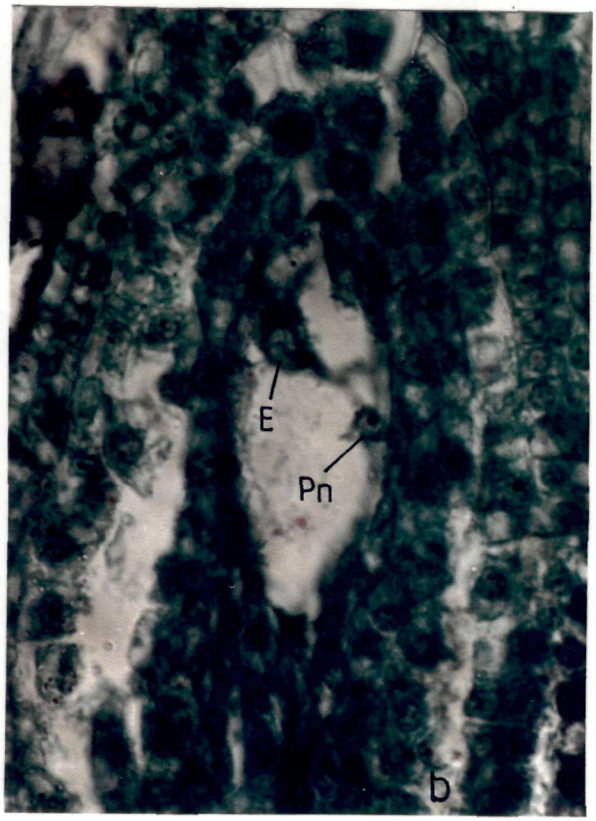
FEMALE GAMETOPHYTE

L.S. of Ovule

Synergids

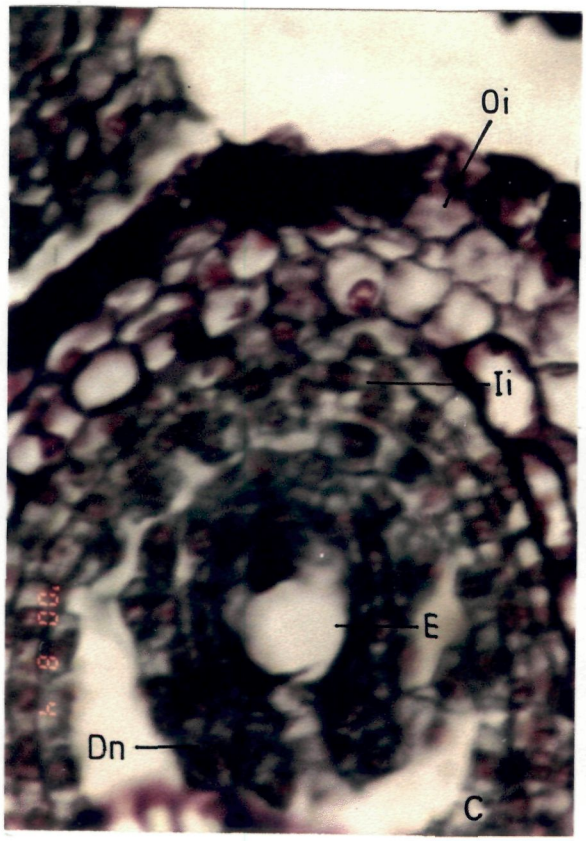


Egg Cell, Polar Nucleus and Antipodals



T.S. of Embryo Sac

Egg Cell



Two Polar Nuclei

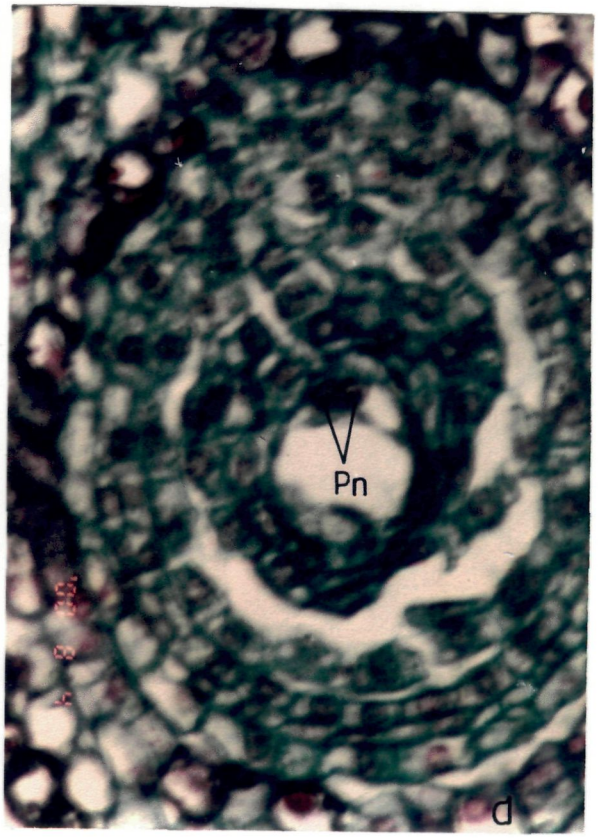


Plate - 6.3.4

a: L.S. of ovule showing the female meiocyte. Note the nucleus and nucleolus are enlarged considerably. x 750.

b: Meiotic division figure with spindles, note the nucleolus still persisting. x 700.

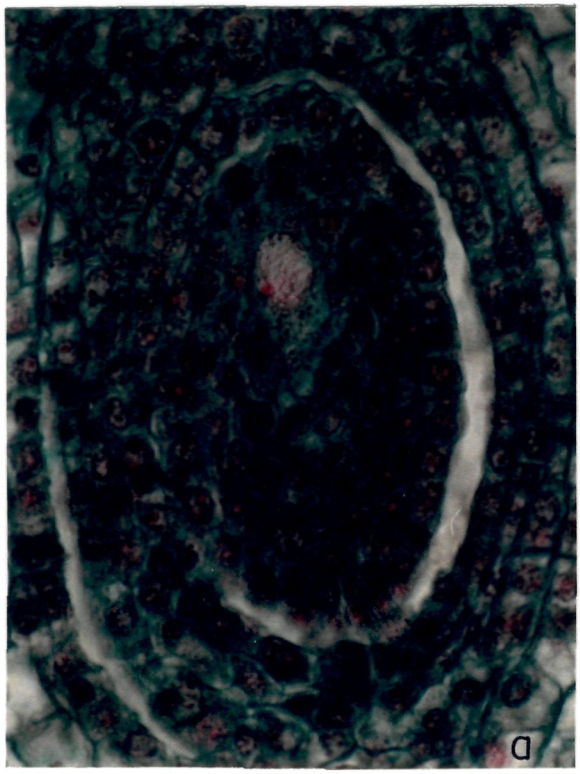
C: After meiosis - I, the two daughter nuclei are positioned toward micropylar and chalazal poles respectively. x 1400.

d: After meiosis - II, the four nuclei constitute the coenomegaspore (Com), two at each micropylar and chalazal poles. x 1250.

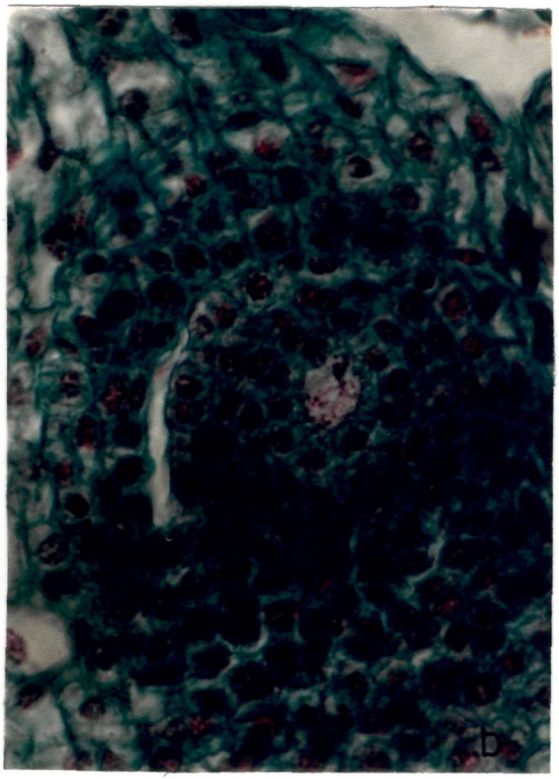
Plate – 6.3.4

TETRASPORIC DEVELOPMENT OF THE EMBRYO SAC

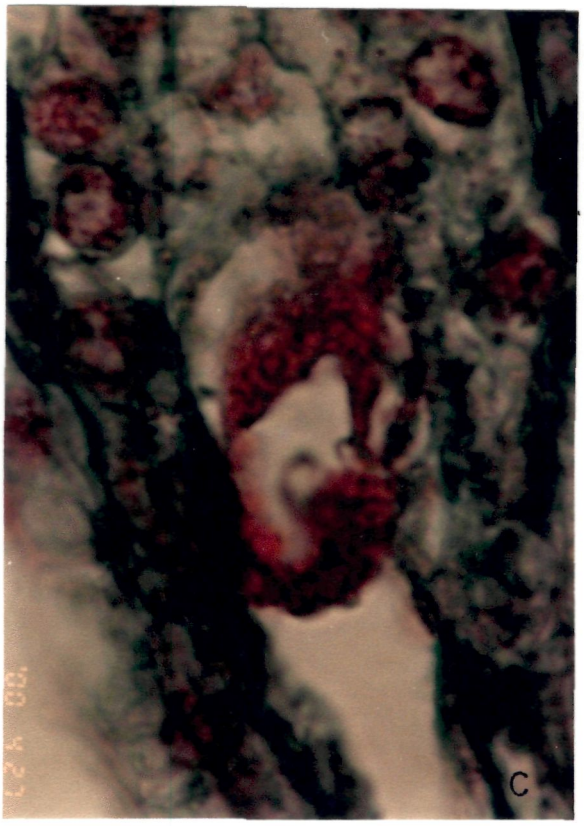
Meiocyte



Meiotic Division



Binucleate Stage



Coenomegaspore Four Nucleate Stage

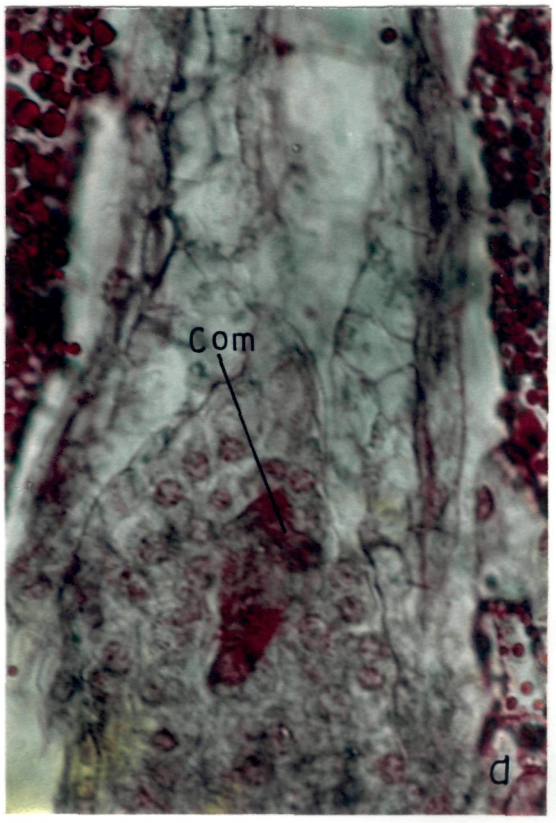


Plate - 6.3.5

a: The coenomegaspore gives rise to Drusa type of embryo sac Organization: remnants of pollen tube (pt); Synergid (Sy); Zygote (Zy); Primary endosperm nucleus (Pen) and eight antipodalcells are the chalazal end (Ap). x 850.

b: Fertilization stage showing the two degenerating synergids (Sy); Egg nucleus (En) and two male gametes (Mg). x 1000.

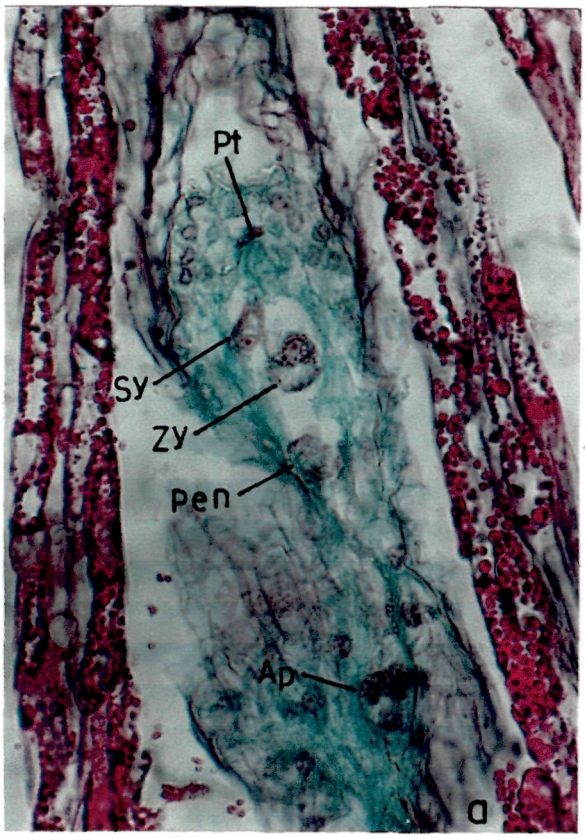
C: Showing the persistent pollen tube (Pt); Zygote (Zy) and degenerating synergids (Dsy). x 1000.

D: L.S. of ovule depicting the zygote (Zy), note the nucleus and most of the cytoplasm occupied in the chalazal end (apical portion) and primary endosperm nucleus (Pen). x 900.

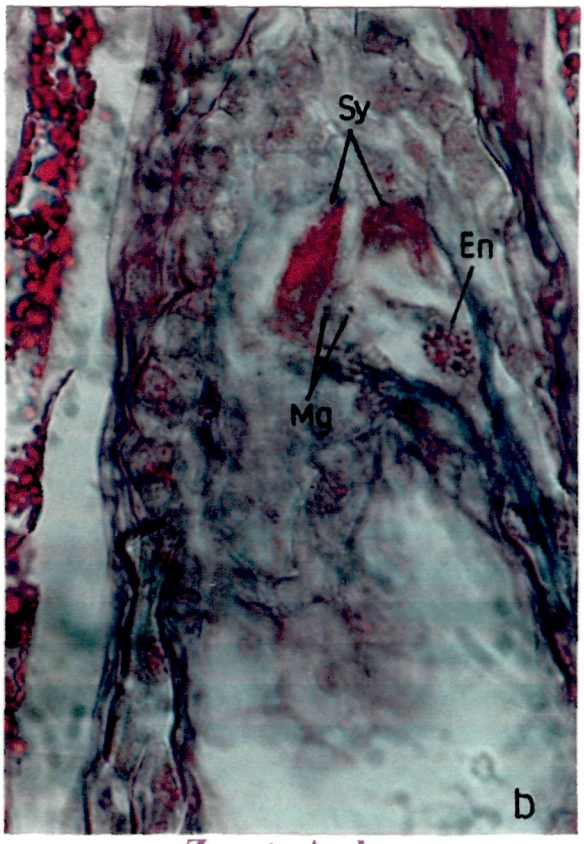
Plate– 6.3.5

L.S. OF OVULE

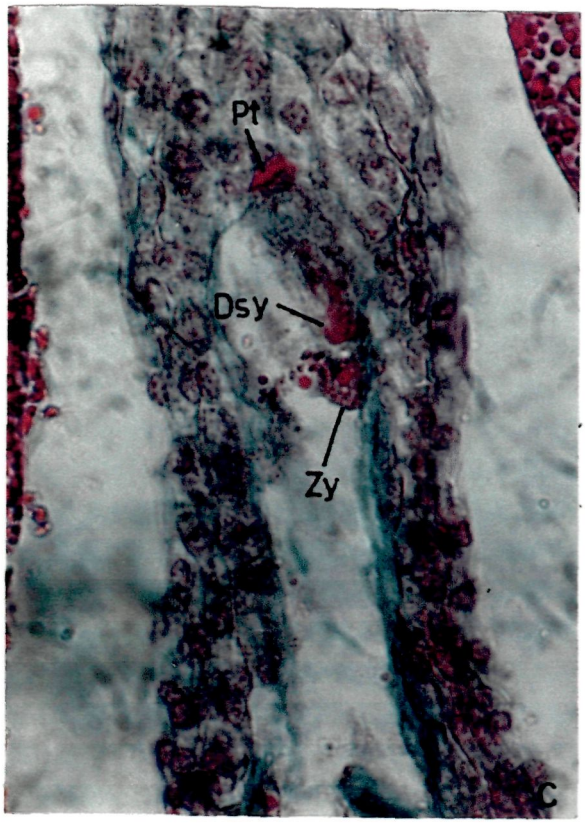
Drusa Type of Embryo Sac



Fertilization. Intermediate Type



Disappearence of Synergids



Zygote And Polar Nuclei

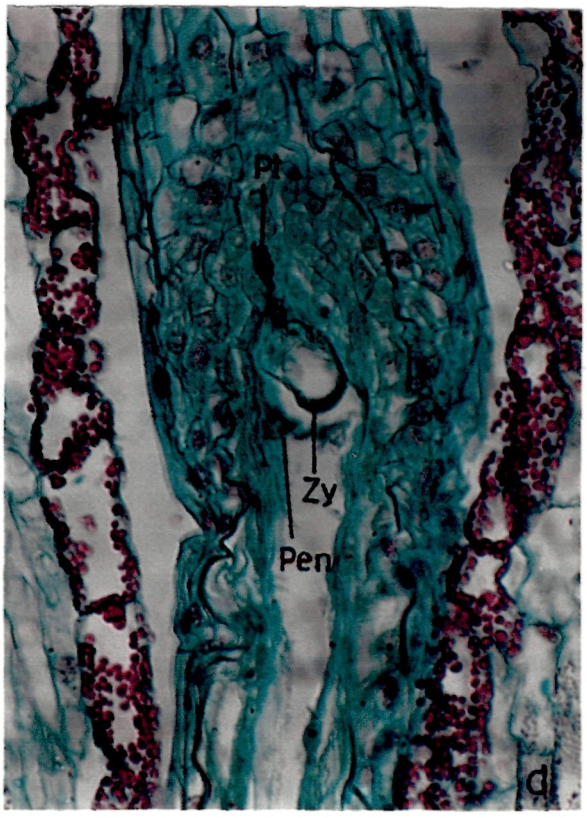


Plate - 6.4

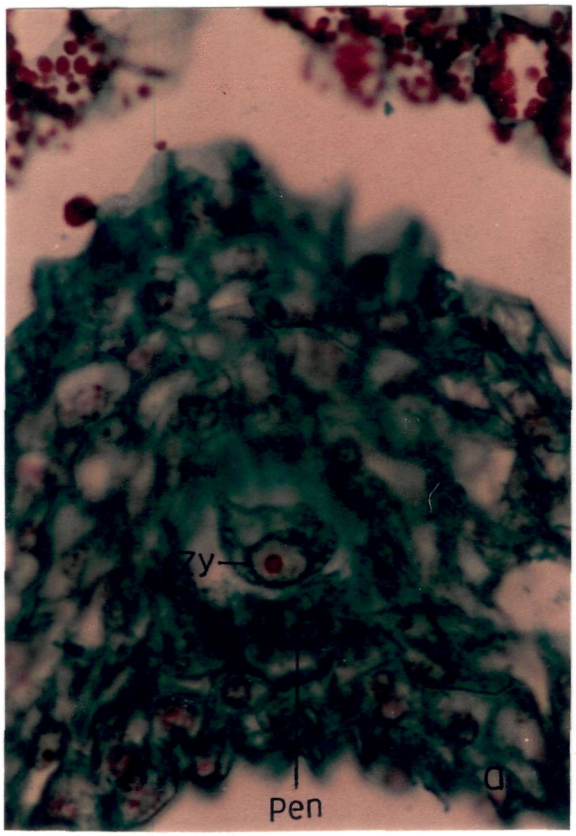
a and b: **L.S. of ovule (slightly oblique section) showing the zygote (Zy) and primary endosperm nucleus (Pen). Note the polarized appearance of nucleus and cytoplasm. a = x 1800; b = x 2600.**

C: **L.S. of ovule showing the two celled proembryo: apical cell ('ca') and basal cell ('cb'). x 1600.**

Plate-6.4

EMBRYOGENESIS

L.S. of Ovule - Zygote and Primary Endosperm Nucleus (PEN).



Two Celled Proembryo

An Enlarged View of Zygote and PEN

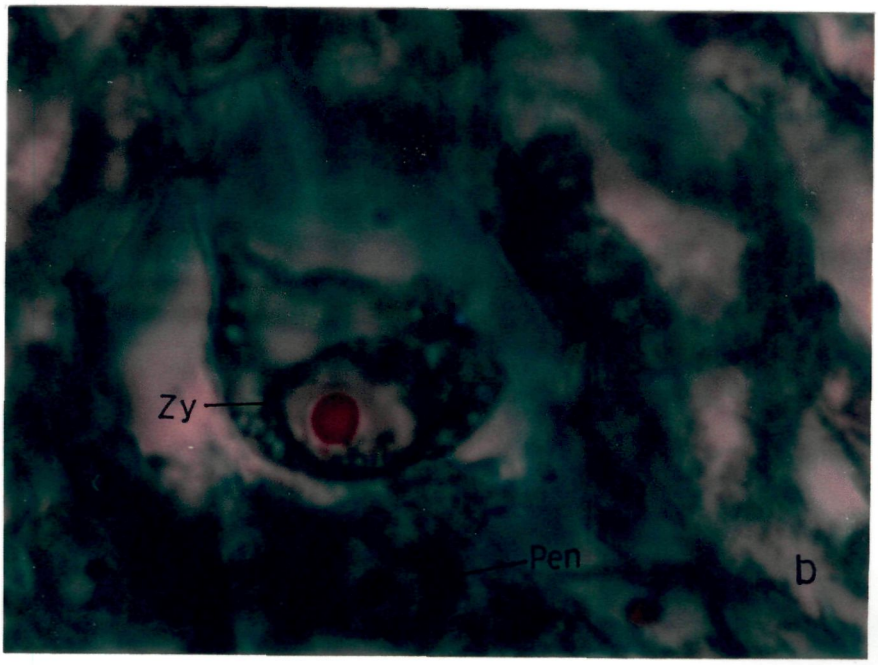


Plate - 6.5

a and b: **Linear phase of embryo development. Note the basal cell enlarges and it won't divide and further development of embryo only from the apical cell 'ca'. a = x 3000; b = x 3500.**

C: **Note the derivatives of apical cell pushes towards the nuclear endosperm. x 1750.**

d: **The Globular proembryo with nuclear endosperm. Note the dove-tail relationship. x 2500.**

Plate – 6.5

EMBRYOGENESIS

Linear Phase of Growth

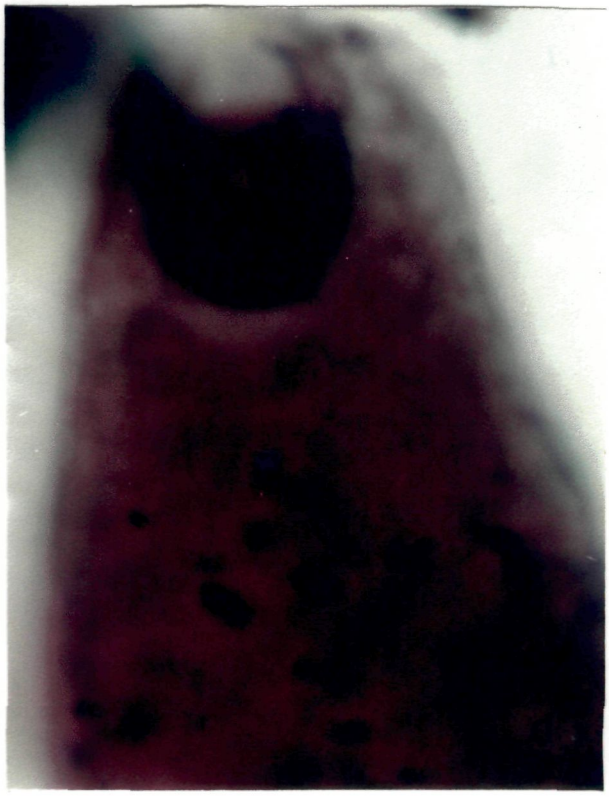


Plate - 6.5.1

a and b: **Tetrad stage of proembryo. Note the endosperm nuclei (Endn). a = x 1250; b = x 3800.**

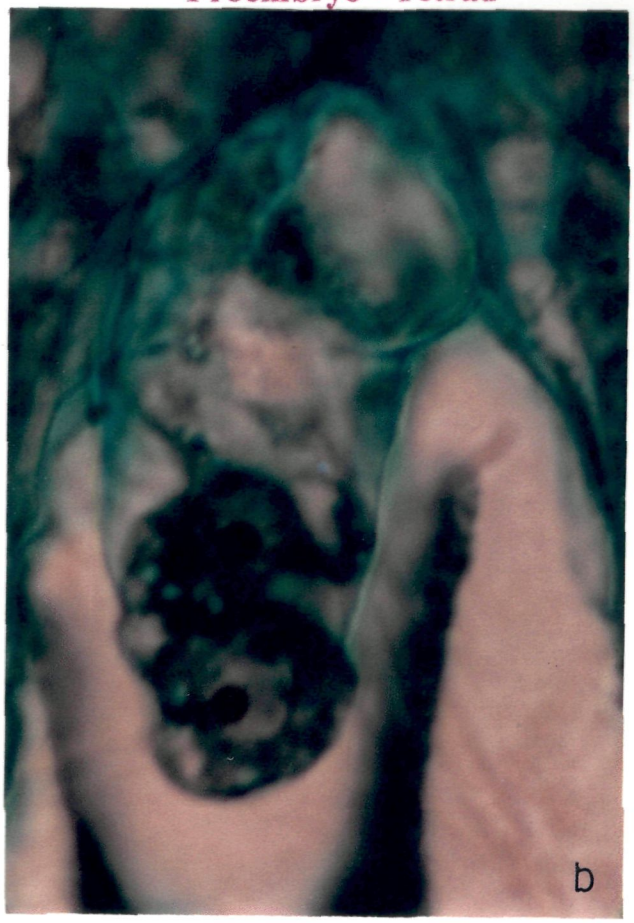
C: **T. S. of ovule showing the tetrad in cross section (T); endosperm (En) and degenarating inner integument (DIi). x 500.**

d: **Octant stage of proembryo. x 4000.**

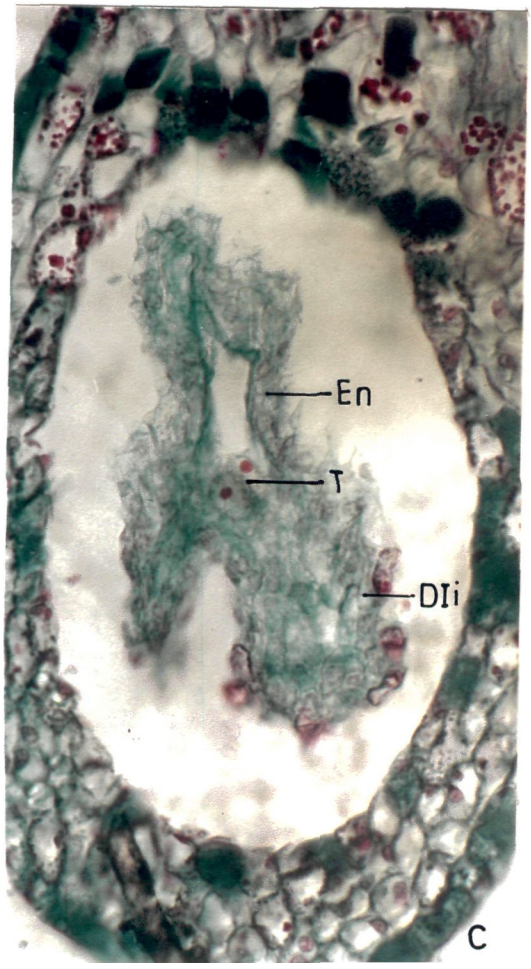
Plate–6.5.1

Proembryo - Tetrad

An Enlarged View of Proembryo - Tetrad



T.S. of Tetrad



Proembryo - Octant

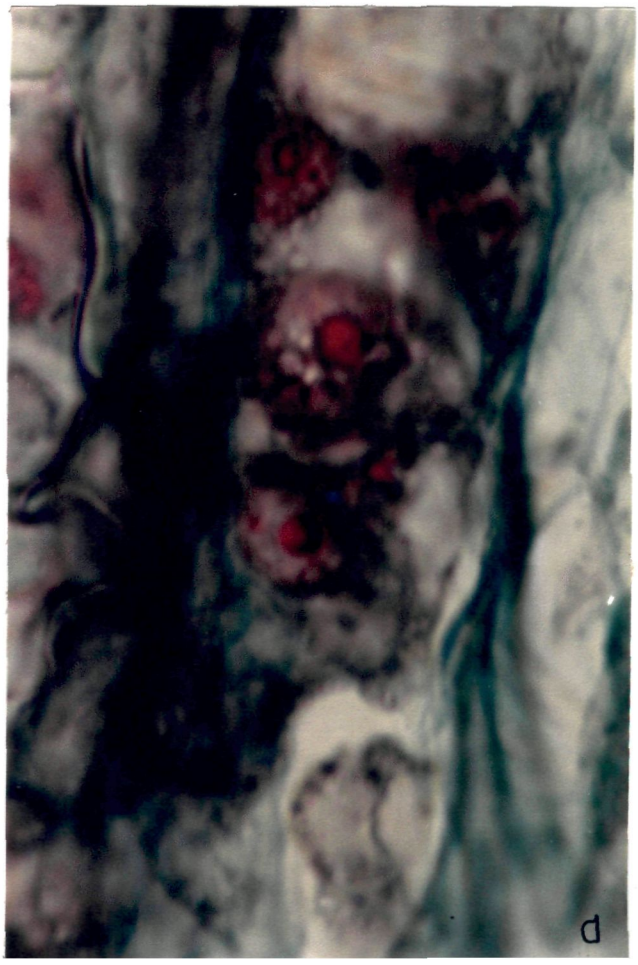


Plate - 6.5.2

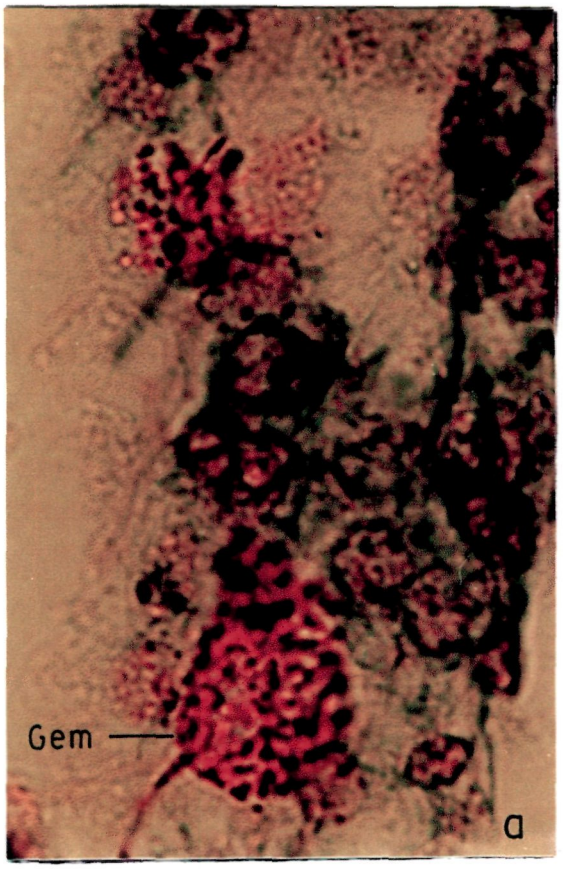
a and b: **Globular proembryo (Gem). x 2500.**

C: **Heart shaped embryo (Hem) with suspensor (S). x 1800.**

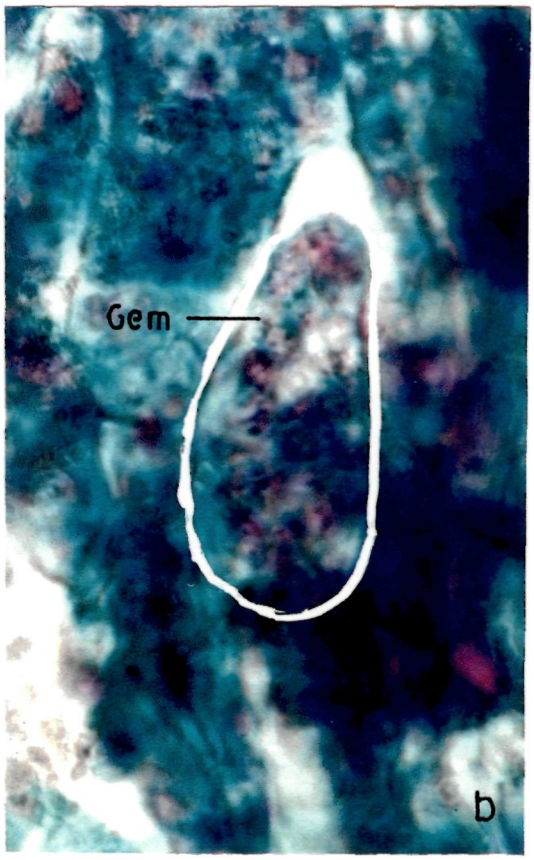
d: **Dissected mature embryo with two cotylendons (Coty) and hypophyseal region (Hy). x 800.**

Plate-6.5.2

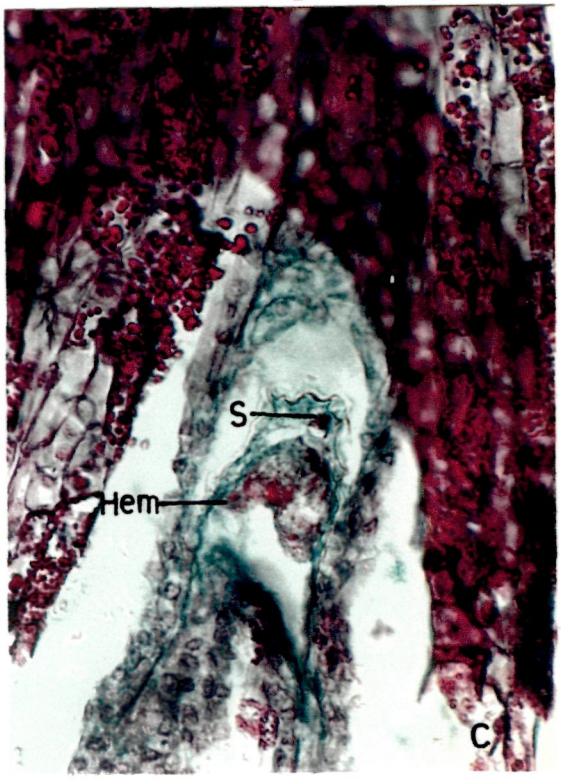
Globular Embryo



An Enlarged View of Globular Embryo



Heart Shape Embryo



Mature Embryo

Plate - 6.6

a and b: T.S. of ovule with two polar nuclei (Pn). Note the two polar nuclei are attached with one another. a = x 300; b = x 500.

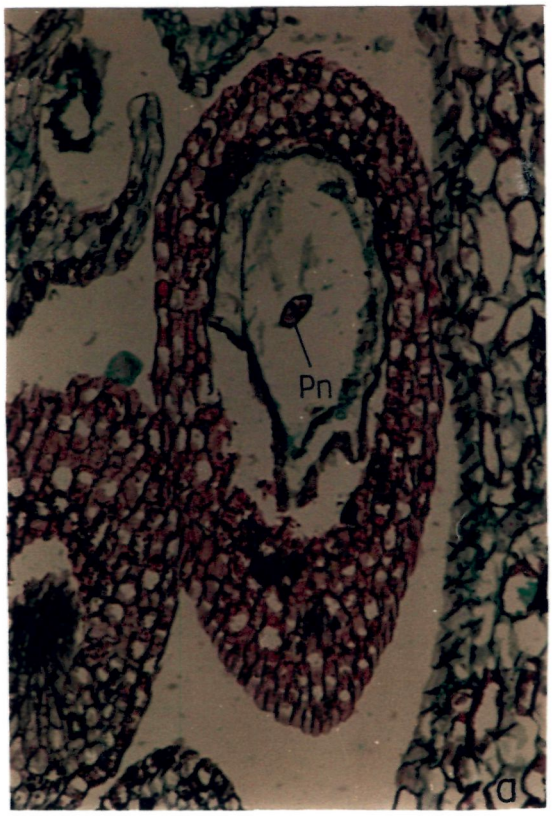
C: T.S. of ovule showing the Zygote (Zy) and Primary endosperm nucleus (Pen). x 900.

d: The primary endosperm nucleus (Pen) is suspended in a thin cytoplasmic strand. x 1450.

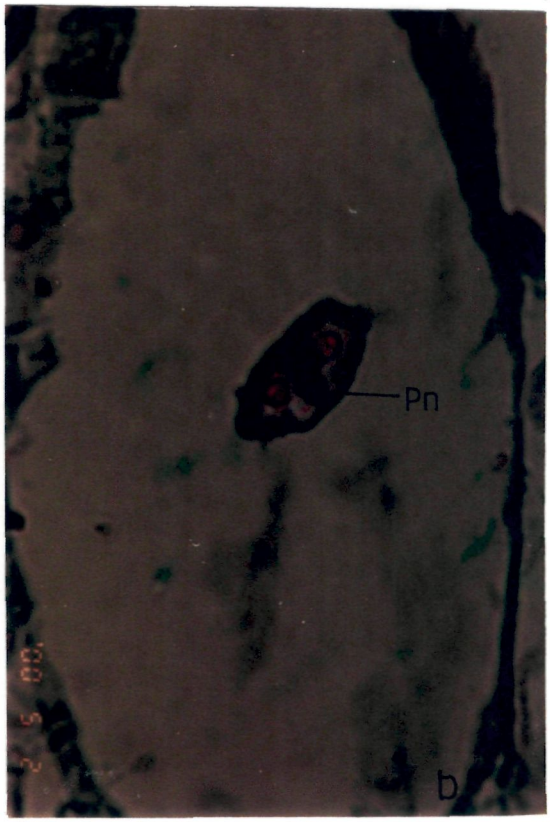
Plate–6.6

DEVELOPMENT OF ENDOSPERM

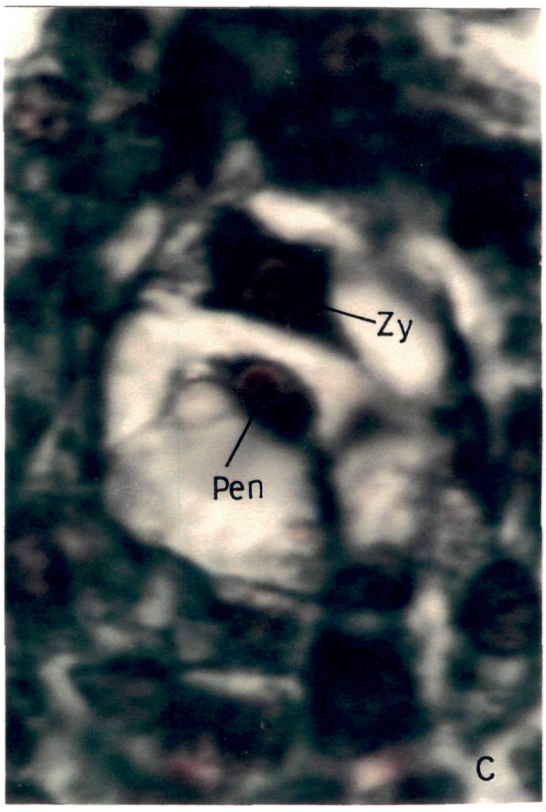
T. S. of Ovule with
Two Polar Nuclei



An Enlarged View
of Polar Nuclei



Zygote and Primary
Endosperm Nucleus



L.S. of Ovule with
Primary Endosperm Nucleus

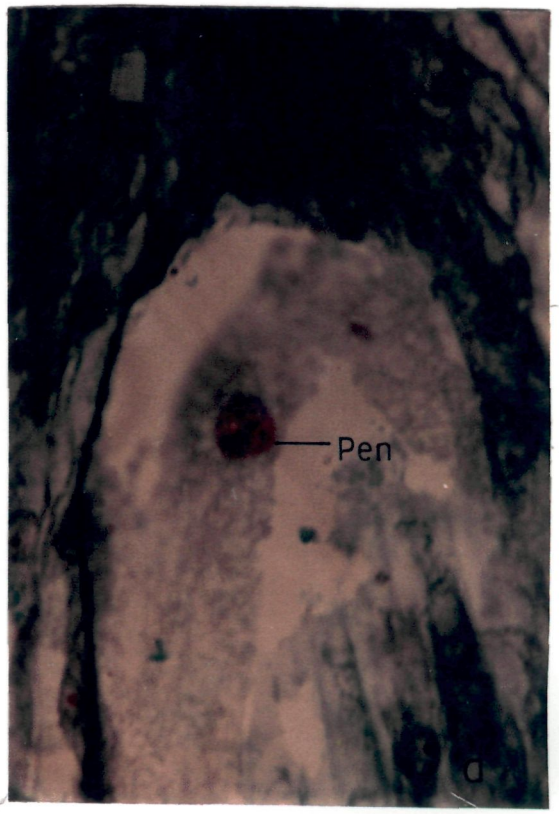


Plate - 6.6.1

a: Four nucleate stage of the endosperm. x 1100.

b: Many endosperm nuclei are suspended in a thin cytoplasmic strand (Cys). x 200.

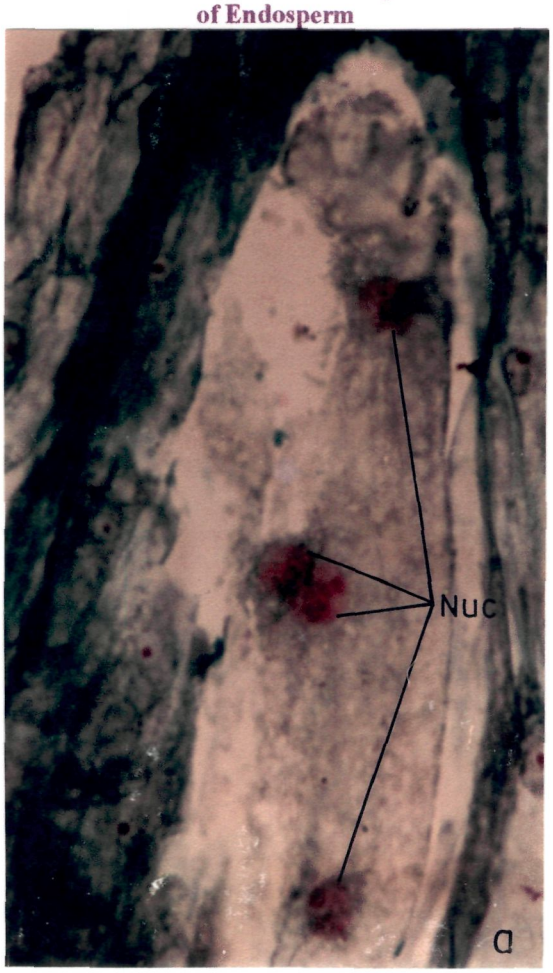
C: Dissected nuclear endosperm (End) without haustorium. x 1000.

d: L.S. of ovule with proembryo and a long chalazal haustorium (H). x 215.

e: Endosperm showing the chalazal haustorium. Endosperm (End); Haustorium (H) and starch grains in the integument (sg). x 900.

Plate-6.6.1

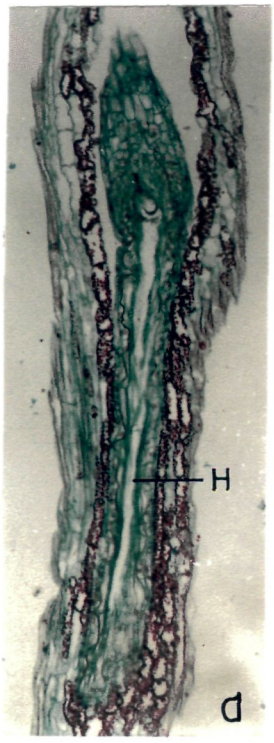
Four Nucleate Stage of Endosperm



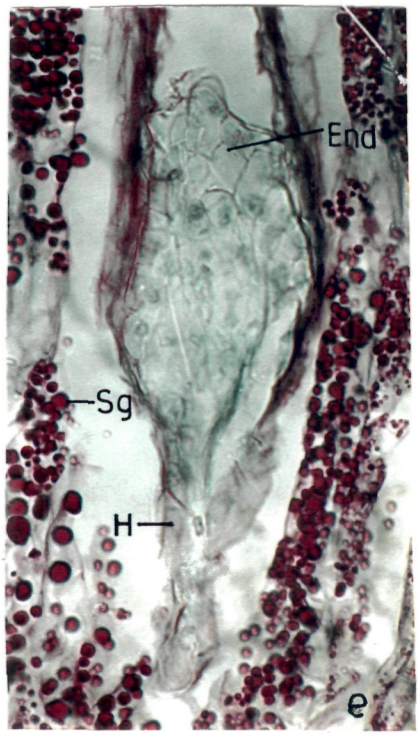
Several Free Nuclei of Endosperm suspended in thin Cytoplasmic strand

Dissected Nuclear Endosperm without Haustorium

L.S. of Ovule with Proembryo & Long Chalazal Haustorium



Mature Cellular Endosperm with Haustorium



CHAPTER - 7

Seed viability

7.1 *Introduction*

Viable seeds must retain the power of germination. Germination is the process by which the embryo wakes up from the state of dormancy and takes to active life. When a viable, non-dormant seed is provided with a wetted substratum, oxygen and suitable temperature, water is imbibed, respiration and metabolic events increase and after a certain period of time the radicle emerges from the seed. Thus, moisture, temperature and oxygen are considered essential factors for seed germination though light is not usually essential e.g. *Metrosideros excelsa* (Gabriele Schmidt-Adam et al., 1999), *Carmichaelia* sp. (Ingrid Gruner and Heenan, 2001), it may be required for some kind of seed e.g. *Lactuca sativa* (Agrawal and Dadlani, 1992), *Dysoxylum spectabile* and *Alseusmia macrophylla* (Burrows, 1999). Some seeds of carnivorous plants require a period of damp cold for germination (*Sarracenia alata*, *S. flava*, *S. purpurea*, *Drosera filiformis*, *D. anglica* and *D. intermedia*. Other seeds may require treatment with hormones (*Drosera burmanni* and *D. indica*). Some species like growing in sphagnum moss (*Drosera rotundifolia*, *Sarracenia*, *Nepenthes ventricosa*, *N. bicalcarata* and *N.*

gracilis), peat and sand mix (*Drosera aliciae*, *D. binata*, *D. capensis* and *Utricularia* sp.) Since informations on the seed viability of *Nepenthes khasiana* are lacking, an attempt was made to find out the percentage of seed viability by germination test which may be helpful in seed quality programme.

7.2 *Results*

Table-7.2 revealed the effect of temperature and light on seed germination of *Nepenthes khasiana*. *N. khasiana* seeds germinated at 25°C of temperature only whereas no germination occurred at 20°C and 30°C respectively (Table - 7.2.1).

Nepenthes plants need 1000-1250 lux of light. *N. khasiana* seed germination was observed under 16h dark and 8h light condition (Plate-7.2, 7.2.1; Table-7.2.1). Under continuous light and dark condition, seed germination was not observed in *N. khasiana* (see Table-7.2.1).

Seedlings of *Nepenthes khasiana* emerged after 27 days when seeds were germinated in 16h dark and 8h light on moist Whatman filter paper at 25°C in BOD incubator (Plate-7.2a,b; 7.2.1a, b, c) and the emergence was completed after 65 days (see Plate-7.2.1; Table-7.2). The percentage of germination is 78.34 in Whatman filter paper whereas, seeds of *N. khasiana* germinated on sand showed seedling emergence

Table - 7.2: Germination of seeds of *Nepenthes khasiana* Hk.f. under Laboratory condition at 25°C of temp. at 16h dark and 8h light condition.

Culturing method	Total no. of seeds / petridish	Days to start germination	Days to complete germination	Germination (%) after 115 days
Filter paper	150	27	65	74.34 ± 2.02
Sand	150	32	60	80.67 ± 2.4

± = SE. The mean difference is not significant at the 0.05 level.

Table -7.2.1: Effect of Temperature and Light conditions on seed germination of *Nepenthes khasiana* Hk.f.

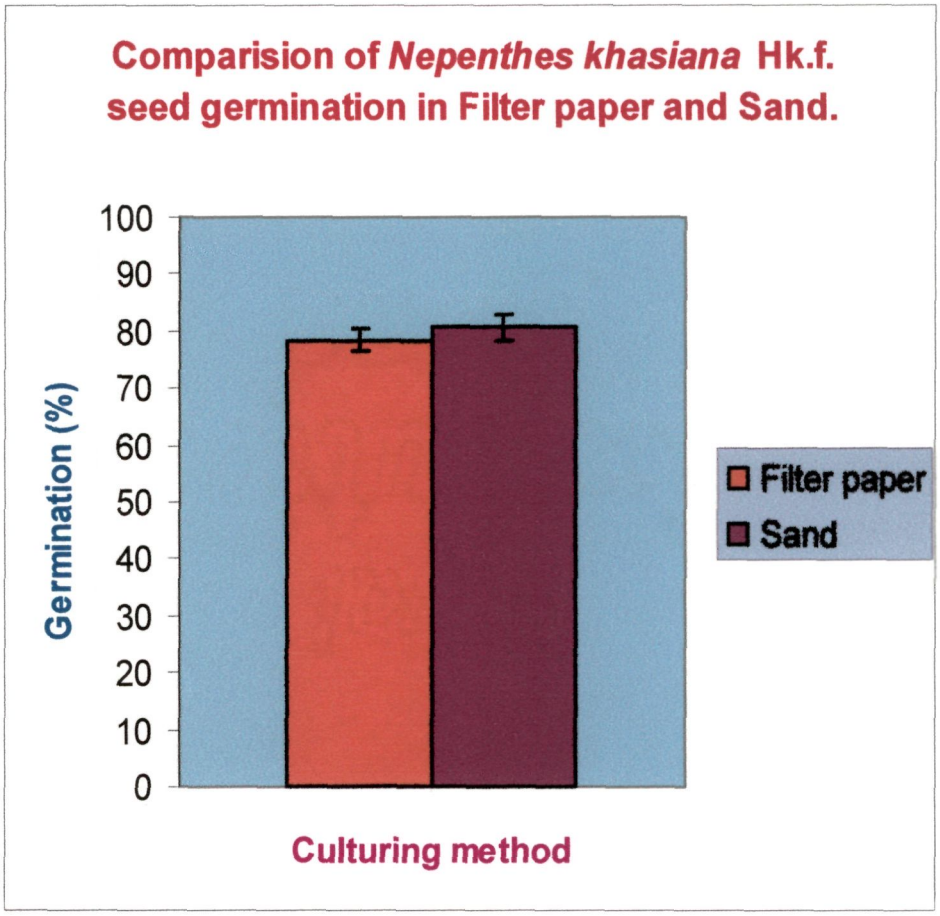
Temp. condition / Light condition	20°C Germination (%)	25°C Germination (%)	30°C Germination (%)
Light	-	-	-
16h Dark + 8h Light	-	80.67 ± 2.4	-
Dark	-	-	-

after 32 days and the germination was completed after 60 days under the same light and temperature condition (Plate-7.2c). The germination percentage was slightly higher on sand i.e. 80.67% (Table-7.2). However, the percentage of germination on sand is not significantly different from that of filter paper one (see Plate-7.2a, b, c; Fig. 7.2; Table-7.2). Emergence of seedling on sand is slower (5 days) than that on filter paper one. In otherwords, the germination on sand was completed 5 days before the filter paper one.

7.3 *Discussion*

Endospermic seeds are reported in Nepenthaceae (Watson and dallwitz, 1992; Subramanyam et al., 1995). But, in *N. khasiana*, the seeds are ex-endospermic (I used the term ex-endospermic where the endosperm is formed initially but it is completely used up by the developing embryo i.e. the seeds are without endosperm). Kaul, 1982 also observed no endosperm in the seeds of *N. villosa* and *N. gracilis*. However, endospermic seeds are reported in Sarraceniaceae and Droseraceae (Johri et al., 1992; Watson and Dallwitz, 1992). Seeds are small, spindle shaped in Nepenthaceae, winged in Sarraceniaceae and Nepenthaceae (Hooker, 1886; Watson and Dallwitz, 1992; Johri et al., 1992).

Fig. 7.2


Comparision of *Nepenthes khasiana* Hk.f. seed germination in Filter paper and Sand.
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
Culturing method
Filter paper
Sand

Germination phanerocotylar in Nepenthaceae and Sarraceniaceae whereas both the phanerocotylar and cryptocotylar seed germination was reported in Droseraceae (Watson and Dallwitz, 1992).

In general most of the plants do not usually require light but *Nepenthes* plants need 1000-1250 lux of light to germinate. The plants should be in light shade or under fluorescent lights. Many Carnivorous plants grow very well under broken sunlight, under a tree or patio cover is ideal (ICPS Seed Bank, 2002). *Nepenthes khasiana* seeds germinated at 8h light and 16h dark only in the vitro germination. In the continuous light and dark no germination of the *Nepenthes khasiana* seeds was observed. Germination of *Weinmannia, Alseuosmia macrophylla* seeds were inhibited in the dark but germinated well when put in the light (Burrows, 1999). *Dysoxylum* seeds died in the dark. *Carmichaelia* species (Ingrid Gruner and Heenan, 2001), *Metrosideros excelsa* (Gabriele Schmidt – Adam et al., 1999) showed no specific light requirements for seed germination.

Temperature plays an important role in germination. Imbibed seeds of *Pittosporum eugenioides*, *P. obcordatum* and *P. tenuifolium* showed almost no germination and marked a loss of viability after 3-4 months in storage under light condition at 21°C. In contrast, low temperature stratification storage for 8 weeks resulted in complete

germination of seeds in the above mentioned species (Simon Moore, 1994). Germination percentages of four members of Asteraceae viz. *Millotia myosotidifolia, Podotheca gnaphalioides, P. chrysantha* and *Ursinia anthemoides* seeds were highest at average temperature 25°C. It is reported that *Nepenthes* seeds germinated in warm 32°C plus temperatures e.g., *N. ventricosa, N. bicalcarata* and *N. gracilis* (ICPS Seed Bank, 2002), but, *N. khasiana* seeds showed germination at 25°C ± 1°C temperature only (see Table-7.2.1). Generally, seeds start to germinate after four or five weeks in the carnivorous plants (Mark Pogany, 1999). *Sarracenia leucophylla, S. flava, Pinguicula vulgaris* and *Drosera intermedia* seeds need a stratification period for good germination percentage except *N. ventricosa.*

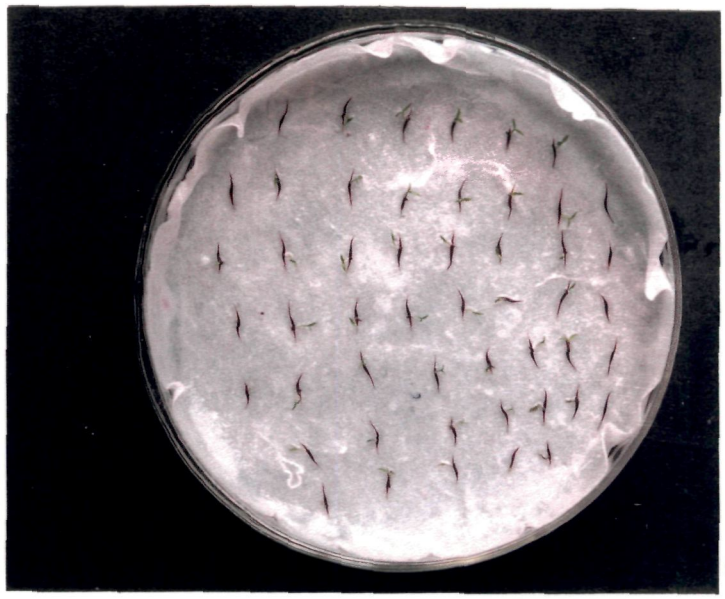
Much of seed viability depends upon storage conditions. The ideal storage condition for seed is somewhere cool and dry. A capped jar in the refrigerator serves the purpose (Sherry Rindels, 1995). *Nepenthes seeds* will last upto 12-14 months provided the seeds are treated correctly. The seed of *N. fusca* showed at least 90% germination after 14 months (Phill Mann, 1998). Seeds of species such as *N. bicalcarata* and *N. ampullaris* seem to have a very short life span and it is a matter of weeks only (Phill Mann, 1998).

Nepenthes seeds are slow to germinate. Germination can take from four weeks to almost a year (ICPS Seed Bank, 2002). In vitro germination usually takes eleven days to three months. Some *Nepenthes* seeds still germinate after six to seven months. It may be mainly depends on how long we can keep the cultures going before they dry (Phill Mann, 1998). *N. khasiana* seeds took 27 days to 65 days in filter paper and 32 days to 60 days on sand to germinate. *Sarracenia leucophylla, S. flava, Pinguicula vulgaris* and *Drosera intermedia* seeds also start to germinate after four or five weeks (Mark Pogany, 1999). The fresher the seed, the stronger the seedlings and higher the rate of germination (ICPS Seed Bank, 2002). But, *N. fusca* showed 90% germination after 14 months (Phill Mann, 1998). In *N. khasiana* the germination percentage is 80.67 ± 2.4% which showed high seed viability after 8 weeks. The *N. khasiana* seeds need moisture, 1000-1250 lux of light and 25°C of temperature. Therefore, planting medium should be moist but not soaking wet for the germination of seeds in *N. khasiana.*

Plate – 7.2

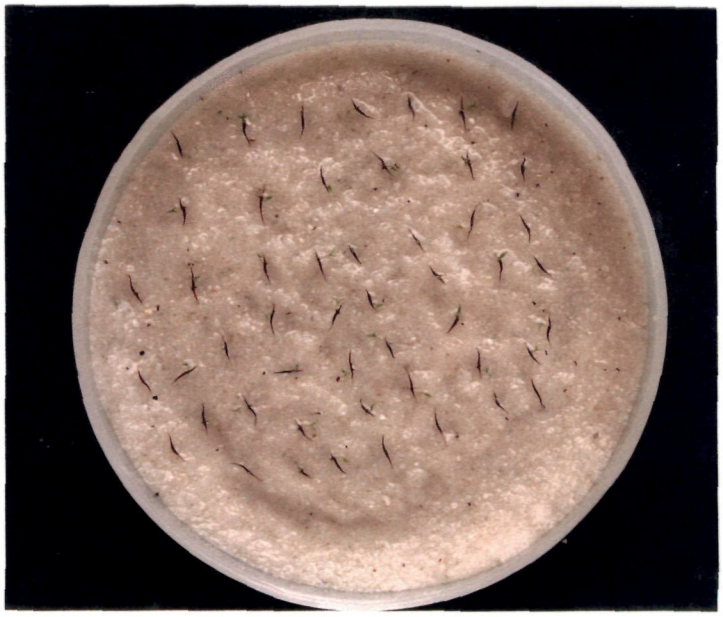
Nepenthes khasiana Hk. f.

SEED GERMINATION



a

b



c

Plate–7.2.1

Nepenthes khasiana **Hk.f.**

STAGES OF SEED GERMINATION

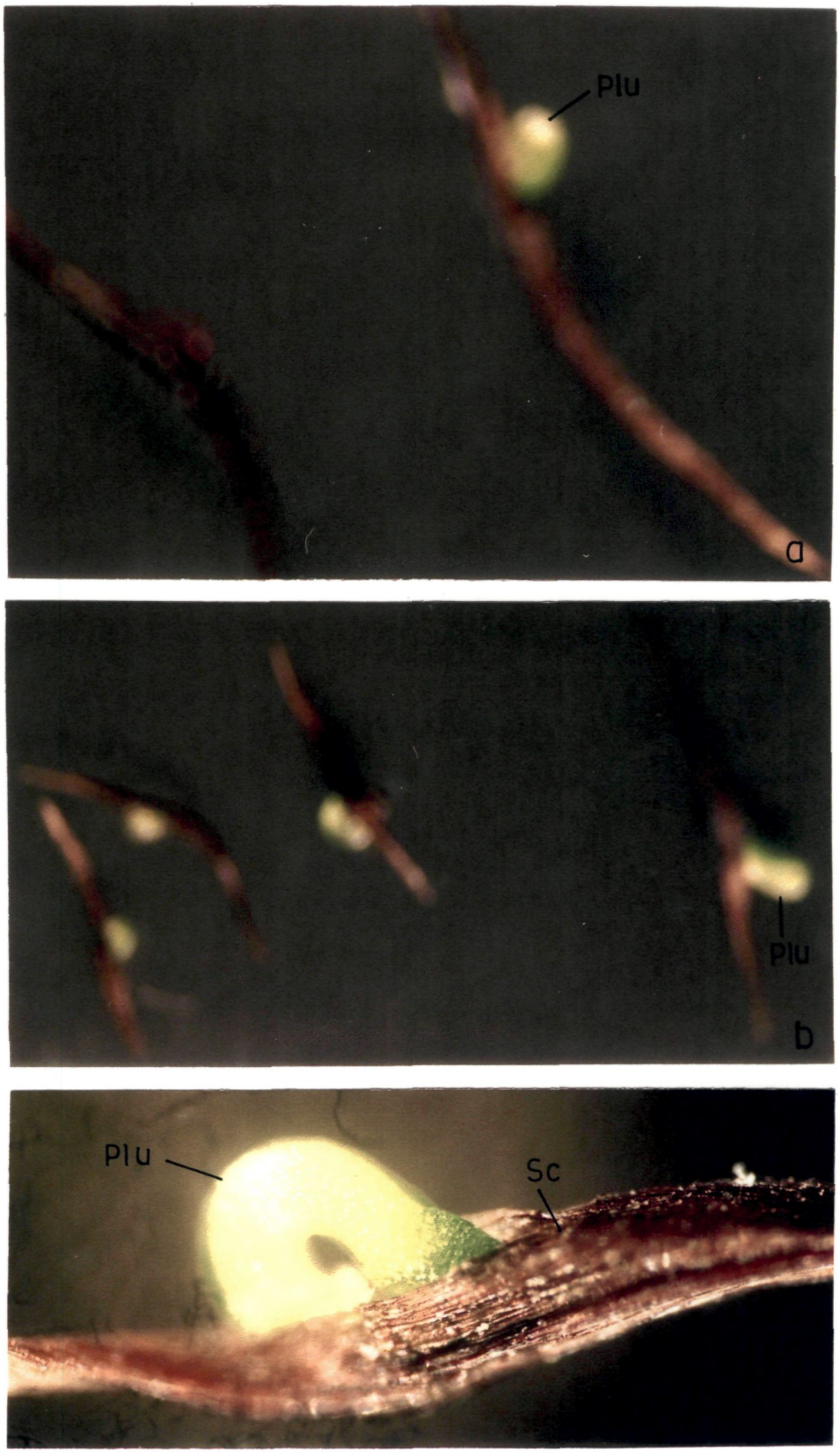
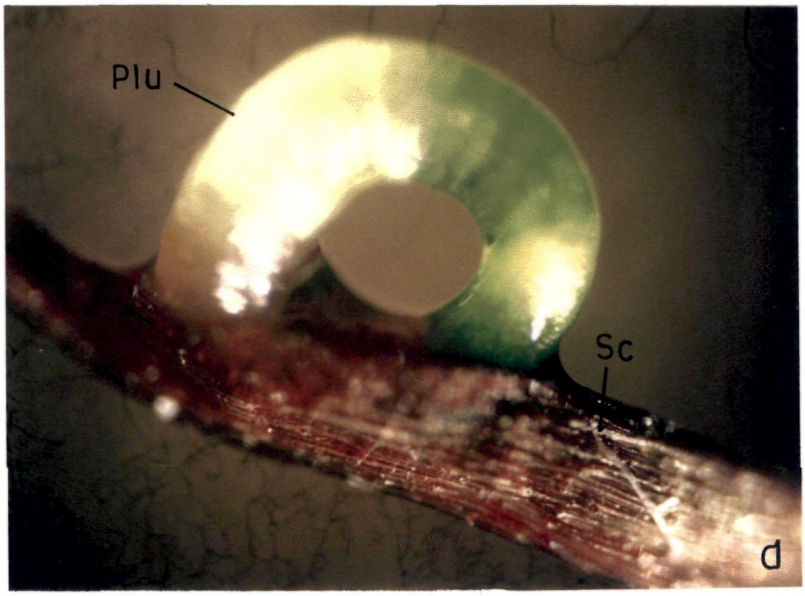
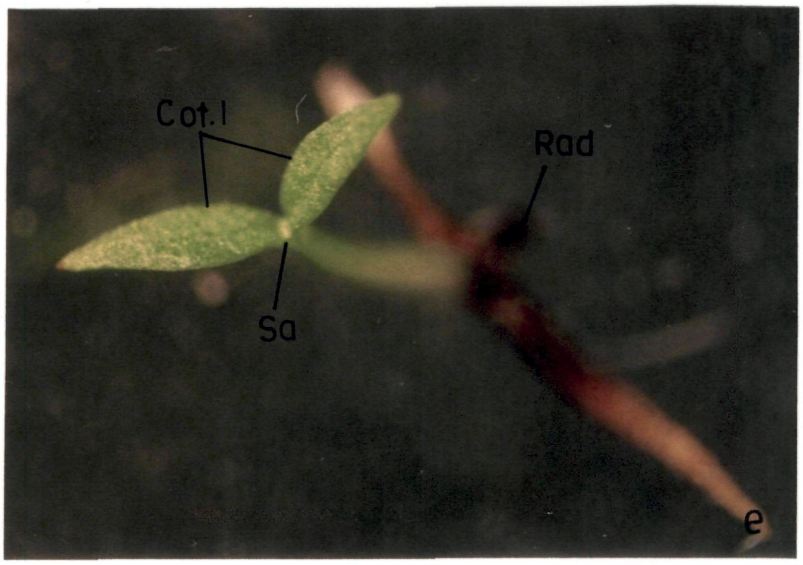
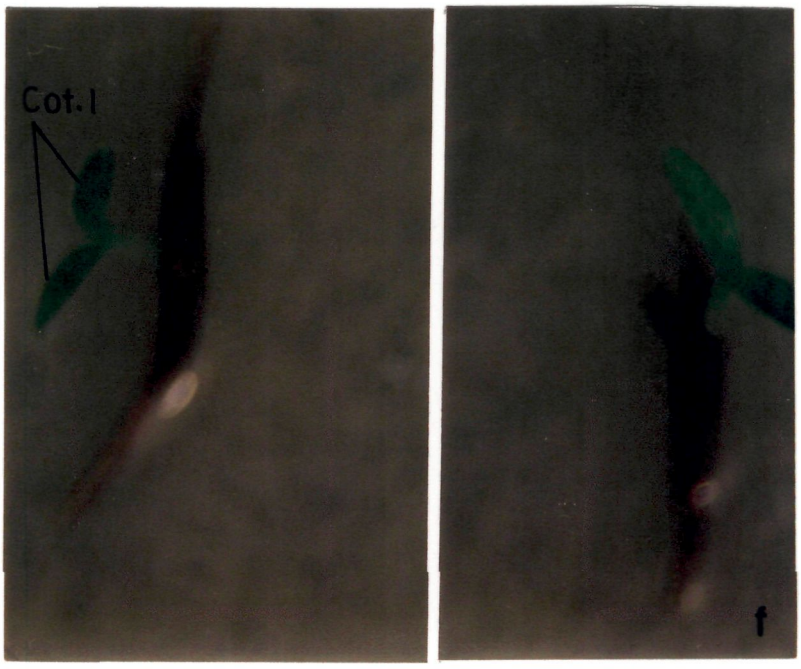


Plate – 7.2.1







CHAPTER-8

General Discussion

8.1 *Flowers*

Nepenthes khasiana Hk.f. is a dioecious straggler, distributed in certain isolated provenances of Meghalaya. It is an endemic and endangered plant (Hooker, 1885; Jain and Sastri, 1980; Joseph and Mani, 1982). The staminate and pistillate flowers are borne in pairs, similar to that of *Nepenthes lowii*. The peduncles, pedicels, outer side of the tepals, androphore (when young) and gyneocium are invested by ferruginous coarse hairs. In both sexes, the four tepals are free, decussate and provided conspicuously on their adaxial surfaces with embedded nectar glands. Endress (1994) reported that the nectaries of *Nepenthes* fall under Type II, where often the epidermis is differentiated as a pronounced epithelium. These extrafloral nectaries are device in genus *Nepenthes* to attract pollinators.

In the staminate flowers, the elongation of androphore is brought about by the intercalary meristamatic activity in the receptacle. Therefore, the androphore is not exactly the monoadelphous condition of filaments. This fact was reported by Stern as early as 1917. Kaul (1982) also suggested that the androphore is receptaclular in nature, because the

four vascular traces directly enter into the androphore without any branching.

8.2 *Staminate flower*

The bisporangiate anther has been reported only in few families (Bhandari, 1984). The insectivorous families Nepenthaceae, Droseraceae and Sarracenaceae show a tetrasporangiate condition (Watson and Dallwitz, 1992). But, an interesting feature in *N. khasiana* is that it shows a bisporangiate condition. Davis (1966) reported the co-occurrence of bi- and tetrasporangiate anthers in four monocotyledon and twelve dicotyledon families. Such a co-occurrence feature is absent in *N. khasiana* Hk.f. The number of sporangia ranges from 25-30 on each androphore in *Nepenthes khasiana* Hk.f. The same condition has been reported in *Nepenthes lowii* (Kaul, 1982). The plate of archesporial cells, hypodermal in origin, has been reported in many plants (Maheshwari, 1950; Periasamy and Swamy, 1959; Periasamy and Kandasamy, 1981; Swamy and Krishnamurthy, 1990). The anther development is dicot type (Davis, 1966; Poddubnaya, 1976; Subramanyam et al., 1985). The origin of the tapetum is dual in origin; a part of it is derived from the primary parietal cell, which is a single layer whereas another part, which is abutting the androphore, is derived from

the connective tissue of the androphore, which is multilayered, multinucleate and the nuclei are hypertrophid. These tapetal cells are much larger in size than the parietal tapetum (Periasamy and Swamy, 1966). However, the dual origin of tapetum has not been reported in *Nepenthes lowii* and *Nepenthes villosa* (Kaul, 1982). Subramanyam et al. (1985) also did not mention this fact. The secretory type of tapetum also confirms the observation of Subramanyam et al. (1985). The connective tapetum degenerates earlier than the parietal tapetum. The further differentiation of the androphore and its elongation take place only when the pollen grains are in tetrads. The same phenomenon has also been observed in *Nepenthes lowii* and *Nepenthes villosa* (Kaul, 1982). But the activity of an intercalary meristem, which is responsible for the elongation of androphore, has not been observed in the above-mentioned species.

As the microspore mother cells enter into reduction division, there is a distinct callose wall around each microspore mother cell. The endothecium acquires wall thickenings at the end of meiosis II, simultaneously the callose wall around the tetrads starts to disappear.

In general, the type of cell division in the microspore mother cell (meiocyte) can be classified as successive or simultaneous. *N. khasiana* exhibits simultaneous type of cytokinesis, but there are two variations

within it. 1. Simultaneous centripetal constriction furrows at the end of meiosis II. 2. It is intermediate where in the first furrowing that commences after the first nuclear division is not completed until the second nuclear division. As a result the completion of cytokinesis may be simultaneous although the initiation of the division furrow is successive. A similar condition was reported in *Magnolia* (Farr, 1918; Stoudt, 1960) and in *Annona* (Juliano, 1935).

In view of the unique morphological, anatomical and embryological fluxes exhibited within the relatively few members of insectivorous families of the order Sarraceniales studied so far with regard to various aspects of the microsporangium, a study of more members of this group may bring to light other features that may be of value in the understanding of the morphology and evolution of the angiosperms as a whole.

8.3 *Pollen viability and pollen germination*

Nepenthes khasiana Hk.f. shows high pollen viability (89.62%). In all the angiosperm species the pollen viability decreases with time (Pacini et al., 1997). Binucleate pollen generally survives longer than trinucleate and germinates readily in vitro (Stanley and Linskens, 1974). Entomophilous species also generally have longer pollen viability

(Pacini et al., 1997). But, in *Nepenthes khasiana* the pollens seem remain viable for only a short period. This suggests that the pollen grains are in a highly active metabolic state and, as a result, the biochemical changes probably occur over time.

Pollen germination can be substantially altered by the temperature. Pollen of most plants show optimum germination and tube growth between 20 - 30°C (Johri and Vasil, 1961). In the case of *Nepenthes khasiana,* pollen grains germinated only at 25°C. The sucrose concentration required for optimal germination varies from species to species e.g., *Solanum aviculare*, *S. laciniatum*, *S. sisymbrifolium* require 10% sucrose; *Corylus avellata* require 10 to 25% sucrose (Kim et al., 1985). In *Nepenthes khasiana*, 5% sucrose enhances pollen germination and tube elongation (see Table-5.2 and 5.2.1).

The pollen germinability is influenced by flower age, drying time and pollen source (Randolf and Robert, 1986). No pollen germination and tube growth were occurred in the stored pollens in *N. khasiana* and collected few hours after anthesis. However, in *Asclepias exaltata*, flower age has little effect on pollen germinability (Shannon and Wyatt, 1986). Fresh pollen grains collected during the anthesis gave optimal pollen germination and tube elongation in *Nepenthes khasiana.* Pollen grains must be among the shortest-lived independent bodies in nature,

for there are few which can remain alive for more than a few days after they have been shed, while some can live for only a few hours (Echlin, 1968; Sporne, 1974). Pollen grains germinate in a wide range of pH but the best results are obtained at pH 5.5-6.5 (Johri and Vasil, 1961). But, in *Nepenthes khasiana* optimal germination and tube elongation were seen at pH 8.0 under dark.

Lights of different colour influenced pollen germination and tube elongation in *Schima wallichii* (Chauhan and Katiyar, 1996). Similarly, in *Nepenthes khasiana* also dark had significant effect on pollen germination. However, there was not much difference in tube elongation as compared with the light treatment.

As such the pollen viability and germination percentage were high, which result into a high degree of pollen fertility in *N. khasiana*. Therefore, one could expect more viable seeds after fertilization. Usually, high fruit set is also reported in *Nepenthes gracilis* (Holttam, 1954; Makoto Kato, 1993). Probably the maximum fruit set in these two species may be due to the higher percentage of pollen viability and pollen germination. In *N. khasiana* also a high rate of seed viability has been observed.

8.4 *Pistillate flower*

In Nepenthaceae the development of Crassinucellate ovule, initiation of ovule primordium and inner integument is similar to that of other angiospermous families. But the initiation and derivation of inner integument is 5-6 cells below the apex of the ovular primordium. Thus, the initiation of inner integument demarcates the funiculus and the nucellus. The development of both the integuments confirms the observations of Bouman (1984). The outer integument develops later when the inner integument directly covers three fourth of the nucellar tissue in *Nepenthes khasiana*. But, the micropyle is formed by the inner integument alone.

The archesporial initial is always single and it originates from the third layer of the nucellus but not hypodermal. I did not come across any multicellular archesporium, as reported by Subramanyam et al. (1985) in *N. khasiana*. But the present study confirms the observations of Kaul (1982) and Subramanyam et al. (1985) with regard to the chalazal end of the ovule exhibits pronounced growth (see Plate-6.2.1d). Davis (1966) reported the formation of a single parietal layer in Nepenthaceae and in *Drosera burmanni* (Narasimhachar, 1949; Patankar, 1956). Kuhl (1933) and Kaul (1982) also reported the derivation of parietal cell and megaspore mother cell from the archesporial initial. But in *N. khasiana*

parietal tissue is absent. The archesporial initial directly functions as megaspore mother cell which is similar to that of Sarraceniaceae (Shreve, 1906), *Drosera rotumdifolia*, *D. indica* and *D. peltata* (Venkatasuban, 1950; Narasimhachar, 1951).

The megasporemother cell or meiocytes undergoes meiosis I and II result into 'linear' and 'T' shaped tetrads. In the former the micropylar megaspore is functional while in the later the functional megaspore is chalazal one. The 'T' shaped tetrad has also been reported in *Aldrovanda vesiculata* (Batygina and Yakovlev, 1985). Subramanyam et al. (1985) reported linear tetrad only in the same plant *N. khasiana*.

The embryo sac formation is polygonum type, eight nucleate in *N. khasiana*. Similar feature has been reported in Sarraceniaceae and *Droseraceae* (see Johri et al., 1992).

Apart from the Normal type or Polygonum type of female gametophyte development, an interesting feature which has also been observed in *N. khasiana*, is the formation of the tetrasporic embryo sac development which has not been reported so far in the order Sarraceniales. More than one type of embryo sac formation has also been reported in members of Ranunculaceae (Vijayaraghavan, 1970). Kapil and Prakash (1966) recorded five different types (Polygonum, Sndymion, Drusa, Penaea and Adoxa) of development in *Delosperma*

cooperi (Aizoaceae). Dharamadhaj and Prakash (1978) observed twin embryo sacs, one monosporic and the other bisporic, in the same nucellus of an ovule of *Capsicum annum* var. *acuminatum*. According to Swamy and Krishnamurthy (1975), Supra - Homeotypic (Polygonum type) category and Homeotypic category II (tetrasporic) have been observed in *N. khasiana*.

In majority of the angiosperm the three cells of the egg apparatus share the common wall surface, to form triangular arrangement (Willemse and Van Went, 1984; Raghavan, 2000).

By contrast in *N. khasiana* the two synergids are lying side by side and do not share common wall. Therefore in *N. khasiana* the two synergids are distinct and their filiform apparatus are oval in shape at the micropylar end. The egg is slightly projecting beyond the two synergids. The egg cell and two synergids are highly vacuolated and strongly polarized cells in *N. khasiana* (see Schulz and Jensen, 1968 in *Gossypium*; Mansfield et al., 1991 in *Arabidopsis*).

8.5 *Seed germination*

Endospermic seeds are reported in Nepenthaceae (Watson and Dallwitz, 1992; Subramanyam et al., 1995). But, in *N. khasiana*, the seeds are ex-endospermic (I used the term ex-endospermic where the

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Temperature plays an important role in germination. Imbibed seeds of *Pittosporum eugenioides*, *P. obcordatum* and *P. tenuifolium* showed almost no germination and marked a loss of viability after 3-4 months in storage under light condition at 21°C. In contrast, low temperature stratification storage for 8 weeks resulted in complete germination of seeds in the above mentioned species (Simon Moore, 1994). Germination percentages of four members of Asteraceae viz. *Millotia myosotidifolia, Podotheca gnaphalioides, P. chrysantha* and *Ursinia anthemoides* seeds were highest at average temperature 25°C. It is reported that *Nepenthes* seeds germinated in warm 32°C plus temperatures e.g., *N. ventricosa, N. bicalcarata* and *N. gracilis* (ICPS Seed Bank, 2002), but, *N. khasiana* seeds showed germination at 25°C ± 1°C temperature only (see Table-7.3). Generally, Seeds start to germinate after four or five weeks in the carnivorous plants (Mark Pogany, 1999). *Sarracenia leucophylla, S. flava, Pinguicula vulgaris* and *Drosera intermedia* seeds need a stratification period for good germination percentage except *N. ventricosa.*

Much of seed viability depends upon storage conditions. The ideal storage condition for seed is somewhere cool and dry. A capped jar in the refrigerator serves the purpose (Sherry Rindels, 1995). *Nepenthes seeds* will last upto 12-14 months provided the seeds are treated correctly. The seed of *N. fusca* showed at least 90% germination after 14 months (Phill Mann, 1998). Seeds of species such as *N. bicalcarata* and *N. ampullaris* seem to have a very short life span and it is a matter of weeks only (Phill Mann, 1998).

Nepenthes seeds are slow to germinate. Germination can take from four weeks to almost a year (ICPS Seed Bank, 2002). In vitro germination usually takes eleven days to three months. Some *Nepenthes* seeds still germinate after six to seven months. It may be mainly depends on how long we can keep the cultures going before they dry (Phill Mann, 1998). *N. khasiana* seeds took 27 days to 65 days in filter paper and 32 days to 60 days on sand to germinate. *Sarracenia leucophylla, S. flava, Pinguicula vulgaris* and *Drosera intermedia* seeds also start to germinate after four or five weeks (Mark Pogany, 1999). The fresher the seed, the stronger the seedlings and higher the rate of germination (ICPS Seed Bank, 2002). But, *N. fusca* showed 90% germination after 14 months (Phill Mann, 1998). In *N. khasiana* the germination percentage is 80.67 ± 2.4% which showed high seed viability after 8 weeks. The *N. khasiana*

seeds need moisture, 1000-1250 lux of light and 25°C of temperature. The planting medium should be moist but not soaking wet for the germination of seeds in *N. khasiana*.

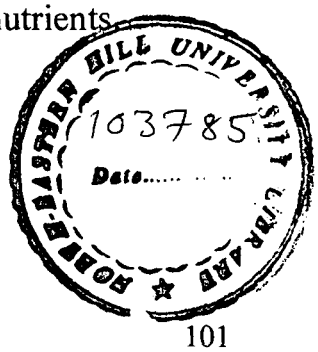
CHAPTER - 9

Summary and Conclusion

- The developmental events of anther formation and pollen release are exquisitely timed and choreographed; cell differentiation and dehiscence events occur in a precise chronological order that correlates with floral bud size.

- In *Nepenthes khasiana* Hk.f. a unique condition in which all the anthers are borne on a stalk called androphore and the number of anther range from 25-30. The anthers are bisporangiate. The tapetum is dual in origin. Microspore mother cells undergo meiosis within the callose wall, and the pollen grains are dispersed in tetrads.

- The ovules are crassinucellate, bitegmic, anatropous. The hypostase acts as a connecting link between the vascular strand and the embryo sac and helps in transportation of nutrients.



- The female archesporial cell directly functions as megaspore mother cell which gives rise to the megamametophyte (embryo sac) after meiosis.

- Two types of megagametophyte (embryo sac) development are seen in *Nepenthes khasiana* Hk.f.

1. Monosporic type	Polygonum type of embryo sac	8 nucleate condition
2. Tetrasporic type	Drusa type of embryo sac	16nucleate condition

- Inside the embryo sac, the egg apparatus, secondary nuclei and antipodals are located along the micropylar to the chalazal axis and within the egg apparatus the distribution of nuclei, vacuoles and other cell organelles show distinct polarity. which ones.

- The early embryogenesis is following the Caryophyllad type irrespective of the embryo sac organization.
- Caryophyllad type: ca: pco + pvt + phy + iec + co + s

  cb: does not contribute to the embryo proper

- There is a significant correlation among the pollen viability, pollen germination and seed germination percentage.

- Taxonomic consideration:

Johri et al. (1992) pointed out that scanty data are available on the embryology of Nepenthaceae. Based on the present investigations the following conclusion has been arrived:

Bentham and Hooker (1862-1893) put Nepenthaceae under Monochlamydae along with Aristolochiaceae and Cytinaceae.

Engler and Diels (1936) placed the family Nepenthaceae under the order Sarraceniales which includes Sarraceniaceae, Nepenthaceae and Droseraceae.

Dahlgren (1981) erects three orders: Sarraceniales (Sarraceniaceae); Theales (Nepenthaceae); Droserals (Droseraceae).

Based on the tetrad pollen grains, crassinucellate ovule, absence of endothelium, nuclear endosperm and Caryophyllad type of embryogeny the families Nepenthaceae and Droseraceae are mostly akin which can be placed together under the separate order Nepenthales in the subclass Dilleniidae as proposed by Cronquist (1988) and the family Sarraceniaceae as the sole family in the order Sarraceniales (as per Takhtajan, 1980).

The present study provides overall developmental events of Reproduction in *Nepenthes khasiana* Hk.f. Though a variety of experimental approaches have led to the characterization of gene expression patterns from flower to fruit formation (The plant cell, 1993; Developmental biology of flowering plants, Raghavan, 2000). However, as far as insectivorous plants are concerned only negligible works have been carried out so far. Therefore, further progress will require an integrated combination of approaches like physiological and molecular level to understand the reproductive mechanisms of insectivorous plants which will pave the way to know the evolutionary relationship within group as well as the other angiosperms, reproductive ecology and the attainment of insectivory etc.

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