STUDIES ON THE DEVELOPMENTAL AND HISTOCHEMICAL ANALYSIS OF DIGESTIVE AND SECRETORY GLANDS OF SOME INSECTIVOROUS PLANTS OF MEGHALAYA

BY

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DECLARATION

I, Ksh. Raseshowri Devi, hereby declare that the subject matter of this thesis entitled ***"Studies on the developmental and histochemical analysis of digestive and secretory glands of some insectivorous plants of Meghalaya"*** 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

Carnivorous plants are angiosperms which possess special morphological, anatomical and physiological properties. They attract their victims by means of scent, colouration and nectar (Llyod, 1942), and can trap and digest arthropods as well as some other small animals. After digestion, they take up minor quantity of the digested prey (Llyod, 1942; Juniper et al., 1989). This whole process is carried out in modified plant parts or traps to capture the preys, such as, pitchers in *Nepenthes*, modified leaves in *Drosera* and bladders in *Utricularia* spp. Therefore, different traps of insectivorous plants play key role in benefiting the supplements of essential nutrients.

Plant carnivory is one of the many possible adaptational strategies to overcome the unfavorable condition of micro habitats, such as, soil condition in which moisture content is very high, acidic and deficient in essential nutrients mainly nitrogen and phosphorous. Besides, carnivorous plants obtain water and minerals from the soil; they also extract nutrients from captured animals. This phenomenon of carnivory has fascinated the scientific community ever since Darwin (1875), who drew attention to it in his remarkable book "Insectivorous Plants". The general features of carnivorous plants have been reviewed in detail by Juniper et al. (1989).

On the basis of their trapping mechanism, Lloyd (1942) divided the carnivorous plants into two different categories, such as, carnivorous plants bearing *active traps* and carnivorous plants bearing *passive traps*. Later on, Heslop-Harrison (1976) grouped the genera of angiospermic carnivorous plants into four classes, such as, i) pitcher plants (e.g. *Nepenthes*), in which glandular surfaces bearing the secretory glands are immersed in the pitcher fluid which contain digestive enzymes. The fluid is secreted by the glands during the early growth phase of the leaves, and the products of the digestion are taken up continuously by the gland tissues which supplement nutrient requirements for several months; ii) plants with small traps in which microscopic preys are drawn together with water by active suction process (e.g. *Utricularia*), or passively by water currents (e.g. *Genlisea*) where, the digestive glands release their enzymes after such type of stimulation of the traps; iii) plants in which the secretion accumulates on the surface of the individual gland head forming globules (e.g. *Drosera*), where digestion of the prey begins when the prey comes in contact with the globules, and the digested products are absorbed through the glands and iv) plants in which the digestive glands remain dry until stimulated by the preys which are being captured (e.g. *Dionaea*).

Approximately 645 species of insectivorous plants have been reported so far from all over the world, and their distribution is mainly restricted to the tropical and sub-tropical belts (Venugopal et al., 2007). The Eastern Himalayan region of India has been identified as one among the 25 biodiversity “hotspots” of the world (Swaminathan, 1991;

Mohan Ram et al., 2001). Out of the six families of insectivorous plants available in the world, India has contributed three families namely: Droseraceae, Lentibulariaceae and Nepentheceae.

The Lentibulariaceae of insectivores has 4 genera, of which *Utricularia* and *Penguincula* occur in India. Lentibulariaceae accounts for *ca.* 280 species (Janarthanam and Henry, 1992). The 'Bladderworts' with nearly 210 species is the largest and widely distributed genus among the insectivorous plants. They are widely distributed in tropical, subtropical and a few species in temperate zones. Taylor (1989) included 33 species of insectivorous plants from India under the genus *Utricularia* in his taxonomic monograph, and later on, Janarthanam and Henry (1992) reported a total of 35 species of *Utricularia* from India. Out of the 35 species of insectivorous plants available in India, 18 species are endemic to particular localities.

North-Eastern Himalayan region of India is rich in insectivorous plants with maximum number, where, Joseph and Joseph (1986) reported eleven species of *Utricularia*, two species of *Drosera* and the unigeneric species *Nepenthes khasiana* Hk. f. *Nepenthes khasiana* Hk. f. of the family Nepentheceae is predominantly distributed only in Meghalaya state. Janarthanam and Henry (1992) also reported thirteen endemic species of *Utricularia* from Peninsular India and five species from the Eastern Himalaya particularly from Meghalaya state. The species reported from Meghalaya state include *U. brachiata* Oliver, *U. furcellata* Oliver, *U. kumaonensis* Oliver, *U. multicaulis* Oliver and *U. recta* Taylor.

In case of angiosperms, all the endemic species are considered neoendemics as they have taxonomically interrelated species complements and occur in the same or adjacent areas (Nayar, 1980). The genus *Drosera* (Droseraceae) commonly called 'Sundew' consists of approximately 170 species. In India three species of the genus *Drosera* namely, *D. indica* L., *D. burmanii* Vahl. and *D. peltata* Sm. var. *lunata* Cl. have been reported from different regions (Jayaram and Prasad, 2006). *D. peltata Sm.* var. *lunata* and *D. burmanii* Vahl. grow throughout the plains as well as on the hills up to an elevation of 3000 m asl. Mathew (1999) reported that in Western Ghats of India, *D. indica* flourished in open habitats during the rainy season. According to Kanjilal and Bor (1940) and Raseshowri and Venugopal (2006), *D. peltata* is commonly distributed in Assam, Meghalaya and Mizoram states of North-east India, which grows in similar habitats to *D. indica*. The distribution of *D. burmanii* Vahl and *D. peltata* var *lunata* are also being reported from Jowai, Jarain, Pynursla, Shillong and Raliang area of Meghalaya (Joseph and Joseph, 1986).

The unigeneric family Nepentheceae comprises of approximately 74 species (Willis, 1966; Kaul, 1982) of which *Nepenthes khasiana* Hk. has been reported from Khasi, Jaintia and Garo hills of Meghalaya state (Jain and Sastri, 1980; Joseph and Mani, 1982; Hooker, 1886). Few population of this species was rediscovered from certain other parts of Meghalaya such as Jarain, Sutnga, Magheshkola and Lawbah (Haridasan and Rao, 1985; Rogers and Gupta, 1989; Choudhury, 2000).

Rathore and Tandon (1995) conducted shoot bud culture of *N. khasiana*. While studying the vegetation dynamics in coal mines of Jaintia hills in Meghalaya, Lyngdoh et al. (1992) reported that *N. khasiana* could not regenerate even after ten years in those areas due to the unscientific coal mining activities. This mining led to the degradation and destruction of the natural vegetation of the hills including the suitable micro habitats of *N. khasiana*. Studies on the reproductive biology of the family Nepentheceae is very meager, however, the development of anther and microsporogenesis was studied by Venugopal and Rashi Devi (2003).

Venugopal (1999) observed two types of glands in the pitcher - one for secretion of digestive enzymes and another for the absorption of digested products. Pant and Bhatnagar (1977) studied the detail anatomy and morphology of *Nepenthes khasiana, N. gracilis, N. raffleisana and N. ampullaria*. However, studies on the developmental aspects of glands in *Nepenthes khasiana* Hk. f., *Drosera peltata* Sm., *Utricularia bifida* Sm. and *U. pubescens* Sm. have not been carried out so far. Numerous studies have been published on foliar structure and development (Hooker, 1859; Fenner, 1904; Macfarlane, 1908; Kuhl, 1933; Roth, 1953; Schmid-Hollinger, 1970, 1979), glandular structure and function in the pitchers (Llyod, 1942; Luttge, 1971; Amagase, 1972a; Amagase et al., 1972b; Toekes et al., 1974; Heslop-Harrison, 1976; Adams and Smith, 1977; Fahn, 1979), and on the animal inhabitants (nepenthebionts) and prey in the pitchers (Beaver, 1979; Wirth and Beaver, 1979; Erber, 1979).

The potential medicinal uses of the insectivorous plants found in North-East India still remain unknown. Many ethnic groups of Meghalaya have been using the liquid of unopened pitcher as remedy for stomach pain and cough. The herbal practitioners of Khasi and Jaintia hills prescribe the fluid of the pitcher for diabetic patients as well as to cure patients suffering from urinary tract diseases. Cystitis patients and patients with vaginal tumors or menopause are also treated with this fluid. For the external use, the fluid of both open and unopened pitchers is applied on infected parts of the body.

The local people of Garo hills grind the pitcher into powder along with the shells of molluscs and are used for the treatment of leprosy. However, application of parts of such insectivorous plant for the treatment of diseases mentioned is not scientifically proved. This needs detail scientific study which may throw some light on the pharmaceutical potentiality of the plant. Many of the secondary products of secretory glands may have varied types of biological activities including insecticidal and pesticidal property which lead to better understanding of plant protection mechanisms to crops.

Studies on all the aforesaid aspects, particularly digestive enzymes secreted by the glands of insectivorous plants are important. There are only a few studies and reports on the developmental aspects of digestive glands and histochemical localization in the gland tissues (Heslop-Harrison and Knox, 1971; Fahn, 1979; Heslop-Harrison and Heslop-Harrison, 1980, 1981). Therefore, this study aims to investigate the development of secretory and digestive glands, and histochemical localization in the gland tissues of the four representative species of the three families viz. Nepentheceae, Droseraceae and Lentibulariaceae.

The thesis embodied particular emphasis on the following objectives:

- to study the development of digestive and secretory glands in *Nepenthes khasiana* Hk. f., *Drosera peltata* Sm. Var. lunata Cl., *Utricularia bifida* Sm. and *U. pubescens* Sm.,
- to study the histochemical localization in the different types of traps of the above mentioned plants.

CHAPTER – 2

Review of Literature

Darwin (1875) and his son Francis Darwin (1878) and Busgen (1883) proved that *Drosera* benefited from supplementary nutrition from insects. Pringsheim and Pringsheim (1962, 1967) and Harder (1963) on *Utricularia* and of Harder and Zemlin (1967, 1968) on *Pinguicula* grown in axenic culture, also showed that insectivory may contribute to the supply of other mineral elements like phosphorous and nitrogen. The first observation on the structure and function of the trap in *U. dichotoma* was conducted by Thomson (1880). The vegetative organs produced by *U. dichotoma* are bladders (traps), runner stolons, anchor stolons (rhizoids) and foliage leaves (Lloyd, 1942; Taylor, 1989). Carnivorous plants do not require a high rate of supply of mineral nutrients from soils, as they are able to store and re-utilize nutrients efficiently from the digested preys (Dixon et al., 1980). A weakly developed root system is a common characteristic feature of most of the carnivorous plants (Luttge, 1983; Juniper et al., 1989). In different carnivorous plants, the ratio of root biomass to the total plant biomass ranges from 3.4% to 23% (Karlsson and Carlsson, 1984; Karlsson and Pate, 1992b; Adamec et al., 1992).

Insectivorous plants generally grow in moist, sunny but nutrient-poor acidic soils (Juniper et al., 1989; Albert et al., 1992; Ellison and Gotelli, 2002; Ellison and Farnsworth, 2005; Ellison, 2006). Few carnivorous plant species can survive in water stress condition (Lloyd, 1942; Dixon and Pate, 1978) and even in fire (Roberts and Oosting, 1958; Carlquist, 1976a; DeBuhr, 1976; Dixon and Pate, 1978). Physico-chemical properties including construction costs of the assimilatory organs i.e. leaf and pitcher of the lowland *Nepenthes* species were studied by Osunkoya et al. (2007). Resistance to heavy metal stress of the carnivorous plants of serpentine habitats, such as, *Darlingtonia californica*, *Nepenthes rajah*, *N. xalisaputrana*, *N. villosa* and *Stylidium* species have been reported by Kaul (1982); Gibson (1983b); Juniper et al. (1989); Clarke (2001a, b) and Darnowski (2002, 2003).

Adamec (2002) reported that the uptake of inorganic nutrients by the roots of *Drosera* species has to be stimulated by nutrients transported from the leaves, i.e. by successful prey capture. Recently, Fuchs and Haselwandter (2004) showed the association of vesicular arbuscular mycorrhiza in *Drosera intermedia* Hayne, an endangered carnivorous plant in Austria. There is some evidence of mycorrhizal association in the roots of *Nepenthes* (Clarke, 2001a, b). More information on the roots of carnivorous plants has been reported by Adlassnig et al. (2005). They summarized the available information on the morphology, contribution of the roots to the nutrient supply of the plants with the acquisition of nutrients via the traps and their tolerant ability to stress conditions in their habitats. Aquatic carnivorous plants e.g. *Utricularia*, *Genlisea* etc. lack root system completely, and other carnivorous plants, such as, *Drosera, Pinguicula, Dionaea,* etc. are hygrophytic in nature with poorly developed root systems (Adlassnig et al., 2005).

The developmental morphology of seedling and stolon of *Nepenthes alata* was briefly described by Merl (1915) and Lloyd (1942). Numerous studies on foliar structure and their function in the pitchers have been published (Lloyd, 1942; Luttge, 1971; Amagase, 1972a, b; Toekes et al., 1974; Adams and Smith, 1977; Fahn, 1979), and on the animal inhabitants (Nepenthebionts) (Beaver, 1979; Erber, 1979; Wirth and Beaver, 1979). Studies on the flowers, fruits and seeds of many insectivorous plants have been carried out, especially on the hybrids (Macfarlane, 1908; Stern, 1917; Danser, 1928; Daumann, 1930; Kuhl, 1933; Roth, 1953; Schmid-Hollinger, 1970). Kaul (1982) have reported on the flowers, fruits and inflorescences of *N. lowii* Hk. f. and *N. villosa* Hk. f.

Pant and Bhatnagar (1977) studied the anatomy and morphology of *Nepenthes khasiana, N. gracilis, N. rafflesiana* and *N. ampullaria.* Their study revealed that, in *N. gracilis* and *N. khasiana* the digestive glands occur on the inner surface towards the lower half of the pitcher, whereas in *N. rafflesiana* and *N. ampullaria* the distribution of digestive glands extend much higher towards the rim of the pitcher. The digestive glands In the pitchers of *N. gracilis* are larger towards the orifice of the pitchers, and they gradually narrowed near the bottom. SEM observation of pitcher in *N. alata* by Owen et al. (1999) revealed that the mature glands were distinct with oval depressions at their bases. They also observed autoflorescence of cutin or suberin in the endodermal layer using toluidine blue stain.

Different views were provided on the origin of the glands in *Nepenthes*. Three such views are: (i) the glands were originated from the subepidermal layer of pitcher (Meyen, 1837; Kothals, 1839 and Lloyd, 1942), (ii) these glands were strictly epidermal in origin (Oudemanns, 1864; Macfarlane, 1908 and Lloyd, 1942) and (iii) some underlying parenchyma cells also involve in the formation of these glands (Fenner, 1904). Heslop-Harrison (1975) divided the gland head portion into three main components viz. secretory, endodermal and communicatory which are made up of relatively massive tissues in the glands of *Drosera, Drosophyllum* and *Nepenthes,* and much simpler ones in *Pinguicula, Genlisea* and *Utriculatria.* The study also revealed that, the secretory cells in *Drosera* and *Drosophyllum* form a group of several layers and the layer is glomerulus in *Nepenthes.* In *Pinguicula*species the gland complex is derived from a single epidermal initial (Heslop-Harrison and Heslop-Harrison, 1980). The endodermal layer in *Nepenthes* is sub-spherical in shape, whereas in case of *Drosophyllum* it forms a shallow cup-like structure. The endodermal cell in *Pinguicula, Genlisea* and *Utricularia* is represented by a single cell, whereas in the case of *Dionaea* it is composed of two cells.

Drosera peltata is the most widely distributed carnivorous plant species in the United States (Gibson, 1992) which extends from the coast to the western plains, up to 1500 m asl. It occurs in all the states of Australian, New Zealand and Southeast Asia, from Japan to India (Erickson, 1968; Marchant and George, 1982). A comprehensive anatomical feature of glands of carnivorous plants was developed during the late 19th and beginning of 20th centuries (Goebel, 1891-1893; Fenner, 1904; Macfarlane, 1908). Literatures on such area were enriched by Lloyd (1933) by providing excellent reviews on insectivorous plants (Lloyd, 1942). Many species of *Drosera* have been cultured *in vitro* using various explants. Seed, leaf, shoot, flower bud, pedicel, stem and internode were used as explants for micropropagation of different species of *Drosera*, such as, *D. intermedia, D. pygmaea, D. rotundifolia, D. natalensis;* D. *burmanii* and *D. indica* L. (Burger, 1961; Harder, 1964a, b; Small et al., 1977; Simola, 1978 a, b; Van Waes, 1985; Cruoch and Van Staden, 1988; Kukulczanka and Czastka, 1988, 1991; Crouch et al., 1990; Bobak et al., 1995; Nalini and Murali, 2002; Jayaram and Prasad, 2005). Direct regeneration of plantlets from the leaf and shoot tip as explants in *Drosera anglica*, *D. binata* and *D. caueifolia* was carried out by Kawiak et al. (2003).

Fahn (1979) studied the stalked glands or tentacles present on the margin and adaxial surface of the leaf of *Drosera* species. According to him, the marginal tentacles of *Drosera* species are bisymmetrical, whereas those occurring on the leaf surface are radially symmetrical. Each stalked gland consists of a stalk and an oval-shaped head. The stalk consists of two layers of cells surrounding the central column of tracheids. The cells of the inner layer are longer than that of the epidermal cell. Ragetli et al. (1972) revealed the presence of intercellular spaces in the tentacle stalks which play an important role in the movement of the tentacles. The dorsal surface of leaf of *Drosera* species also contains small sessile glands (Fahn, 1979).

The wall protuberances of the sessile digestive glands in *Pinguicula* secrete mainly the slime for trapping the prey (Schnepf, 1961a), whereas the stalked glands or tentacles of *Drosera* species secrete mucilage as well as digestive enzymes which also absorbs the digested products (Fahn, 1979). The sessile digestive glands of *Pinguicula* do not secrete enzymes until they are stimulated by the captured prey (Darwin, 1875).

Scanning and transmission electron microscopic studies carried out by Heslop-Harrison (1970, 1975), and Heslop-Harrison and Knox (1971) further provided more understandings on the external morphology and internal organization of the traps of the secretory glands present in insectivorous plants. The walls of secretory cells possess spongy inner surfaces which act as transfer cell type (Heslop-Harrison, 1976). In *Pinguicula* and *Genlisea*, the cell wall ramification is more on the radial walls compared to that of tangential walls. Apart from the studies on wall ramifications in *Drosera*, *Drosophyllum*, *Dionaea* and *Pinguicula* carried out by many authors (Vogel, 1960; Schnepf, 1961a, b, 1963a, b, c; Scala et al., 1968; Schwab et al., 1969; Heslop-Harrison and Knox, 1971; Dexheimer, 1972 and Ragetli et al., 1972). Heslop-Harrison (1975) reported some other distinctive features on secretory cells. The cuticle of the epidermal cells in *Pinguicula* is uniform, whereas it shows discontinuity in the gland portions. In *Drosera* and *Drosophyllum,* the central core of tracheids is abutted directly from the endodermal layer of gland head. These tracheal cells connect to the vasculature of the stalk, whereas in the simpler sessile glands of *Pinguicula, Genlisea* and *Utricularia,* there is no direct connection between the vascular system and the endodermal cells. Fenner (1904) observed flat, elongated bell-shaped parenchymatous cells devoid of chloroplasts in the third layer of the tentacle. However, a very small number of plastids with very few lamellar structures are present in the secretory cells (Ragetli et al., 1972). Schnepf (1961a) and Williams and Pickard (1974) found that the radial cell walls of the head cells in *Drosera* are completely cutinized and is regarded as an endodermis. Schnepf (1976) also confirmed the presence of wall protuberances on the secretory cell walls. In *Pinguicula* the nucleus of the endodermal cell, contain massive protein bodies of distinct fibrillar organization (Schnepf, 1961b; Heslop-Harrison, 1975), but in case of *Drosera,* the endodermal cells contain many osmiophilic bodies (Heslop-Harrison, 1975).

Juniper and Gilchrest (1976) postulated two possible pathways of digested substances in *Drosera,* both starting with diffusion through the cuticular pores by the apoplast of the outer layer of secretory cell walls, and then into the cytoplasm via the symplastic movement into the endodermis. Helder (1967) revealed that the tentacles in *Drosera capensis* represent an active transport for the absorption and translocation of substances. The secretion product passes through the cuticle via pores in *Drosophyllum* (Schnepf, 1965b) and *Drosera* (Williams and Pickard, 1969), but it passes directly in *Pinguicula* (Schnepf, 1961a).

Utricularia khasiana is an allied species of *U. cymbantha* Oliver, which is a rare flowerless, rhizoidless, unobtrusive, filamentous, free-floating like algae. It grows in entangled masses in shallow water along the margins of a fresh water lake, (Wards lake, Shillong), which may also propagate vegetatively (Joseph and Mani, 1983). Fineran (1985) studied on the trapping mechanism in *U. dichotoma* in relation to the structure of its glands using transmission electron microscopy. Afterwards, Richter (1990) has studied the morphology of the bladders of *Utricularia* using scanning electron microscopy. Most of the work on the genus *Utricularia* is restricted to its physiology (Pringheim and Prinsheim, 1962; Sorenson and Jackson, 1968; Syderham and Findlay, 1975; Sasago and Sibaoka, 1985); seed biology (Farooq, 1964; Abraham and Subramanyam, 1965) and taxonomy (Taylor, 1964).

The ultrastructure and development of external glands in *U. minor* L. has been examined by Kristen (1974). Fineran and Lee (1974a, b) investigated the ultrastructure and organization of hairs and external glands in traps of *U. monanthos* Hk. f. (Fineran, 1980). Cheema et al. (1992) explored the developmental and histochemical localization of the bladder of *U. stellaris.* According to them, the mature bladder of this plant species shows bifid and quadrifid hairs with two antennae at the mouth region. The progressive developmental sequence of the bladder and its histochemistry revealed that the opening and closing mechanism of the orifice is controlled by the various types of bladder cells. During the development and differentiation of the terminal cells of the gland of *U. monanthos*, the nucleoli are prominent and are actively engaged in RNA and protein synthesis (Fineran and Lee, 1975). The Architecture and development of the transfer cell walls in the secretory hairs of *U. intermedia* and pavement epithelium of *U. volubilis U. stygia* and *U. intermedia* have been studied in detail by Plachno and Jankun (2004) and Plachno et at. (2005a, b).

Insectivorous plants secrete extracellular digestive enzymes including amylase (Jones, 1971; Heslop- Harrison and Knox, 1971; Varner and Mense, 1972), pectinases (Morre, 1968), cellulases (Fan and MacLachlan, 1967; Thomas et al., 1974) and other carbohydrases (Jones, 1971; Cortat et al., 1972; Stieglitz and Stern, 1973; Reid and Meier, 1973b), nucleases (Jones and Price, 1970; Heslop- Harrison and Knox, 1971), proteases (Schwab et al., 1969; Heslop- Harrison and Knox, 1971). Secretion via vesicles derived from Endoplasmic reticulum has been suggested for α- amylase (Vigil and Ruddat, 1973), glucanase (Cortat et al., 1972), wall degrading enzymes (Bal and Payne, 1972) and other extracellular materials (Franke et al., 1972a). Secretion via vesicles derived from Golgi apparatus has also been suggested for ribonuclease (Jones and Price, 1970), proteases (Schwab et al., 1969), and phosphatases (Palisano and Walne, 1972; Dauwalder et al., 1969, 1972).

Luttge (1964) concluded that the fluids present in the closed pitchers of *Nepenthes* contain enzymes, whereas the digestive glands of *Drosera, Drosophyllum* and *Pinguicula* release enzymes only after stimulation by the captured prey. Pitcher plants use a passive method of attraction and entrapment of preys (Heslop-Harrison, 1976; Fahn, 1979; Slack, 1980; Juniper et al., 1989). Specifically, the traps of pitcher are modified epiascidiate leaves, in which the adaxial surface curls around and fuses to form the inner wall of the pitcher tube, also the digestive glands are covered with the individual cuticular droplets (Juniper et al., 1989). In *Nepenthes*, nectars are secreted by the double-edged rim or collar called peristome of the pitcher (Hooker, 1859; Lloyd, 1942) and acidic fluid is secreted at the bottom of the pitcher (Morrissey 1955; Higashi et al., 1993; An et al., 2001).

The acidic fluid of the pitcher plays an important role in the digestion and absorption of prey based on the following facts: (i) the pH level of pitcher fluid is reduced when preys are accumulated in the fluid (Luttge 1964), (ii) the optimum pH of proteolytic enzymes in pitcher fluid is around 2-3 (Steckelberg et al., 1967; Nakayama and Amagase, 1968; Athauda et al., 1998), and (iii) proton gradients across the plasma membrane are generally used to derive uptake of many solutes, ions, metabolites etc. (Michelet and Boutry, 1995). Vines (1901) suggested the name 'nepenthin' for the digestive enzymes present in *Nepenthes*. The pitcher fluid contains proteases and chitinases (Vines, 1901; Amagase et al., 1969; Amagase 1972a; Amagase et al., 1972b; Toekes et al., 1974). These enzymes are presumably secreted by the plant, although bacterial activity or a combination of these two is not neglected (Prankevicius and Cameron, 1991; Lennon, 1995). The glands of *N. alata* secrete degradative enzymes, including ribonucleases and phosphatases (Matthews, 1960), proteases (Amagase, 1972a; Jentsch, 1972) and possibly chitinases (Amagase et al., 1972b), as well as ions including chloride (Luttge, 1971) and calcium (Massa, 1998). However, the origin of these enzymes in pitcher plants has been recently brought into question (Luciano et al., 1998; Santo et al., 1998). The physiological activities of these glands are similar to the specialized salt glands (Thomson, 1975) and the absorptive trichomes of Bromeliads (Benzing et al., 1976).

Vassilyev and Muravnik (1988) described the ultrastructural changes in the sessile glands of *Pinguicula vulgaris* L. from their initiation up to a fully mature condition in the unstimulated leaves. Ultrastructural studies indicate that the digestive enzymes are synthesized on the rough ER of the head cells, and are transferred to the cell wall, particularly into the slime layer and vacuoles. Heslop-Harrison and Heslop-Harrison (1980, 1981) concluded that in *Pinguicula vulgaris* L., hydrolytic enzymes e.g. acid proteases, acid phosphatase, esterase etc. which involve in the digestion of prey are synthesized prior to stimulation, namely, during the maturation of sessile digestive glands, and are stored until the prey is captured.

Vines (1897) showed that fluid from unopened pitchers digested protein when 0.2% HCl was added. On the other hand acidified water does not digest protein. Vines (1897) demonstrated that digestion occurred even when bacterial poisons were present. Couvreur (1900) claimed that the results of Vines were due to the interaction of reagents, but he neither offered rigorous support for his theory, nor his arguments were sound.

The expression of transporter genes for nitrogenous compounds (ammonium, amino acids and peptides) has been reported in the pitchers of *Nepenthes alata* (Schulze et al., 1999). The mRNA of the ammonium transporter has been shown to be localized in the head cells of the digestive glands. It was reported that the transport of ammonium depends on the proton motive force (Ninnemann et al., 1994). These findings also suggest that the acidification of pitcher fluid is an important need for the nutrient uptake in the pitchers, in which a plasma membrane proton pump is thought to be involved in the acidification of pitcher fluid.

An et al. (2002) found that the fluid pH decreased when prey accumulates in the pitcher fluid of *Nepenthes alata*. This study also revealed that plant cells have three types of proton pump, namely, the plasma membrane H^+-ATPase (PM H^+-ATPase), responsible for acidification of the pitcher fluid; the vacuolar H^+ -ATPase (V- ATPase), and the vacuolar H^+ -pyrophosphatase (H^+-PPase). Presence of aspartic proteinase was reported in the pitcher fluid (Athauda et al., 1998; Toekes et al., 1974). It was recognized that pitchers consists of morphologically distinct zones with different functions (Lloyd, 1942; Juniper et al., 1989). Amino acids are always present in floral nectaries (Baker and Baker, 1975, 1983), and are generally consistent with species specific (Baker and Baker, 1977, 1986). The overall concentration of amino acids differs among species and related to the principal pollinator (Baker and Baker, 1973, 1986).

Pitcher nectar usually contains carbohydrates (Juniper et. al., 1989), but quantitative study on sugar content is very meager. William et al. (1997) identified and quantified nine amino acids in extra floral nectar produced by pitchers of *S. purpurea* using reverse phase high performance liquid chromatography with phenyl-isothiocyanate derivatization. Insects are attracted by extrafloral nectar, flower fragrance (Moran, 1996), or UV light absorption patterns near the pitcher opening (Moran, 1996; Moran et al., 1999). The upper portion of the pitcher is frequently lined with an exfoliating epicuticular wax (Phillipps and Lamb, 1996) that creates surface slippery to arthropods (Lloyd, 1942; Juniper et al., 1989; Riedal et al., 2003; Gaume et al., 2004). Observations of insects placed on *Nepenthes alata* pitchers suggested that the inner waxy pitcher wall is the most important surface zone for the initial capture of insects (Cresswell, 1998).

High contrast UV patterns have also been identified in the trapping structures of several carnivorous plant genera (Joel et al., 1985). Two studies have shown UV patterning in Old world pitcher plants of the family Nepentheceae. Glossner (1992) identified a high-contrast UV pattern in a pitcher of *Nepenthes alata* Blanco, and Moran (1996) demonstrated the presence of UV patterns on the pitchers of *Nepenthes rafflesiana* in Borneo, Indonesia. In *N. rafflesiana* the rim of the pitcher mouth, or peristome (which contains the main nectaries of the pitcher and is the site of primary interest to most of the visiting invertebrates) is UV absorptive and stands out in contrast to other areas of the pitcher, which are reflective. Jonathan et al. (1999) studied the pitcher morphology and spectral reflectance characteristics in the six species of *Nepenthes* from northwest of Borneo (*N. ampullaria, N. albomarginata, N. bicalcarata, N. gracilis, N. mirabilis var. echinostoma and N. rafflesiana*). The six species showed a wide range of reflectance patterns. The pitcher of N. raffleisana possesses the greatest degree of fitness between the contrast maxima and insect sensitivity maxima, in the UV, blue and green regions of the spectrum. Based on the morphological and reflectance analyses Jonathan et al. (1999) hypothesized that the pitchers of *N. rafflesiana* would be more attractive to anthophilous (flower-visiting) invertebrates than the sympatric *N. gracilis*.

Pad- bearing insects deliver secretory fluid onto the contact zone to increase the attachment force between the pad and substrate (Ishii, 1987; Kosaki and Yamaoka, 1996; Gorb, 1998; Eisner and Aneshansley, 2000; Voetsch et al., 2002; Federle et al., 2002). Insects are thus able to increase the capillary and viscous forces contributing to overall adhesion.

The cytochemical evidence of cell wall ingrowths in the secretory cells of Drosophyllum (Schnepf, 1963a) and in *Drosera* (Dexheimer, 1976) suggests that these ingrowths in these genera have a composition similar to those of *Pinguicula*. The staining properties show that they are rich in pectic polysaccharides. Dexheimer (1972,

1976) found numerous dictyosomes in the cytoplasm of the secretory cells of *Drosera* during the production of the mucilage droplet. But in *Dionaea*, Schwab et al. (1969) found appearance of large vesicles only during the phase of secretion-resorption-regeneration cycle. Histochemistry has gained a momentum to investigate the localization of enzymes in the tissues and organs (Jensen, 1962). The cryostat technique is superior to earlier used methods for cytochemical studies of enzymes (Avers, 1958; Avers and King, 1960; Vanden Born, 1963; Koenigs, 1966). Heslop-Harrison (1975) revealed the integral function of glandular portion in the production of hydrolytic enzymes. In *Drosera capensis,* Dexheimer (1978a, b) cytochemically demonstrated the presence of acid phosphatases in the mucilage secreted by the digestive glands.

Biochemical studies on *D. rotundifolia* (Clancy and Coffey, 1976) have shown that gland stimulation increases production of acid phosphatase. Acid phosphatases have been implicated in polysaccharide transport in gland cells of *D. capensis* (Dexheimer, 1978 b) and protein secretion in the glands of *Dioneae* (Robins and Juniper, 1980). Thus, wall associated acid phosphatase activity in unstimulated glands of *D. rotundifolia* may have a role in mucilage transport while the increased production of phosphatases following stimulation may primarily be involved in prey digestion and inward transport of digested products. Studies of Gahan and Maple (1966) on *Vicia faba* L. roots, reported particulate activity of acid phosphatase in the root meristem. The absence of enzyme activity in the promertistem region in most roots indicates metabolically inactiveness of the particular region. This support the concept of quiescent centre in roots (Clowes, 1956). Intense activity of acid phosphatase was demonstrated in the procambial strands and in the differentiating treachery elements and these observations were found similar to the observations of the earlier workers (e.g. Gahan and Maple, 1966; McLean and Gahan, 1970; Benes and Opatrna, 1964).

Sirova et al. (2003) studied enzymatic activity of five hydrolases fluorometrically present in the fluid collected from traps of four aquatic *Utricularia* species and the water medium in which the plants were cultured. Highest enzyme activity of phosphatases and β-glucosidases was recorded, while the activities of α-glucosidases, β-hexosaminidases and aminopeptidases were usually lower.

DeZeeuw (1934) and Steckelberg et al. (1967) reported proteolytic activity in both sterile and contaminated pitcher fluid. Based on repeated chromatography on highly cross linked Sephadex and Sephadex ion exchanger, Jentsch (1972) isolated and purified of the proteolytic enzyme and other unknown proteins in closed pitchers. He named the acid protease in *Nepenthes* as nepenthacin. Enzymatic properties of protease present in the pitcher fluid are very similar to pepsin (Jentsch, 1972). De Zeeuw (1934) and Morrissey (1955) postulated the existence of similarity between the pitchers of *Nepenthes* and the mammalian stomach.

Nakayama and Amagase (1968) described the partial purification of acid protease from opened pitchers. Acid phosphatase activity has been reported in the golgi apparatus of developing pollen (Maruyama, 1974) and other plant cells and tissues (Charvat and Esau, 1975; Bentwood and Cronshaw, 1976; Noguchi, 1976). Acid phosphatase in the vesicles may be involved with the digestion of sequestered cytoplasmic material leading to the differentiation of autophagic vacuoles (Matile, 1975). Toekes et al. (1974) have listed two proteases in the pitchers of *Nepenthes macferlanei*, a major enzyme Nepenthesin with molecular weight of 59000 kda, and a minor one of 21,000 kda.

CHAPTER – 3

Materials and Methods

Location & physiography of the study site

The study was carried out in Meghalaya state of Northeast India, which is a land-locked territory lying between 25°47' and 26°10' North latitudes and 89°45' and 92°47' East longitudes. The state is encompassed with three major hills namely, Khasi Hills, Jaintia Hills and Garo Hills. The altitude of the state varies between 100-1900 m asl. It has a 496 km long international boundary with Bangladesh in the south and west, whereas the state of Assam surrounds it from the north and east. The Meghalaya plateau is highly dissected and has irregular terrain in the western and northern sides, and has a continuous escarpment with steep slopes marking the southern side. The state has seven districts with thirty-eight blocks with a geographical area of 22,429 sq km (Plate- 3.1).

Collection of plant materials

Pitchers of *Nepenthes khasiana* Hk. f., leaves of *Drosera peltata* Sm. var. lunata Cl., bladders of *Utricularia bifida* Sm. and *Utricularia pubescens* Sm. were collected at different developmental stages (from very young to matured stage) from different localities of Meghalaya (Plate- 3.1). Modified leaves of *Drosera peltata* Sm. were collected from Sohrarim, Janiaw, Lawbah and NEHU campus area of East Khasi Hills, whereas pitchers of *Nepenthes khasiana* Hk. f. were collected from Jarain area of Jaintia Hills and Lawbah area of East Khasi Hills.Plant samples of *Utricularia pubescence* Sm. and *Utricularia bifida* Sm. were collected from Janiaw, adjoining areas of Shillong and NEHU campus of East Khasi Hills.

Developmental study of glands

After collection of the plant materials, suitable samples were selected and fixed in FAA (Formalin 5cc: Acetic acid 5cc: 70% Ethanol 90cc) and 2-3% glutaraldehyde (prepared in phosphate buffer at pH 7.2) in the field itself. The plant samples fixed in FAA were used for microtomy by the usual dehydration method using tertiary butyl alcohol series followed by impregnation with paraffin wax (Johansen, 1940; Sass, 1953; Berlyn and Miksche, 1976; William, 2000). The glutaraldehyde fixed samples were dehydrated in propanol and embedded in glycol methacrylate (Technovit 7100). The paraffin and Technovit (7100) blocks were trimmed and sectioned at a thickness of 7-10µm size. The sections were stained by following the staining procedures:

1) Safranin fast green (Johansen, 1940)
2) Methyl green pyronin G (Jensen, 1962)
3) Total insoluble polysaccharides: Periodic acid Schiff's (PAS) method (Jensen, 1962 and Feder and O'Brien, 1968).
4) Aniline blue and Cotton blue (Johansen, 1940; O'Brien and McCully, 1981)
5) Nucleic acids: Feulgen method (Gomori, 1952).
6) Total proteins: Mercuric bromophenol blue method (Mazia et al., 1953).

7) Cellulose: Calcofluor white method (Hughes and McCully, 1975; O'Brien and McCully, 1981).

Morphology and ultrastructure of glands

Studies on morphology and ultrastructure of digestive glands of different traps at different developmental stages have been carried out by using Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM), respectively.

Scanning Electron Microscopy (SEM)

The following procedure was adopted for SEM study:

i) Pitchers of *N. khasiana* and leaves of *D. peltata* were cut into small squares of 1x1mm length with razor blades and fixed in 2-3% glutaraldehyde prepared in 0.1M phosphate buffer (pH 7.2) at 4°C for 4 hrs, thoroughly washed in 0.1 M phosphate buffer and post fixed in 1% OsO4 for 2 hrs. The bladders of *Utricularia* species were fixed in 3% glutaraldehyde in phosphate buffer at pH 7.2 for 2hrs. at room temperature or in ethanol/ acetic acid (3:1).

ii) After fixation, the plant materials were dehydrated using increasing concentration of acetone (30%, 50%, 70%, 80%, 90%, 95%- two changes in every 15 min. in each step; single change in dry acetone for every 15 min.).

iii) Dehydrated materials were dried in Critical point in Tetramethylsaline (TMS) solution (Dey et al., 1989; Dey, 1993; Dey and Deb, 1993).

iv) Dried materials were fixed on Eikon ion sputter, JFC- 1100 and were coated with thin layer of gold vapour (300 Å layer).

v) Gold coated plant materials were observed under Joel (JSM-6360) Scanning Electron Microscope (SEM).

Transmission electron microscopy (TEM)

The following procedure was employed for the TEM study:

i) Pitchers of *N. khasiana* and leaves of *D. peltata* were cut into small squares of 1x1mm with razor blades and fixed in 2-3% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) at 4°C for 4 hrs. and 2 hrs. in case of bladders of *Utricularia*. The samples were thoroughly washed in 0.1 M phosphate buffer and post fixed in 1% OsO4 for 2 hrs. for *Utricularia* bladders and 4 hrs. in case of *N. khasiana* and *D. peltata*.

ii) After post- fixation, the samples were gradually dehydrated with acetone for 10-15 min. in each step. Three changes were made in absolute acetone.

iii) The plant materials were embedded in Araldite CY 212. Ultrathin sections were cut at approximately 60- 90 nm (600 Å - 900 Å) through a Sorvall MT-2 ultramicrotome using a glass or diamond knife, and then stained with 2% aqueous uranyl acetate and lead citrate. Samples were observed under Zeiss EM-109 TEM.

Histochemical study of the glands

Study in fresh material

Since enzymes are very sensitive to chemical fixatives unfixed frozen sections or free hand cut sections of fresh materials were used for the localization of enzyme activities. The plant tissues containing the portion of digestive glands were processed as quickly as possible after its collection. The following procedure was followed to obtain frozen sections by using cryostat:

i) Different developmental stages of pitchers of *N. khasiana*, leaves of *D. peltata* and bladders of the *Utricularia* species were treated with antifreeze 10% Dimethyl Sulfoxide (DMSO) at 4°C for 2 hrs.

ii) Tissue samples were embedded in solidified 15% antifreeze gelatin medium (15 gm of gelatin dissolved in 100 ml of 0.8% DMSO and solidified at 10°C).

iii) Gelatin blocks containing the tissues were cut at 12µm thickness using cryostat.

iv) Frozen sections were kept on slides and subjected to the following histo- enzymological tests.

1. Lipase activity: Tween method (Gomori, 1952):

Freshly hand cut sections of pitcher of *N. khasiana* and leaf of *D. peltata* were incubated in the incubating medium (2 ml of 10% $CaCl_2$, 2 ml of 5% Tween 60 and 40 ml of distilled water were added to 5 ml of 0.5 M tris i.e. hydroxy methyl amino methane buffer of pH 7.2-7.4) at room temperature for 3 hrs. The incubated sections were washed in distilled water and treated with 1% lead nitrate for 15 min. After washing in distilled water, the sections were immersed in 15% ammonium sulphide for 1- 2 min. Sections were again washed in distilled water and mounted in glycerine jelly.

Colour indication: Brownish- black deposits in the tissues indicated the presence of lipase. In case of control test, sections were treated with incubation medium without the substrate.

2. Esterase activity: Indigogenic method (DeJong et al., 1967):

Freshly hand cut sections were incubated in the incubating medium (8 mg of Indoxyl acetate dissolved in 0.5 ml of ethanol, 0.1M of 0.25 ml each of Potassium ferrocyanide and ferricyanide were added in 10 ml of Tris- HCl buffer of 0.1M, pH 7.0) at 35°C for 30 min. After incubation the sections were rinsed in distilled water and mounted in glycerine jelly.

Colour indication: Bluish- granular product in the tissues indicated esterase activity. In case of control test, sections were treated with incubation medium without the substrate.

3. Acid phosphatase activity: Metal salt method (Gomori, 1952):

Freshly hand cut sections were incubated at 35°C for 30 min. in reaction mixture (incubating medium was prepared by mixing 10 ml of acetate buffer at pH 5.0, 30 mg of sodium β- glycerophosphate and 10 mg of lead nitrate). After incubation, sections were rinsed twice in distilled water for 5 min. each and then, transferred to freshly prepared 1% ammonium sulphide solution for 5 min. Finally sections were washed in distilled water and mounted in glycerine jelly.

Colour indication: The reaction product in the tissue sections appeared brown- black. In case of control test, sections were treated with incubation medium without the substrate.

4. Alkaline phosphatase activity: Tetrazolium Method (Lojda et al., 1979):

Freshly hand cut sections were incubated for 20- 30 min. at room temperature in the freshly prepared incubating medium (incubation medium was prepared freshly by dissolving 2 mg of 5-Bromo-4- chloro-3-indoxyl phosphate and 10 mg of Nitro- BT in 0.5 ml of N, N, dimethyl formamide and10 ml of 0.2 M Tris – HCl buffer, pH 9-9.2). The sections were wash in distill water after incubation and mounted in glycerine- jelly.

Colour indication: The reaction product in the tissues appeared blue. In case of control test, sections were treated with incubation medium without the substrate.

5. Peroxidase activity: Benzidine method (DeJong et al., 1967):

Freshly hand cut sections were incubated in the reaction mixture (incubating medium was prepared freshly by mixing 5 ml of saturated Benzidine, 5ml of 0.2% hydrogen peroxide and 5ml of phosphate buffer, 0.1 M, pH 7.0) for 5- 10 min. at 30°C, rinsed in distilled water and mounted in glycerine jelly.

Colour indication: The sites of the enzyme activity indicate blue which after sometimes turns to brown. In case of control test, sections were treated with incubation medium without the substrate or heat inactivated tissues were incubated in the normal incubating medium.

6. Protease activity: Substrate film method (Fratello, 1968):

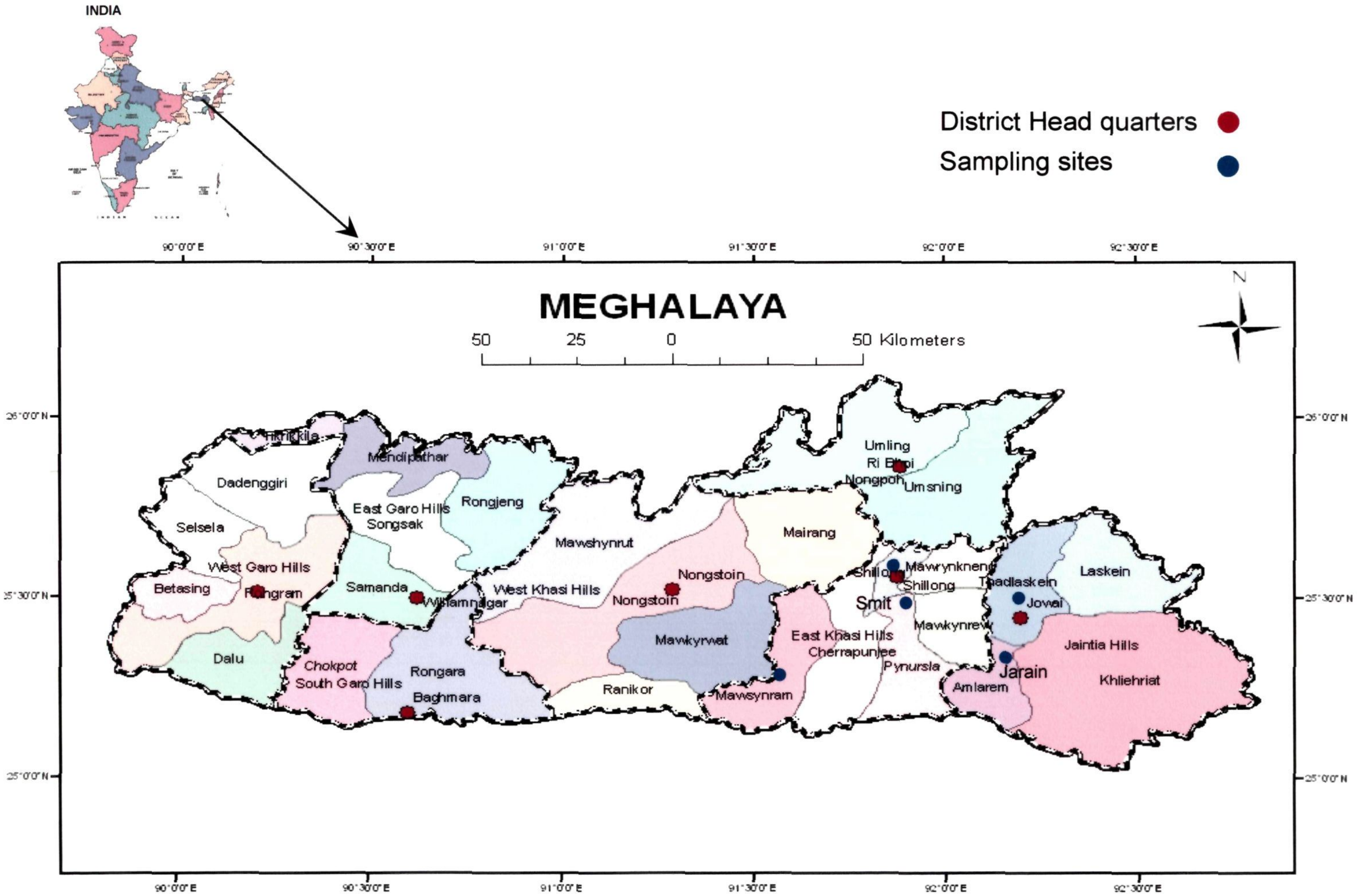
Proteolytic activity of plant tissue sections can be localized by allowing the protease to leak out into a protein- film and demonstrate the affected areas of the film.

Reaction: Freshly collected plant tissues were mounted on a piece of processed black and white films which are previously dampened with 0.2 M phosphate buffer at pH 7.6. The film should absorb the buffer entirely until the gelatin is softened and no free fluid should be present when the tissues were applied. Precautions should be taken up because these free fluid ions may facilitate diffusion of enzymes. After incubation in 35°C for 20- 40 min., the film is allowed to dry under natural conditions. The tissues should be protected from the dust while mounting with Canada balsam.

Indication: The diffusing proteolytic enzyme digests the gelatin layer, hence, sites of enzyme activities show transparency.

Photomicrographs were taken by using Nikon E 600 and Leitz Wetzlar Germany (Type 307-083. 103) fluorescence microscope.

Plate- 3.1



Map of Meghalaya in India showing sampling sites

CHAPTER- 4

Nepenthes khasiana Hk. f.

4.1 Introduction

Pitcher plants (*Nepenthes*) are climbing or scandent evergreen shrubs bearing with cylindrical pitchers (Plate- 4.1 a). They are distributed in tropical region having high humidity and frequent rainfall. There are approximately 74 species under the genus *Nepenthes* which belong to the unigeneric family *Nepentheceae* (Willis, 1966; Kaul, 1982). Clarke and Wong (1997) and Clarke (2001a, b) reported *ca.* 94 species of *Nepenthes* occurring in the tropics of Southeast Asia, which collectively support 52 endemic species of *Nepenthes*.

High degree of endemism of *Nepenthes* in Southeast Asiatic region suggests the center of origin of the genus in and around Borneo (Juniper et al., 1989). In addition to the distribution of 31 species of *Nepenthes* to the island of Borneo, many other species of the genus also extended westward as isolated population such as *N. khasiana* in Assam (India), *N. distillatoria* in Sri Lanka, *N. pervellei* in the Seychelles and *N. madagascariensis* and *N. masoalensis* to Madagascar (Juniper et al., 1989). The distribution of *N. vieillardii* extended eastward and reaches to New Caledonia (Kurata, 1984).

Nepenthes khasiana Hk. f. is an endemic and endangered insectivorous plant growing in Khasi, Jaintia and Garo hills of Meghalaya state of North Eastern India (Hooker, 1886; Jain and Sastri, 1980; Joseph and Mani, 1982). The population of this species is confined in certain localities namely Jarain, Sutnga, Jowai, Magheshkola and Lawbah (Haridasan and Rao, 1985; Rogers and Gupta, 1989; Choudhury, 2000; Raseshowri and Venugopal, 2006). Pitchers are modified leaves in which the adaxial surface curls around and fuses to form the inner wall of the pitcher tube. The pitchers initiated as a tiny, flattened end of a tendril. The youngest pitchers are dark brown in colour which cling on the leaf tendrils, but turned green during maturation. Matured pitchers bear two distinct portions: the upper half with waxy surface and the lower bulbous portion with numerous glands on the inner surface of the pitcher, which secrete several enzymes particularly proteolytic enzyme.

The glands of *N. alata* secrete digestive enzymes including ribonucleases and phosphatases (Matthews, 1960), proteases (Amagase, 1972a; Jentsch, 1972) and chitinases (Amagase et al., 1972b). From the medicinal point of view, Laurent Legendre (1999) manufactured pills of *Nepenthes* as meant for homoeopathic treatment. Local herbal practitioners of Meghalaya have been using the fluid of pitcher of unopened *N. khasiana* as remedy for stomach pain, cough and curing from diabetes.

Varying numbers of digestive glands are present in the pitchers of *Nepenthes*. Danser (1928) estimated *ca.* 6000 glands per sq. cm in *N. sterophylla* and 100 glands in *N. gracillima.* During the elongation phase of development of the pitcher, it starts secreting fluid and approximately 10-15 ml of the fluid is accumulated in a mature pitcher.

The area that eventually forms the opening of the pitcher is smooth and tightly sealed with a flap like lid. Initially the lid is green and later on it turns into reddish green colour. The mouth of the pitcher of *N. khasiana* possesses a single edged rim or collar called peristome (Plate- 4.1 b). Several studies have been carried out on foliar structure and functions of pitcher (Lloyd, 1942; Luttge, 1971; Amagase, 1972a; Toekes et al., 1974; Adams and Smith, 1977; Fahn, 1979), and on the animal inhabitants i.e. Nepenthebionts including spiders, mites, ants, fly, midge larvae and crabs (http://www.nepenthesinfauna/Wikipedia/the freeencyclopedia) and about the existence of preys in the pitchers (Beaver, 1979; Wirth and Beaver, 1979; Erber, 1979). Pant and Bhatnagar (1977) studied the detail anatomy and morphology of *Nepenthes khasiana, N. gracilis, N. rafflesiana* and *N. ampullaria.* This study revealed that the digestive glands in *N. gracilis* and *N. khasiana* occur in the inner surface of the lower portion of the pitcher, whereas in case of *N. rafflesiana* and *N. amullaria* the distribution of digestive glands extend much higher to a level slightly below the rim of the opening. In the pitchers of *N. gracilis* the digestive glands are larger in size towards the upper glandular portion which becomes gradually smaller in size towards the bottom of the pitcher. There are three views regarding the origin of the glands in the genus *Nepenthes.*

Heslop-Harrison (1975) observed three main components of digestive gland viz. secretory, communicatory and endodermal layers. He observed that the glands of *Drosera, Drosophyllum* and *Nepenthes* were made up of relatively massive tissues, whereas the glands of

Pinguicula, Genlisea and *Utricularia* were ontogenically simple. The study also revealed that in *Nepenthes* the secretory cells are glomerulus and the endodermal layer is sub-spherical in shape. In *N. alata*, the formation of the gland appears to be secretion prior to maturity. However at maturity, secretion is blocked by an endodermal layer which limits the function of the gland to absorb nutrients (Owen and Lennon, 1999). Thus, the glands of *N. alata* are specialized for the bidirectional transport of materials.

Various studies has been conducted on *N. khasiana* such as reproductive biology (Rashi Devi, 2004), different types of glands (Venugopal, 1999), shoot bud culture (Rathore and Tandon, 1995). But, less attention has been given on their developmental aspects. Therefore, this chapter deals with the structure, ontogeny and histochemical localization of digestive enzymes in the digestive glands of the pitcher.

4.2 Gland architecture: Number and position of Gland

The digestive gland appears glomerulus on the inner surface of the pitcher of *Nepenthes khasiana*. The glands occupy more than half of the pitcher, which can be discernible externally by a slightly elevated circular line demarcating the inner waxy and glandular surfaces of the pitcher (Plate-4.1 b). The average number of glands per sq. cm ranges from 100– 120, and the average diameter of the matured digestive glands ranges from 140- 150 µm. The density of the glands in matured pitchers is considerably higher in the distal part of the digestive zone than that of the bottom of the pitcher. The depth of the depressions at the base of each gland decreases from top to the bottom of the digestive zone. The glands are sessile discoid or spherical in shape, attached to a disc- shaped depressions or groove partially covered with an overlapping epidermal flap or outgrowth (Plate-4.2 a, c). In other words, the digestive zone bears small oval like depression, and each having a single gland at its base. In cleared leaf, the digestive glands are dispersed in the areoles (the smallest areas which are bounded by the thinnest branches of the veins), mostly at the tip of the terminal vein endings. Occasionally, two glands may occur within a single areole (Plate-4.2 b).

4.3 Internal organization of the gland

The glands are multicellular body consisting of three different cell layers. The outermost columnar single layered secretory head portion which secretes the digestive enzymes; two to four communicatory or the middle layers consisting of rectangular shaped cells and single layered endodermal of foot layer (Plate- 4.3 a). The endodermal layer controls the movement of the secreted products and at the same time it serves as a pathway for the absorbed digested materials into the leaves. There is direct communication between the middle layers and the inner vasculature through the radial walls of some cells of the endodermal layer (Plate- 4.3 a). It is also observed in the florescent micrograph using calcofluor white.

4.4 Outermost secretory or head cells

The secretory layer is a group of several cells (100 - 150 cells approximately) in a single tier. The cells are radially elongated and columnar shaped. Irrespective of their numbers and position, each secretory cell is characterized by possessing walls with a spongy inner surface of the transfer cell type (Plate- 4.4 a). The wall ramifications are present on the radial walls thereby the total surface area is increased by a plate- like extensions, visible with SEM micrographs (Plate- 4.2 c & Plate- 4.3 c). The outer surface of the secretory head cells are covered with thick cuticle which extends the outer walls of the adjoining epidermal cells as well. The outer surface of the secretory cells possesses pores of various sizes ranging from 4 to 6.0µ in diameter (Plate- 4.3 c).

4.4.1 Ultrastructure of secretory head cells

In the young stage, the secretory cells are with dense cytoplasm because of abundant ribosomes. The nucleus generally located in the middle of the cytoplasm which is granular. The nucleolus is electron dense. The vacuoles are smaller and distributed throughout the cytoplasm. These vacuoles also contain smaller vesicular bodies or multivesiculate bodies. The proplastids are variously shaped with few starch grains. The Endoplasmic reticulum is rough ER. The mitochondria are distributed throughout the cytoplasm, whereas the plastids are few in number.

Mitochondrion with numerous cristae indicates that they are physiologically active in releasing energy in the form of ATP. Some of the mitochondria are lobed in structure. The cytoplasm contains numerous vesicles of various sizes which might have probably derived from the golgi apparatus. These vesicles contain digestive enzymes and possibly released its contents in between plasmalemma and cell walls i.e. the periplasmatic space. Microtubules are found parallel to the plasmalemma (Plate- 4.4 c). The widths of the microtubules are 200 Å in diameter. The movement of the vesicles towards the plasmalemma is probably through the mediation of microtubules oriented longitudinal axis of the plasmalemma (Plate- 4.4 b). Electron dense spherical to oval spherosomes or granules are present through out the cytoplasm of the secretory cells. Vesicles of different sizes are present within the granules which might probably be the storage sac of digestive enzymes secreted by the golgi bodies and endoplasmic reticulum (Plate- 4.4 d). Because of the fusion of the vesicles with plasmalemma, the plasmalemma gives a highly convoluted structure (Plate- 4.4 a, e). At this stage there is no secretion of digestive enzymes. The cell walls between the adjacent head cells are thin. The intracellular spaces are filled with cell wall pectic materials. This stage indicates the absence of secretory process in the head cell.

In the matured secretory cells, the cytoplasm is dense as in the case of young stages. The vacuoles are larger in the mature stage. The plastids are well differentiated with granular lamellar structures and two or three amyloplasts (Plate- 4.4 b). The plasmalemmas are distinct which are not highly convoluted with dense cytoplasm due to the presence of abundant ribosomes. The thickness of the cuticle is 1- 1.5 µm. The outer cellulosic wall is fibrilar and amorphous (Plate- 4.4 f). Before the cells start secreting enzymes, the cuticle is uniformed in thickness throughout the head cells or the secretory cells. At the time of secretion, the cuticular layers break to form irregular openings on the tangential surface of the head cells. Secreted products from the cells are noticed in the radial wall as well as at the outer cuticular cell wall. Generally, the released materials in between the plasma lemma and cell walls (i.e. periplasmetic space) of the radial walls of the secretory or the head cells are released through apoplastic movement (Plate- 4.4 e, f).

4.5 Communicatory layers or the middle layers

Two to three layered communicatory or the middle layers lie in between the secretory head cells and the endodermal layer. The inner basal layer of communicatory tissue also acquires sometimes casparian thickenings. However, tangential walls of middle layer are devoid of casparian strips. In *N. khasiana,* the secretory glands have connection with vascular supply of the pitcher through communicatory and endodermal layers. The communicatory layers sometimes act as specialized "reservoir" cells which serve as intermediate links.

4.5.1 Ultrastructure of the Communicatory or middle layer

The middle layers consist of two to four rows of cells in which the spherosomes are more in number. The vesicles are mostly distributed towards the peripheral region of the cytoplasm. They are also present inside the spherosomes. The nucleus is amorphous with distinct nucleolus. The plastids are distinct with amyloplast and their number is less compared to that of the head cells. On the other hand, the mitochondria are more or less uniform in number and their distribution in the cytoplasm. About two to four vacuoles occupy the middle layers so that the cytoplasm becomes a thin film positioned in the periphery with numerous dictyosomes and its derived vesicles. The radial walls are much thicker than the tangential walls with distinct plasmodesmetal connections between adjacent cells (Plate- 4.5).

4.6 Endodermal or foot layer

The endodermal layer consists of a single row of cells separating the outermost secretory head cells and the communicatory layer from the underlying parenchymatous tissues. The cells of this layer are rectangular in shape and are in continuation with the epidermal layer. Heavy cutinization of the casparian strips of the endodermal cells is demonstrated by florescence microscope (Plate- 4.3 b). Continuity of the casparian strip with the cuticle of the epidermal cells on the one hand and the thinner cuticle of the secretory head cells on the other hand is distinctly traced in *N. khasiana* (Plate- 4.3 b). This layer is directly in contact with the treachery elements. The cell wall composition in the middle layer as well as in the foot layer is of suberin or cutin in nature which is determined by auramine O or the calcofluor fluorochrome (Plate- 4.3 b).

4.6.1 Ultrastructure of Endodermal or foot layer

Electron micrographs show that the cell wall is mainly composed of cellulose in the form of long thin threads called microfibrils. The microfibrils are about 10 nm in diameter with the clear unstained area representing the 'elementary fibrils' of 2.7 nm to 4.0 nm in diameter (Plate- 4.6). The cell wall consists of two different wall lamellae, the innermost wall lamellae adjoining the plasmalemma. The cellulose microfibrils widely dispersed over a wide angle, oriented more or less transversely to the axis of growth. It shows greater disorientation from the plasmalemma. In the outer lamellae, the microfibrils lie either at random or with the tendency to axial orientation.

In *N. khasiana,* the nuclei of the endodermal cells are usually seen to contain massive protein bodies with distinct fibriller organization. Invariably, the mitochondria are larger with well developed cristae and are more or less uniform in number and distribution. The cytoplasm is dense with plastids containing smaller vacuoles and more distinct spherosomes. The tangential wall is comparatively thinner than the radial wall, which implies that the transport of materials is through the plasmodesmetal connections in between the communicatory layers and the inner tissues of pitcher (Plate- 4.6).

4.7 Development of the secretory gland

Since the glands are placed equidistantly, the development of the glands is almost uniform. The glands are radially symmetrical and the initiation of the glands on the inner surface of the pitcher starts when the pitchers height is about of 1.5 mm in length. About seven to ten epidermal cells on the inner surface of the pitcher are involved for the development of glands. These cells are distinct from the adjoining epidermal cells due to their large size with dense cytoplasm and prominent nucleus with nucleolus (Plate- 4.7 a, b). Before the onset of the cell division, these initial cells elongate radially and undergo transverse division (periclinal) to form two rows of cells, which become similar in size and structure after radial elongation and differentiation. In other words, these two rows of cells become isodiametric. During the bilayered stage, the height of the pitcher is 2 mm in length. The division of the upper cells at various stages is presented in Plate- 4.7 c & d. Almost 15 to 18 cells are being differentiated to outer secretory portion. At this stage, due to the upward growth of the glands and the growth of the adjacent epidermal cells to gain the overall pitcher height, a depression or a groove is formed at the base of the gland making the gland more prominent (Plate- 4.7 e). The upper tier once again elongates radially and undergoes one more periclinal division to form three tiered arrangement of the gland cells (Plate- 4.7 g, h). Mostly, the uppermost tier which is facing towards the cavity of the pitcher elongates forming fan shaped structure; occasionally some cells in the secretory layer also undergo transverse division to form four layered structure in some places (Plate- 4.7 i). Simultaneously, some of the secretory cells also divide tangentially to increase the surface area of the gland (Plate- 4.7 j). After attaining the four layered gland structure, differentiation of various layers of the gland starts. The middle tier divides transversely, so that, the middle layers form two tiered arrangement. Further enlargement of the gland is brought by typical longitudinal divisions in the secretory head cells and, both longitudinal and oblique divisions in the middle cells forming the communicatory layers. The innermost single celled layer differentiates into the innermost endodermal or foot layer (Plate- 4.7 k, i). All these patterns of cell divisions are regular and synchronous. In the pitcher, the glands are interconnected by the inner vasculature.

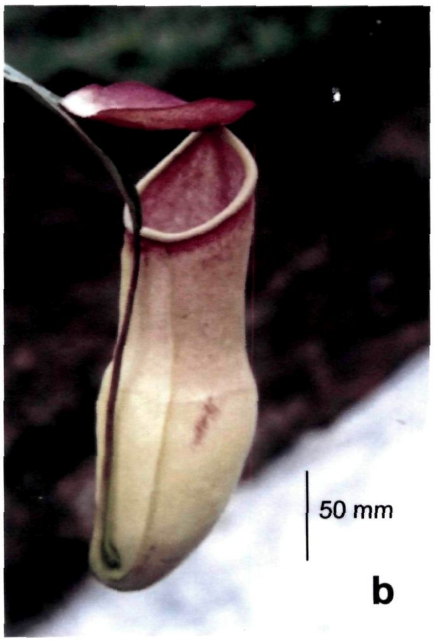
Plate- 4.1

a Habit of *Nepenthes khasiana* Hk. f. The plant is a climbing or scandent evergreen shrub with cylindrical pitchers, which are of modified leaves.

b An enlarged view of pitcher. An opened pitcher with the well demarcation of two regions, the upper waxy and non-glandular region, and the lower glandular region of the pitcher.

Plate- 4.1 *Nepenthes khasiana* Hk. f.

Habit of plant



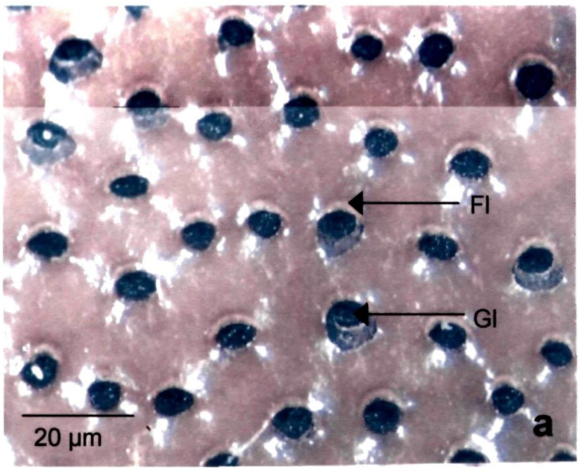
Enlarged view of a pitcher

Plate- 4.2

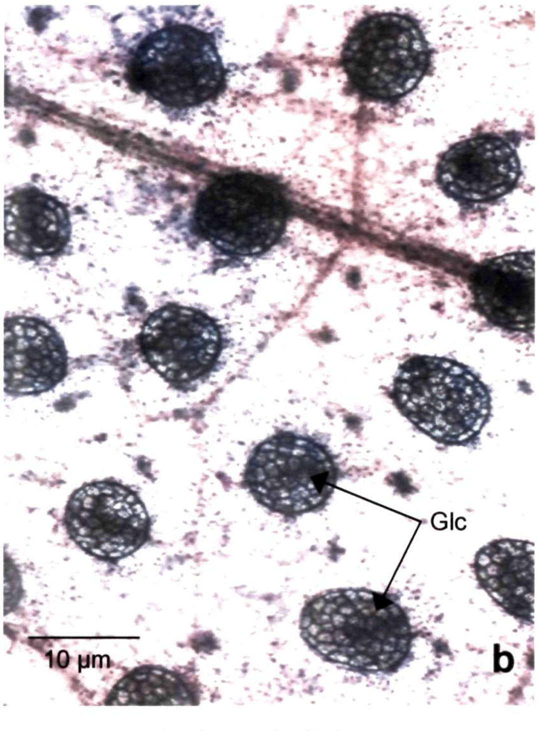
a Glands on the inner surface of the pitcher. Each gland (Gl) is partially covered with the flaps (Fl) which are of epidermal in origin.

b A cleared pitcher. 100- 120 gland cells (Glc) are present on the outer surface of the gland. The glands are uniformed and equidistantly placed.

Plate- 4.2 Number and position of gland

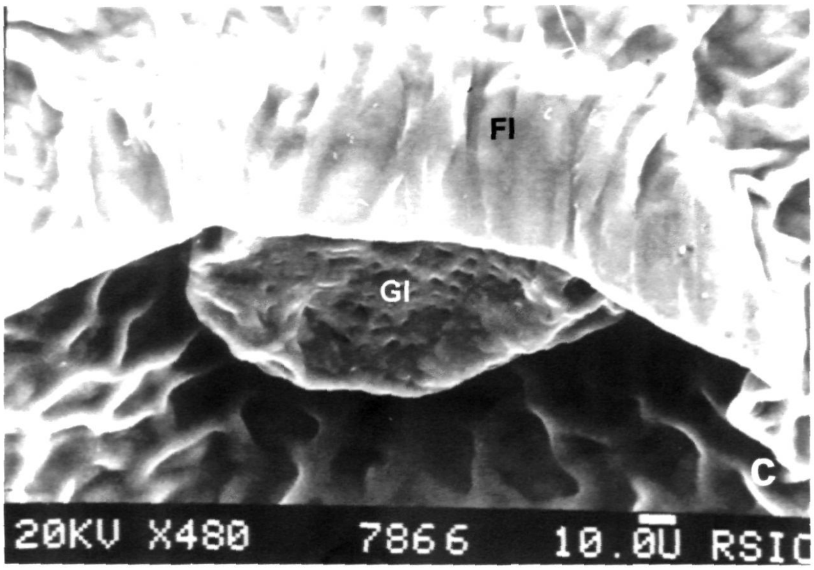


Glands on the inner surface of the pitcher



A cleared pitcher

Plate- 4.2 Number and position of gland



c SEM of a single gland (Gl) showing partially covered epidermal flap (Fl)

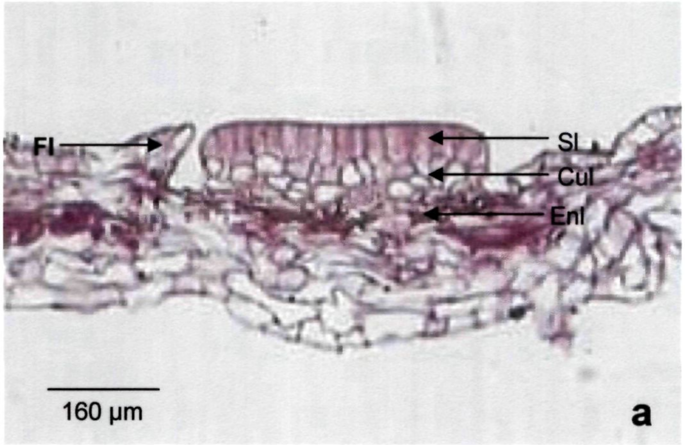
Plate- 4.3

a Longitudinal section (L.S.) of gland showing the three layers of gland viz. the outermost secretory layer (Sl), three to four layered middle or communicatory layers (Cul) and the innermost single endodermal layer (Enl).

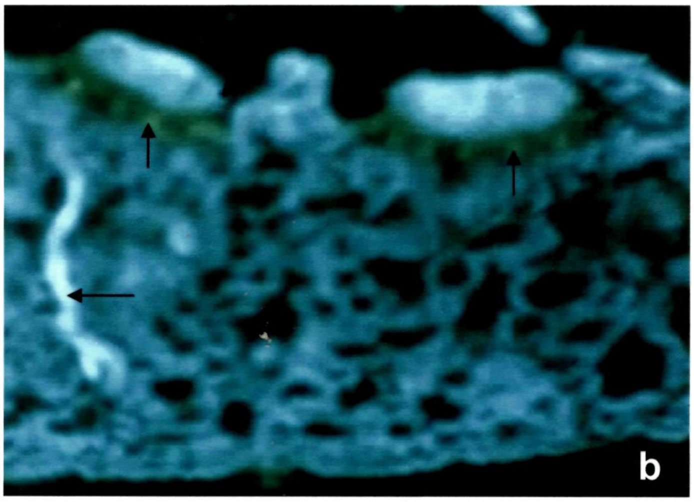
b Fluorescence micrograph of digestive glands. Note the casperian strips on the radial wall of endodermal layer with calcofluor white and the vasculature (arrows ahead).

c SEM of a gland surface showing the undulated outer tangential wall of secretory layer with its porous nature (Pr).

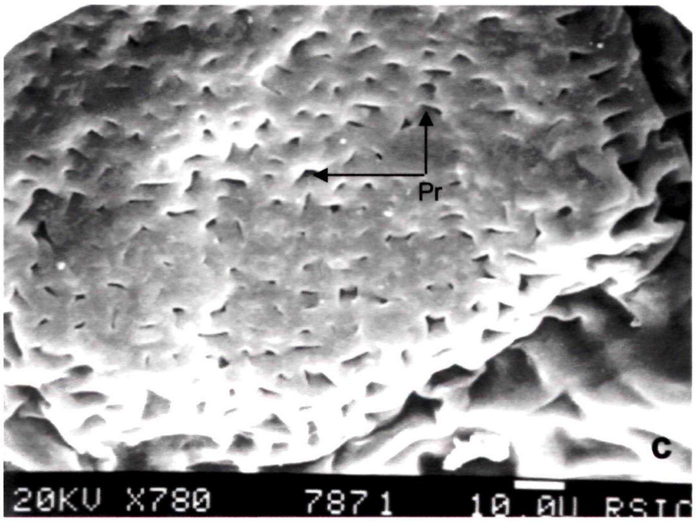
Plate- 4.3 Internal organization of the gland



L.S. of gland



Fluorescence micrograph of digestive glands



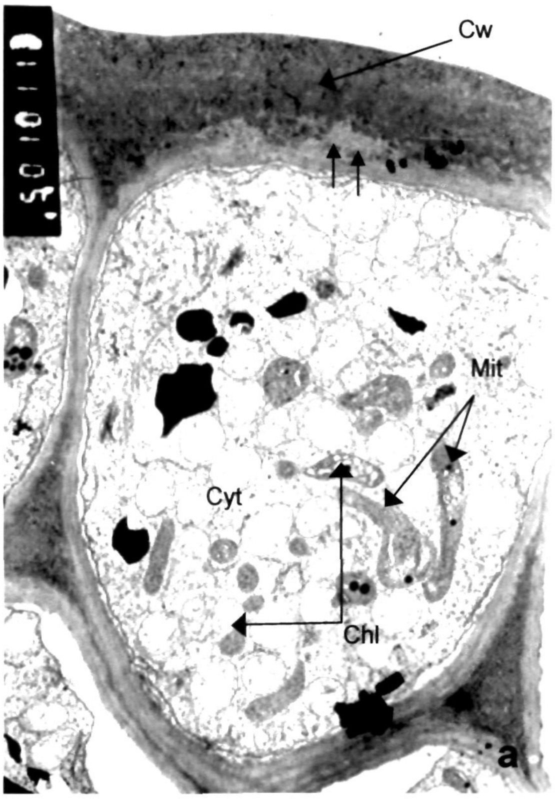
SEM of a gland surface

Plate- 4.4

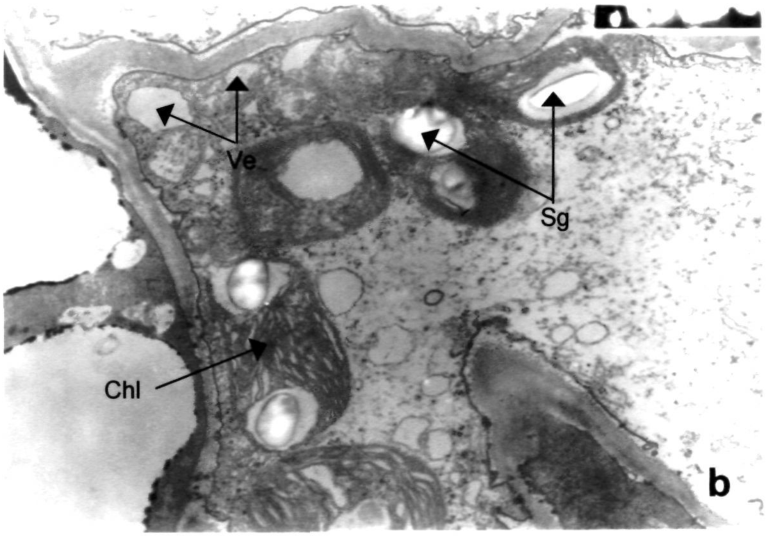
a TEM of single secretory cell with dense cytoplasm, (Cyt) numerous chloroplast, (Chl) mitochondria, (Mit) and thick outer spongy cell wall (Cw) pointed by double arrows ahead.

b TEM of chloroplast. Highly differentiated chloroplast (Chl) with Starch grains (Sg). Numerous vesicles (Ve) located towards the plasma membrane.

Plate- 4.4 Ultrastructure of internal organization of secretory layer



TEM of single secretory cell



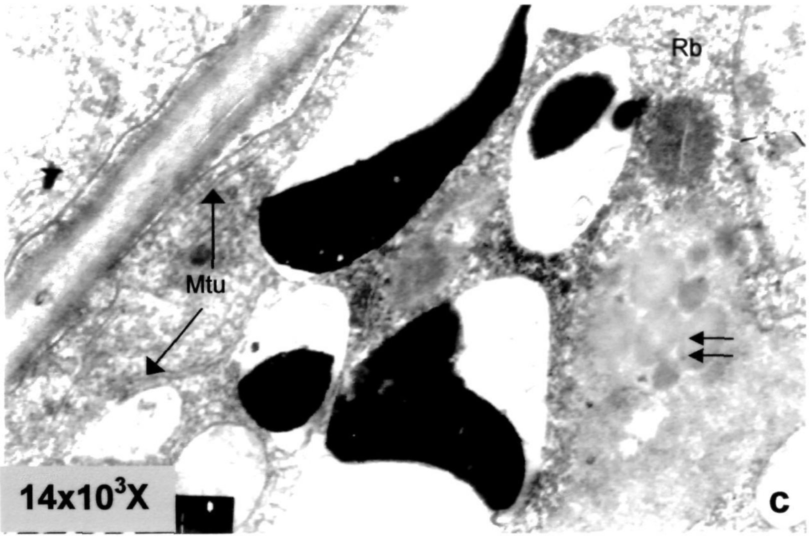
TEM of chloroplast

Plate- 4.4

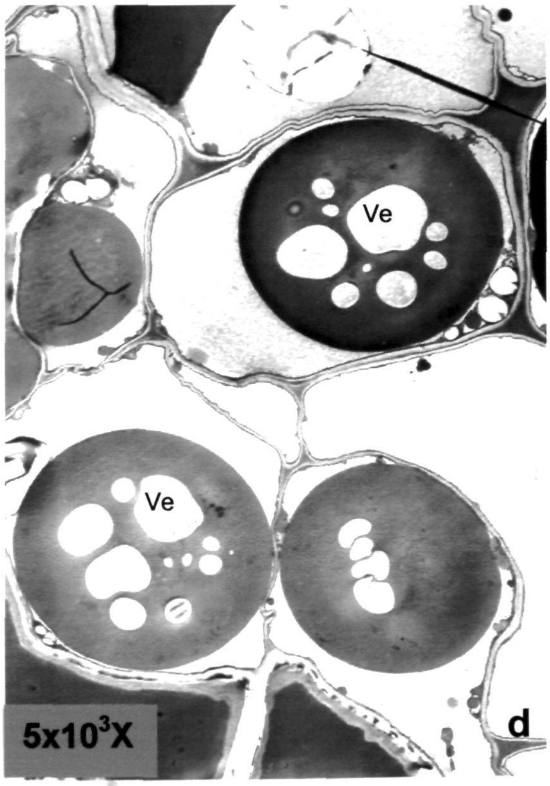
c TEM showing the microtubules. Secretory cell with microtubules (mtu) arrange parallel to the plasmalemma. The dense cytoplasm due to high ribosomal density (Rb) and multivesiculate bodies (double arrow heads).

d TEM of cross section of granules. Granules in the gland cells possess numerous vesicles (Ve).

Plate- 4.4 Ultrastructure of internal organization of secretory layer



TEM showing the microtubules



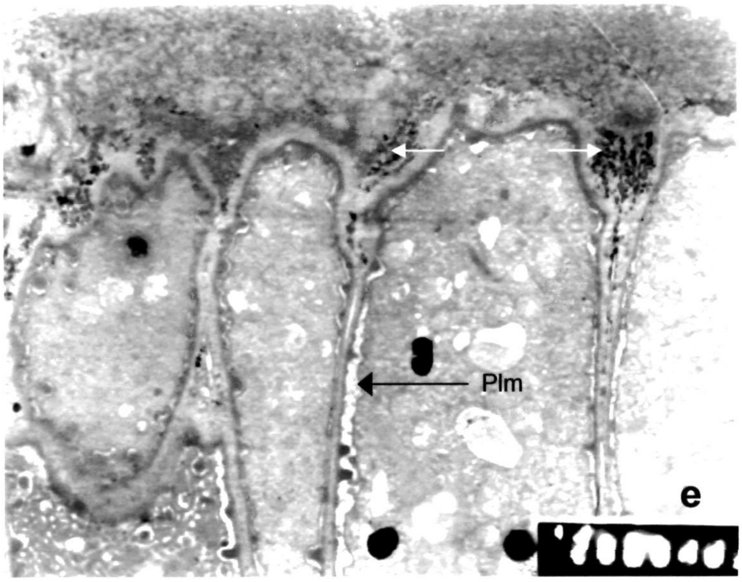
TEM of cross section of granules

Plate- 4.4

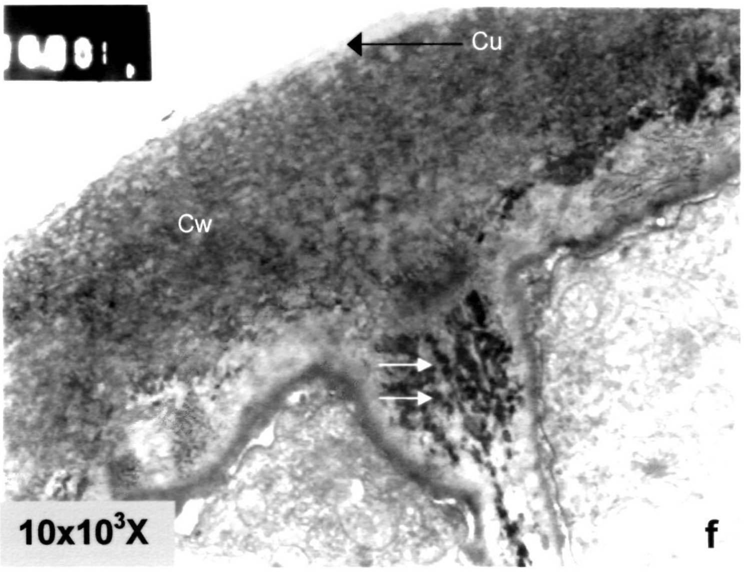
e TEM of secretory cells layer. Secretory cells having the highly convoluted plasmalemma (plm) and apoplastic movement of substances in between the cells of secretory layer (arrows ahead).

f TEM of enlarged cell wall. Cellulosic cell wall (Cw) of the secretory cell with cuticle (Cu) and the secreted materials (double arrow aheads) pass through the radial walls by apoplastic movement.

Plate- 4.4 Ultrastructure of internal organization of secretory layer

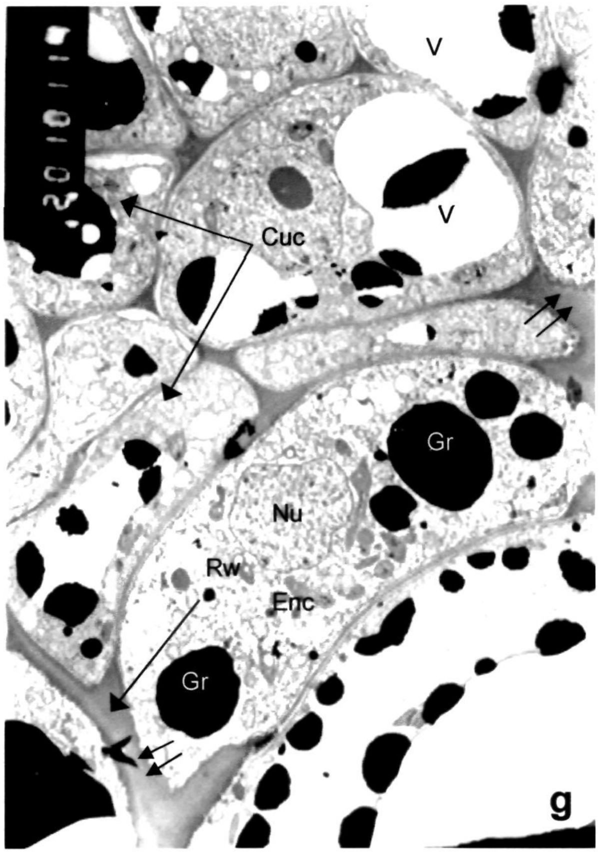


TEM of secretory cell layer



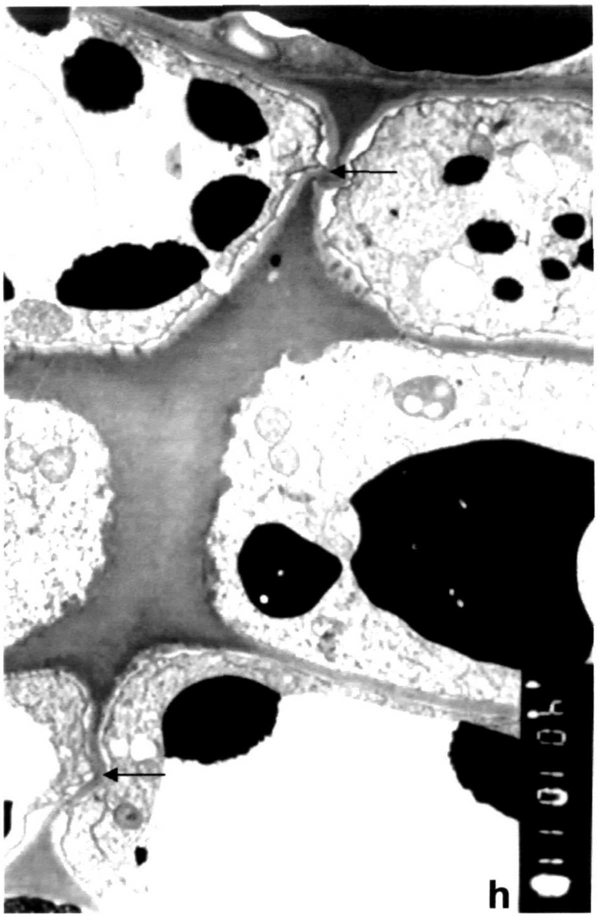
TEM of enlarged cell wall

Plate- 4.5 Ultrastructure of internal organization of communicatory layer



g TEM of the communicatory layer showing communicatory cells (Cuc) with large vacuoles (V). Note more number of electron dense granules (Gr) and prominent nucleus (Nu) in the endodermal cells (Enc) with casparian thickenings (pointed by double arrows) in their radial walls (Rw).

Plate- 4.6 Ultrastructure of internal organization of endodermal layer



h TEM of endodermal layer showing the thick radial wall due to the casparian thickenings and thin tangential cell walls. Note the thin radial cell wall through which the cells are interconnected with plasmodesmata (arrows).

Plate - 4.7

a Initiation of gland. The initiation of the gland starts from the inner epidermal cells of the pitcher. Almost seven to ten cells are involved. All the cells are with prominent nucleus and nucleolus.

b SEM of inner surface of pitcher. Dark spots in the SEM represent the site of initiation of the gland from the inner epidermis of the pitcher.

c Two layered stage of gland. The nucleus of the upper cell is in metaphase stage.

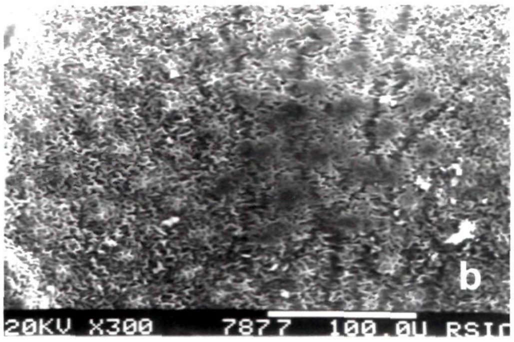
d Dividing inner layer. The inner cells in telophase stage of cell division.

e SEM of gland with groove. Grooves or depressions formation occur when the secretory layer differentiates into 15 to 18 cells.

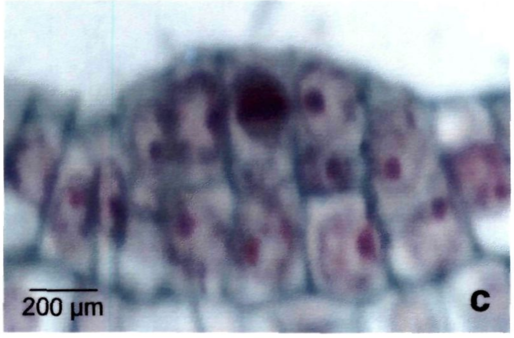
f Complete division of the inner cell. The cells in the inner layer complete the division forming three layered stage.

Plate- 4.7 Development of gland

Initiation of gland

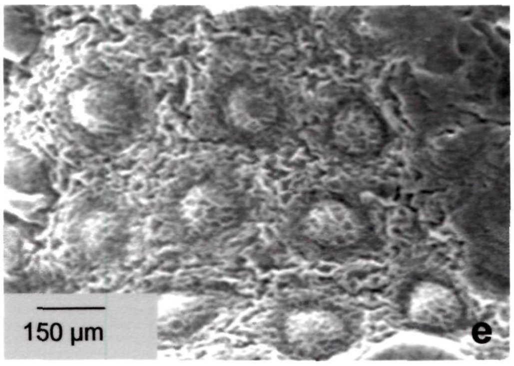


SEM of inner surface of pitcher



Two layered stage of gland

Dividing inner layer



SEM of glands with groove

Complete division of the inner cell

Plate- 4.7

g & h Division in the third layer. Note the metaphase stage in the third layer. All the cells show the dense cytoplasm, prominent nuclei and nucleoli.

i Three layered stage. The gland shows three layers which are ready to divide further.

j Four layered stage. The cells of the outermost layer divide tangentially to increase the surface area of the gland.

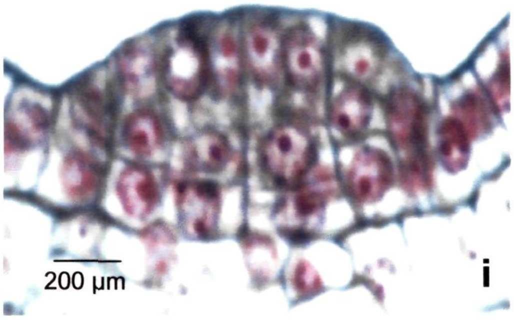
k Differentiated gland layer. A fully Differentiated digestive gland stained with PAS (Periodic acid Schiff's reagent), showing the different layers of the gland viz. the outermost secretory layer (Sl), the middle three to four communicatory layer (Cul) and the inner endodermal layer (Enl).

l SEM of differentiated gland. SEM represents the fully differentiated four layered stage of the gland.

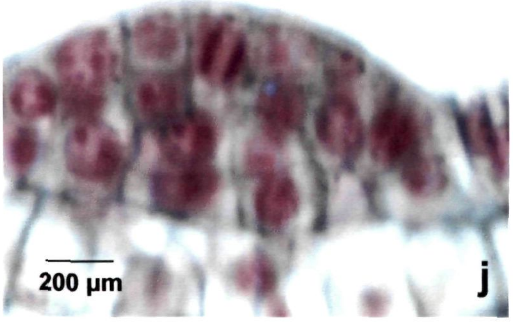
Plate- 4.7 Development of gland

Dividing third layer

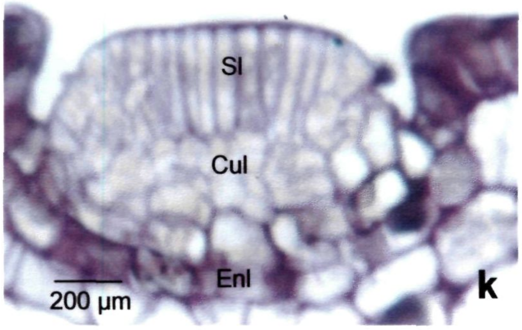
Three layered stage



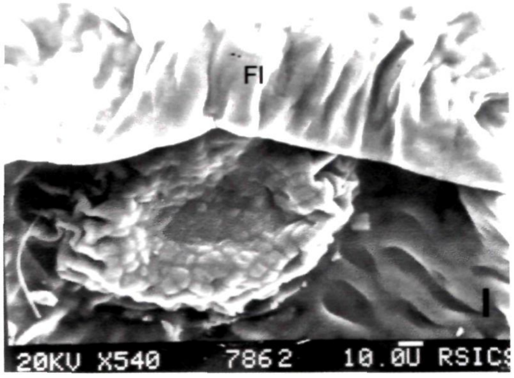
Cells with prominent nucleus and nucleolus



Four layered stage

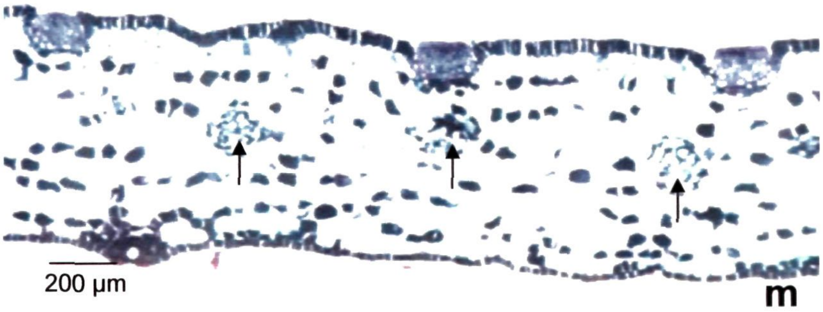


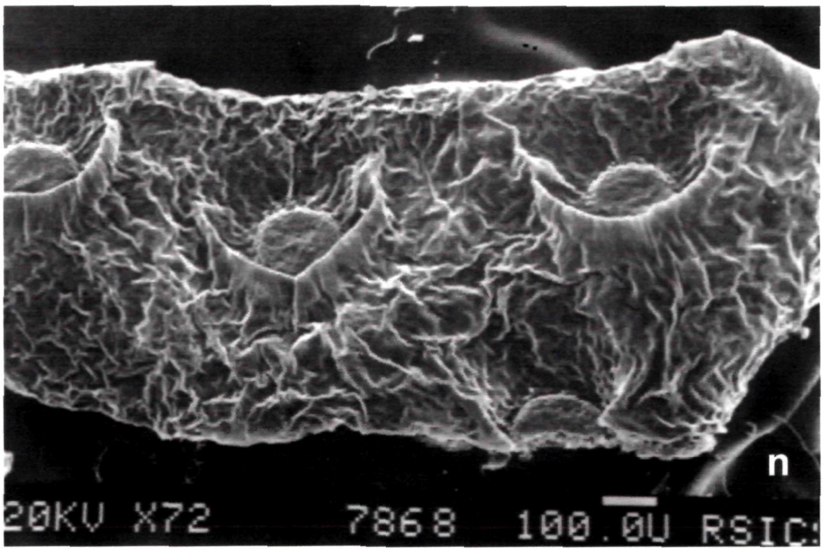
Differentiated gland layers



SEM of differentiated gland

Plate- 4.7 Development of gland





m Transverse section of matured glands stained with Toluidine blue O. The glands are equidistantly placed and each gland is located above vascular bundle of the pitcher (arrows).

n SEM of matured gland.

CHAPTER- 5

Drosera peltata Sm.

5.1 Introduction

Drosera peltata is a delicate herb with erect stem and peltate leaves. It does not have true root system, but possess an underground tuber as perenating organ (Plate- 5.1c). The lateral roots are pseudolateral as it doesn't have true root cap during its ontogeny. It grows along with grasses in open, sandy, moist, rocky and marshy lands (Plate- 5.1 a & b). The leaves are modified organs for capturing insects with attractive colour and glistening glandular hairs, which reflect water drops. These bog plant includes 120 species (Culham and Gornall, 1994; Adamec, 1997), majority of which grow on acidic soils or on substrates mainly deficient in nitrogen and phosphorous (Chandler and Anderson, 1976; Stewart and Nielsen, 1992).

In India, three species of the genus belonging to the family Droseraceae have been reported from different localities. They are *D. indica* L., *D. burmanii* Vahl. and *D. peltata* Sm. var. *lunata* Cl. These three species are also distributed in New South Wales (NSW) of Australia and New Zealand, and they extend up to Southeast Asia mainly to Japan and India (Hooker, 1874; Erickson, 1968; Marchant and George, 1982; Hart, 1987). *D. peltata Sm.* var. *lunata* grows throughout the plains as well as on the hills up to an elevation of 3000 m asl and, *D. burmanii* Vahl. is restricted to eastern and central India.

D. indica Linn. generally grows along the open habitats of the Western Ghats or coastal regions during the rainy season (Matthew, 1999). It is a threatened and endangered insectivorous plant species of Indian origin, which is included in the list of Red Data Book (Reddy et al., 2001). *D. burmanii* Vahl and *D. peltata* var *lunata* occur in wild habitants in Jowai, Jarain, Pynursla, Shillong, Raliang area of Meghalaya state of North east India (Joseph and Joseph, 1986). They are also commonly distributed in Assam, Arunachal Pradesh and Mizoram (Kanjilal and Bor, 1940; Raseshowri and Venugopal, 2006).

Drosera indica L. and *D. burmanii* Vahl. are also considered as medicinal plants in Andhra Pradesh (Jayaram and Prasad, 2006). All the three species of *Drosera* found in India are also used as vital components in the Ayurvedic preparation called Swarnabhasma (Golden ash) is an antisyphilitic. *Drosera* contains naphthoquinones which is the main constituent involved in antimicrobial activity. The occurrence of 'Plumbagin', an antimicrobial compound in large quantity is also reported in many species of *Drosera* including *D. peltata* (Laclercq and Angenot, 1984; Krenn et al., 1995). Medicinal use of extracts of *Drosera* as an important antitussive to cure different respiratory diseases including tuberculosis has been mentioned in the literature of 14th century (Schnell, 1984). Antimicrobial activity of extracts of aerial parts of *Drosera peltata* against bacterial oral diseases has also been reported (Didry et al., 1998). Tincture prepared from the leaves of *D. rotundifolia* was used to cure various respiratory diseases (Blumenthal et al., 1998). Joseph and Joseph (1986) reported the use of powdered leaves of *Drosera* by the local people of Northeast as blistering agent. This preparation is also used to recover from arthritis and rheumatism.

Studies on the development of the tentacles in *Drosera intermedia* have been carried out by Fenner (1904) and Homes (1928). According to them, the development begins with the swelling of the epidermal and sub-epidermal cells. Fahn (1979) studied two different types of glands in *Drosera capensis,* in which tentacles on the margin and upper surface of the leaf blade act as the secretory glands, and numerous sessile glands on the upper leaf surface perform absorptive function. His study gave more emphasis on the cell layers of the glands but not on the developmental aspect of the glands.

Ragetli et al. (1972), Chafe and Wardrop (1973) and Williams and Pickard (1974) reported the presence of many pores in the area of the outer secretory cells of the head of the tentacles. Heslop-Harrison and Heslop-Harrison (1981) studied the digestive glands of *Pingcuicula grandiflora* and observed three functional compartments in the gland, viz., a basal reservoir cell, an intervening cell of endodermal character and a group of secretory head cells. The gland complex is derived from a single epidermal initial. The head cells of the developing gland have labyrinth wall thickenings of transfer cell type composed of pectic polysaccharides. The cuticle is discontinuous without any pore. Besides other cell organelles, they also reported the occurrence of numerous dictyosomes associated with vesicles. Optical and electron microscopic study revealed the synthesis of digestive enzymes in the head cells which later on transferred into the cell walls through vesicles (apoplastic pathway). There is no evidence of a granulocrine mode of secretion.

Secondary products of *Drosera* which are secreted by the glands are medicinally important, although no such attention has been given on the studies of developmental aspects of glands. Therefore, this chapter deals with the structure and ontogeny of tentacle and sessile glands in *D. peltata*.

5.2 Gland Architecture: External structure, Number and position of Glands

Drosera bears five to six leaves which expand more or less horizontally, but the young leaves show vertical orientation. The leaves are reniform with broader base (Plate- 5.2 a, b). The upper surface of the leaves and their margins are covered with tentacle glands. Each gland head bears a drop of externally viscid fluid glistering in the sun, and hence given the name sundew. The marginal tentacles are of 1 to 1.5 mm in height, whereas those on the dorsal surfaces are 0.6 to 0.8 mm in height. The tentacles on the central part of the leaf are short and stand upright, and their stalk is greenish in colour. The tentacles become longer and longer towards the leaf margin, and are more inclined outwards with their stalk. Those on the extreme margin, project perpendicular to the plane of leaf. Each tentacle consists of a thin, straight, flattened hair like stalk, carrying a globular head on its summit (Plate- 5.2 d, e). The head and the stalk regions of the tentacle are demarcated by a distinct endodermal collar (Plate- 5.2 f). The stalk is formed of several rows of elongated cells. In cleared leaves, the leaf veins are found extended to all the tentacles interconnecting the tentacle glands (Plate- 5.2 d & e). Numerous pores are present on the outer surface of the head portion due to the rupture of the cuticle. The secreted products are deposited in the grooves of external surface as small droplets which may be extruded out through the cuticular pores (Plate- 5.2 g). Approximately, 40 to 50 tentacles are present on the margins and 60 to 80 on the dorsal surface, whereas the sessile glands are double the number, approximately 120 to 160 per leaf. On the cleared leaf, the sessile glands appear as dark spots (Plate- 5.2 c).

Sessile glands on the dorsal surface of the leaf are globular in shape. They are also dispersed on the stalk of the tentacles, the main stem and towards axis of leaves. The radial walls also form ridges which is distinctly visible in SEM (Plate- 5.2 h). The matured sessile glands are three-tiered structure having with i) the outer eight-celled layer ii) the middle four-celled layer and iii) the basal layer of two cells. Surface view of outermost eight-cells of sessile gland shows granules of various sizes (small, medium and large size) (Plate- 5.2 i). The sessile glands are apparently meant for the absorption of digested products. The tentacles and sessile glands are radially symmetrical.

5.3 Internal organization of the tentacles

Each tentacle gland consists of a stalk and a head portion. The head portion of the tentacle consists of four layers of cells: i) he outermost single-layered columnar shaped secretory cells which completely ensheathed the head portion, ii) a single or sometimes bilayered cells located below the secretory layer generally known as communicatory layer connected directly with the inner vasculature of the gland, iii) the single layered subspherical to rectangular shaped inverted cup shape endodermal layer which controls the back flow of the secreted fluid and iv) the central core of isodiametric tracheal cell which are shorter and wider compare to the tracheids of the stalk region (Plate- 5.3 a). The stalk region of the tentacle consists of highly elongated and narrow parenchymatous cells which encloses the central vascular strand. The vasculature of the stalk is in direct contact with the central core of tracheids of the head region (Plate- 5.2 d).

5.4 Outermost secretory cell layer

The secretory layer is single layered with approximately 500-700 cells. This layer is completely covered with thin cuticle which extends even inside the endodermal layer. The secretory cells are elongated columnar in shaped with distinct nucleus. The outer tangential walls of the secretory cells have annular or spiral cell wall thickenings (Plate-5.5 p).

5.4.1 Ultrastructure of secretory layer

The primary wall of young secretory cells are fibrilar with microfibrils of 60-70°A in thickness, which run parallel to the outer tangential wall and longitudinally with the radial wall. The microfibrils appear as electron dense and electron translucent region (Plate- 5.4 b). The cell walls of the secretory cells are irregular in thickness when compared to the central portion of the young glands, in which the cell walls are almost uniform in thickness (Plate- 5.4 a). The radial walls are much thinner than the tangential walls. The cuticle is somewhat electron translucent with discontinuities on its surface and this discontinuity probably represent the areas through which the secreted products are exuded out (Plate- 5.4 a & b).

Several electron dense dark granules are present inside the cytoplasm of young secretory cells. They are probably the spherosomes or granules which may contain digestive enzymes (Plate- 5.4 a). The cytoplasm also contains several small, medium and large sized vacuoles. The plasmalemma is highly irregular, convoluted in its margin indicatind the active secretion process (Plate- 5.4 c). The cytoplasm is dense containing all organelles such as mitochondria, proplastids, dictyosomes, nucleus and multivesiculate or polyvesiculate bodies (Plate- 5.4 a). Several single membrane bound vacuoles are present inside the poly or multivesiculate bodies. These multivesiculate vesicles are probably might have derived from the dictyosomes as well as endoplasmic reticulum, which are later on fuses with the plasmalemma and releases its content in the periplasmetic space (space between the cell wall and plasmalemma). The central core of the cells of young head region is more or less similar in organization to that of secretory cells.

5.5 Endodermal and communicatory layers

The endodermal layer is a single layered consisting of approximately *300- 400 numbers of cells. This layer is inverted cup or 'ꓵ' shaped* structure (Plate- 5.3 a). The cells in this layer are subspherical to rectangular in shaped. The radial walls of the endodermal cells are much thicker than the tangential walls, completely cutinized and fibrous in nature forming the casparian strips. There is a continuation of the casparian strips with the cuticle of the secretory head cells. In communicatory layers, the plasmalemma is uniform and not convoluted. The endodermal cell contains many osmiophilic bodies. Cytoplasm is granular with dictyosomes. As in the case of outer secretory cells, the poly or multivesiculate bodies inside the endodermal cells show continuation with the plasmalemma (Plate- 5.4 a, d). Mitochondria are numerous with well developed cristae and are smaller than those found of the secretory cells. The plastids are inconspicuous, and without starch grains. The nucleus is granular. The elongated cells of the endodermal layer are connected to each other by a few plasmodesmata which pass through the constricted regions of the cutinized radial walls. The tangential walls are thin. Rest of the gland cell layers are connected to the central core of trachieds through the endodermal layer. Thus, the secretory glands have continuity with the vascular supply of the leaf. When the glands matured, two to four layered communicatory regions is found shrink due to overgrowth of the endodermal cells (Plate- 5.3 a & b).

5.6 Development of tentacle

Initiation of the tentacle primordium starts from the hypoepidermal cells on the dorsal surface of the leaf when the leaves achieve the size of 0.3 mm diameter. At this stage, some of the hypoepidermal cells show periclinal and anticlinal division. As a result of this, some of the epidermal cells protrude perpendicular to the leaf surface showing more prominent from the rest of the epidermal cells (Plate- 5.5 a & b). At the same time, the epidermal cells divide anticlinally. The dorsal and marginal tentacles are simultaneously initiated as the leaf surfaces attain 1mm in diameter. The tentacles primordial are prominently visible (Plate- 5.5 c & d). The configuration of tentacle primordia confirms the tunica corpus concept. The tentacle primordial cells are with dense cytoplasm and prominent nuclei which indicate that they are in active stage of cell division (Plate- 5.5 e & f). The cells in the central core of the primordium divide more in transverse plane than the anticlinal plane so that they increases in height. The outer protodermal layer (epidermis) of primordium radially divides in order to compensate the growth of the central cells. When the leaves reaches 1.5 to 2 mm in diameter, the single hypodermal cell of the tentacle primordia divides randomly in all direction to increase in the number of cells to form the globose head region (Plate- 5.5 g-k). Differentiation of different layers occurs after the formation of globose head region. Gland layers differentiates when the leaves reaches almost 3 to 4 mm in diameter (Plate- 5.5 l).

The endodermal layer is differentiated simultaneously with the elongation of epidermal cells or the columnar secretory cells. The communicatory layer is differentiated in between columnar secretory and endodermal layers (Plate- 5.5 m, n). The central cells of head region elongate and differentiate into rectangular treachery cells with annular and spiral thickenings. The outer walls of young columnar secretory cells also develop wall thickenings (Plate- 5.5 p). The endodermal layer checks the back flow of the secreted products to the inner tissues of the tentacle. At the stage of fully matured tentacles the endodermal layer becomes inverted cup shape and demarcates the outer secretory and communicatory layers from the inner tracheal cells (Plate-5.5 n).

The complete differentiation of the tentacular head has been observed when the young leaf reaches the diameter of 3 mm (Plate-5.5 n & o). All these layer formation occur concomitantly. A quit number of cells in the endodermal layer do not possess casparian strips or radial wall thickening so that there is a pathway for the transport of secreted products from the tracheidal cells of the head regions into the secretory layer. The vasculature differentiation in the stalk region of the tentacle is acropetal. Connection between treachery cells of the stalk and the central trachedial cells of the globular head of the tentacle form a continuum between the head and the leaf.

5.7 INTERNAL ORGANIZATION OF SESSILE GLAND

Sessile glands are three layered complexes. The three layers are: i) the outermost eight celled layer, ii) the middle layer of four cells and iii) the innermost two celled basal layer (Plate- 5.6 a). Cells are with dense cytoplasm, prominent nucleus and nucleolus. The basal cells are the continuation of the epidermal layer of the leaf. The thickness of cuticle is uniform throughout the epidermis and it also covers the entire sessile gland portion. In other words, there is continuity of cuticle along the sessile gland and the leaf epidermis. The primary wall stains heavily both with the PAS procedure and with calcoflour white. These reactions indicate that the primary wall composed of mainly cellulosic component (Plate- 5.6 b).

5.7 Ultrastructure of sessile glands

Ultrastructure study of the sessile gland shows structural variations of cells during the different developmental stages of the gland. In the young stage, the cells of the outermost layer are less in number. There is continuity of the outer cuticle layer with the epidermis of the leaf surface. The radial wall of the outer cells is comparatively thin than the outer tangential wall. There are no pores in the cuticles of the gland. The cytoplasm is dense with prominent nucleus and nuclear membrane. Small numerous vesicles produce by the Golgi bodies are found distributed on the periphery of the cells of the outer layer (Plate-5.7 a). Probably, these vesicles are the storage site of enzymes. Mitochondria are comparatively less in number in the young stage than the mature stage whereas proplastids are more in number in the young stage.

At young stage, wall ramification is more on the tangential wall. The wall ingrowths are electron transparent, and microfibrils are loosely dispersed. Cell wall ramification on the outermost layer suggests the absorptive function of this gland. At the young stage, vacuoles in the middle layer are large and less in number, and are dispersed in the middle of the cell. Vacuoles are more in the basal cells. Electron dense bodies in the basal cells indicate that the absorbed products are transported to the inner vasculature of the plant via foot layer (Plate-5.7 a & b).

In the mature stage, the cell wall ramification enlarges and ultimately pushes the plasmalemma towards the cell cytoplasm. It also indicates that the cells are active in absorption and translocation of the digested substances. Mitochondria are numerous in number with distinct and elaborate cristae, while the plastids are lesser in number in the outer layer (Plate- 5.7 c). Vacuoles are small and less in number while large vacuoles occupy the cells of the middle layer. The cytoplasmic contents are found in the periphery of the cell. Nucleus is prominent with distinct nuclear envelope at the centre of the cell (Plate-5.7 b). All these features indicate that the outer cells are physiologically active. The primary walls of the outer cells show a microfibrilar structure with electron dense and electron translucent region (Plate- 5.7 d). In matured gland cells, the vesicles derived from the Golgi bodies are in continuation with the cell wall labyrinths (Plate-5.7 a & b).

5.8 Development of sessile gland

The sessile glands initiates when the tentacle primordia attain 600 µ in length. There is a temporal relationship exist between the differentiation of tentacles and sessile glands. A single epidermal cell enlarges with prominent nucleus and dense cytoplasm, divides in transverse plane to give rise to a group of two daughter cells which are termed as inner basal cell and the outer apical cell (Plate- 5.8 a). The apical cell divides twice in radial plane at right angle to each other to form four cells in a single tier (Plate- 5.8 b & c). These four cells divide transversely but in oblique pattern to form four outer and four inner cells (Plate- 5.8 d). The outer four cells undergo vertical division and form eight cells enclosing the central four cells. The basal cell undergoes again vertical division to form two celled basal layer or foot layer. Thus the sessile glands consist of 14 cells; the outermost eight cells, central four cells and foot layer of two cells (Plate- 5.6 a). The growth of the sessile gland is completed. The radial wall thickenings and the formation of casperain strips are absent in the sessile gland. The globules are prominent in the outer layer of sessile glands. In sessile glands, below the foot layer, a parenchymatous reservoir cell is present.

Plate- 5.1

a Habitat of *Drosera peltata* Sm. Plants growing on sandy and marshy soil (1/10 of natural size).

b An enlarged view of few plants showing the water dew on the leaf surfaces(1/5 of natural size).

c A single plant with leafs (Lf), lateral roots (Lr) and the tuber (Tu) (1/2 of natural size).

Plate- 5.1 *Drosera peltata* Sm.

Habitat of *Drosera peltata* Sm.

Enlarged view of a few plants

A single plant

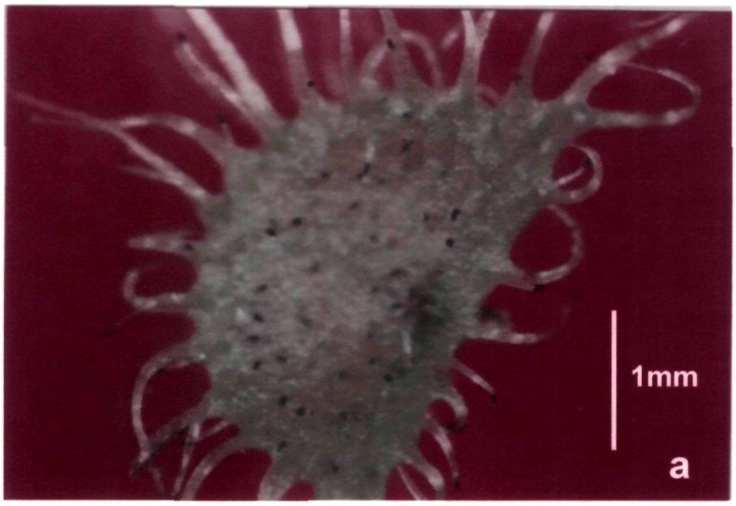
Plate- 5.2

a A single leaf with numerous tentacles on its margin as well as the upper leaf surface along with the sessile glands.

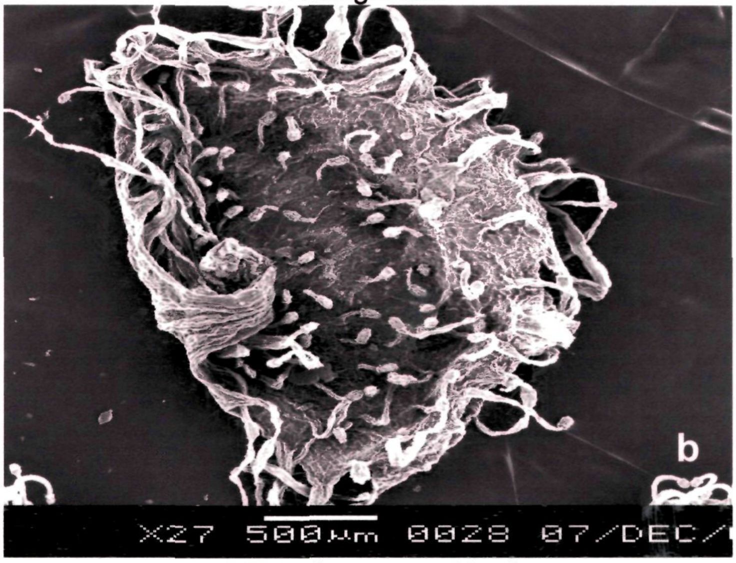
b SEM of a matured leaf showing the marginal and the leaf surface tentacles.

c A cleared leaf showing the interconnections between the tentacles and the sessile glands (dark spots) by vasculature. Nearly, a maximum of two to three sessile glands are found in a single areole.

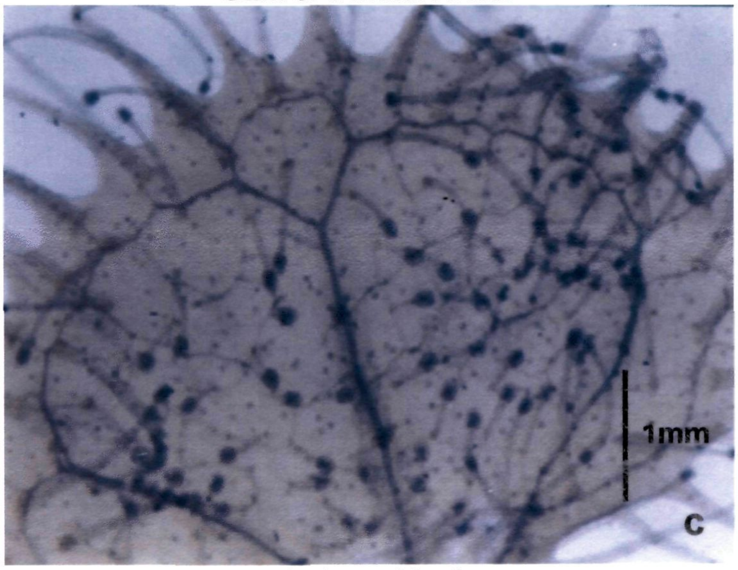
Plate- 5.2 Number and position of glands



A single leaf



SEM of a matured leaf



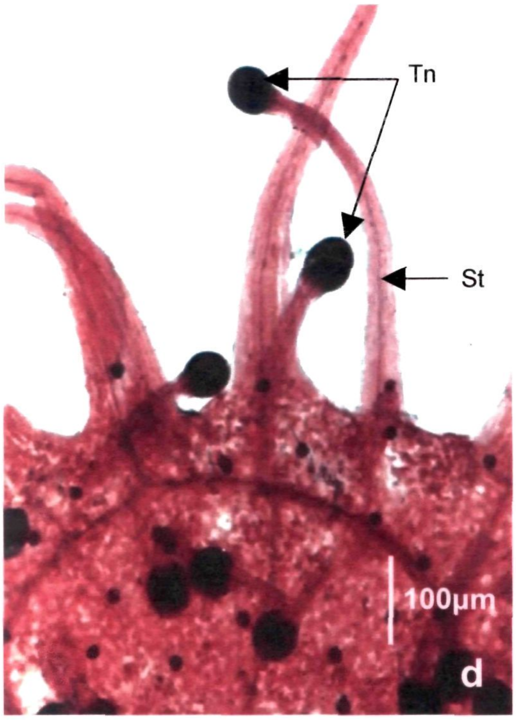
A cleared leaf

Plate- 5.2

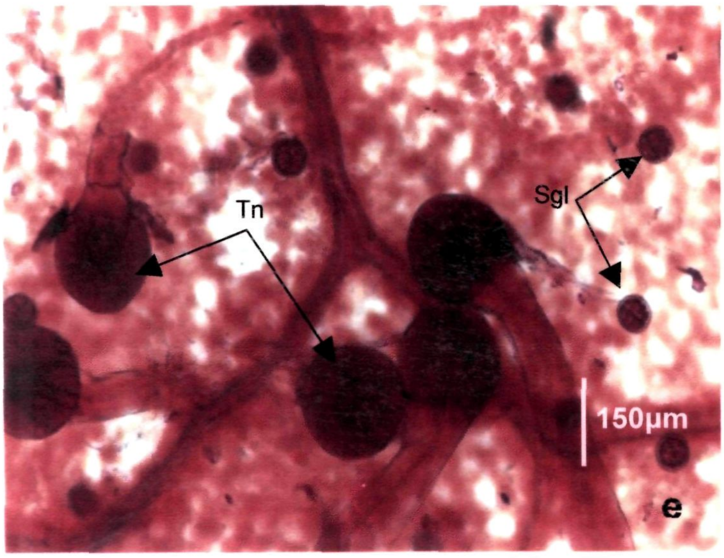
d Marginal leaf tentacles. Cleared leaf stained with saffaranin showing the marginal tentacles (Tn) with distinct head and stalk portions. The surface tentacles of the leaf are smaller than the marginal ones.

e An enlarged view of leaf surface tentacles. The spherical shape structures are the sessile glands (Sgl) on the upper leaf surface.

Plate- 5.2 Number and position of glands



Marginal leaf tentacles



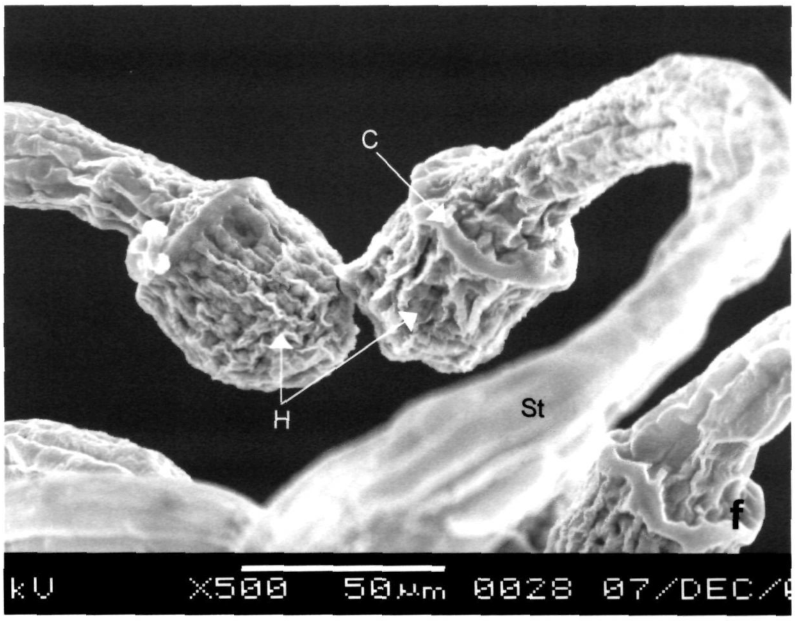
An enlarged view of leaf surface tentacles

Plate - 5.2

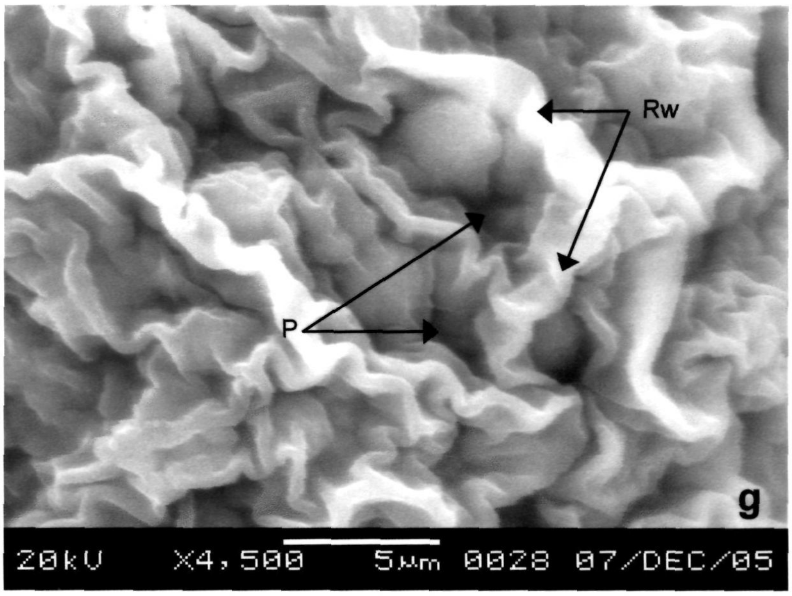
f SEM of marginal tentacles. Tentacles showing the head and stalk portions. The head (H) and the stalk (St) portions are demarcated by the distinct endodermal layer called collar (C).

g SEM of head portion of the tentacle. Pores (P) and radial wall extensions (Rw) of head of the tentacles are visible.

Plate- 5.2 Number and position of glands



SEM of marginal tentacles



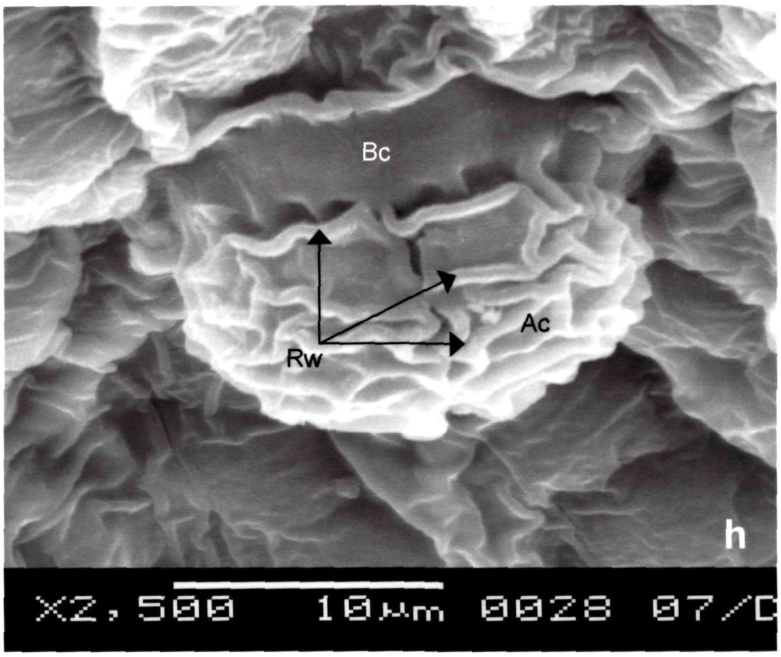
SEM of head portion of the tentacle

Plate- 5.2

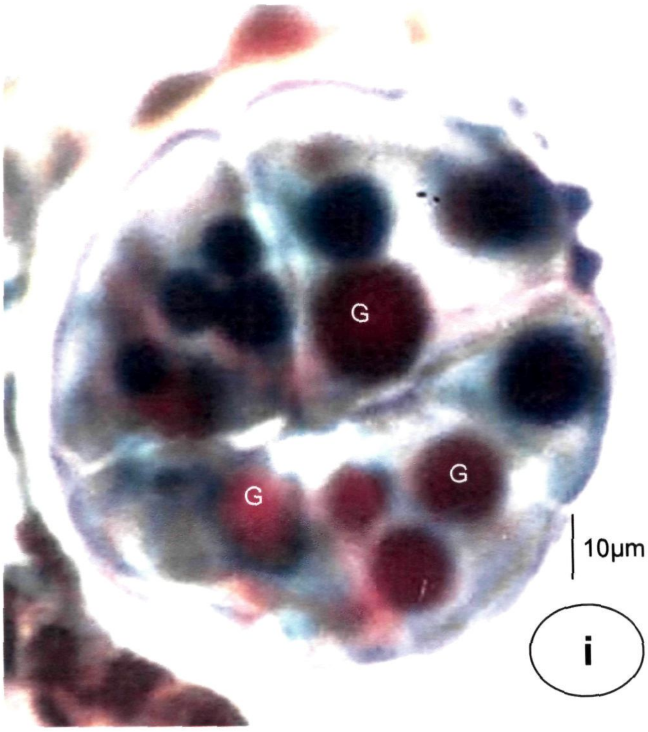
h SEM of a sessile gland. Basal Cell (Bc) and apical cells (Ac) of the sessile gland portions are shown. The apical cell also possesses radial wall extensions (Rw).

i Surface view of a sessile gland. The sessile gland is spherical in surface view with numerous globules (G).

Plate - 5.2 Number and position of glands



SEM of a sessile gland



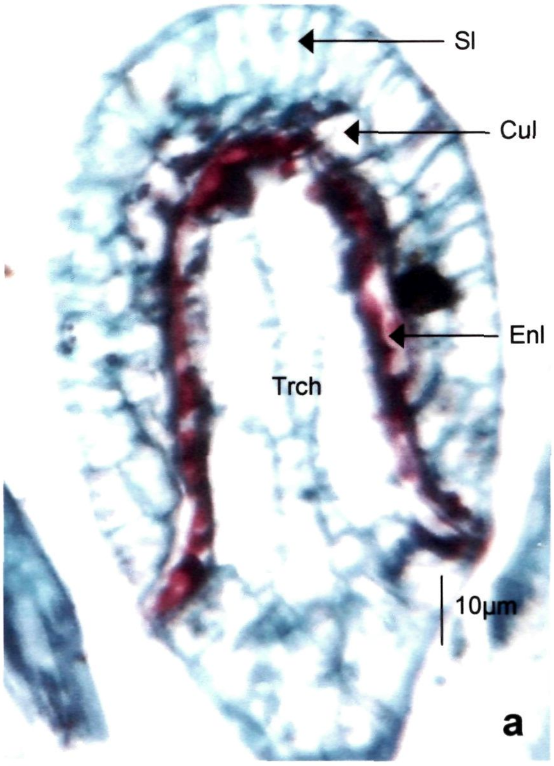
Surface view of a sessile gland

Plate - 5.3

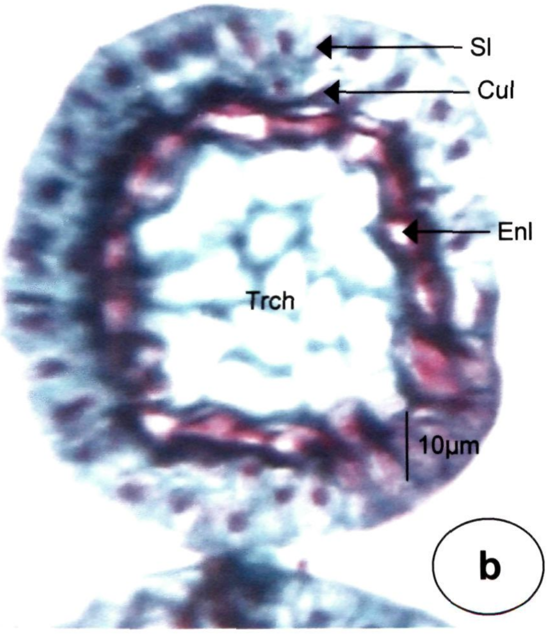
a Longitudinal section of tentacle head region showing the three layers viz. the outer columnar secretory layer (Sl), the middle three to four layers of communicatory cells (Cul) and the inner single layered with rectangular endodermal cells (Enl) below which tracheal cells (Trch) are present.

b Transverse section of head of the tentacle. The three layers i.e. the secretory layer (Sl), communicatory layers (Cul) and the endodermal layer (Enl) of the head of the tentacles is present as in L.S. of the tentacle head. Central tracheal cells (Trch) are more prominent.

Plate- 5.3 Internal organization of tentacle



L.S. of head of the tentacle



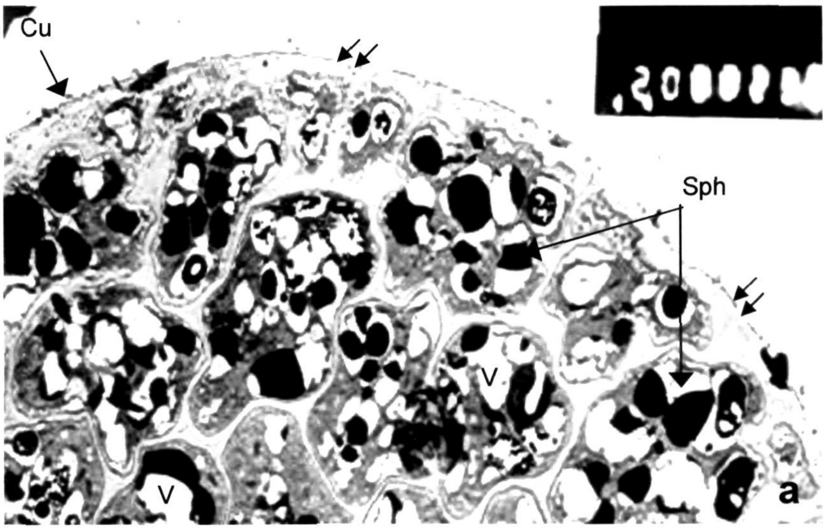
T.S. of head of the tentacle

Plate- 5.4

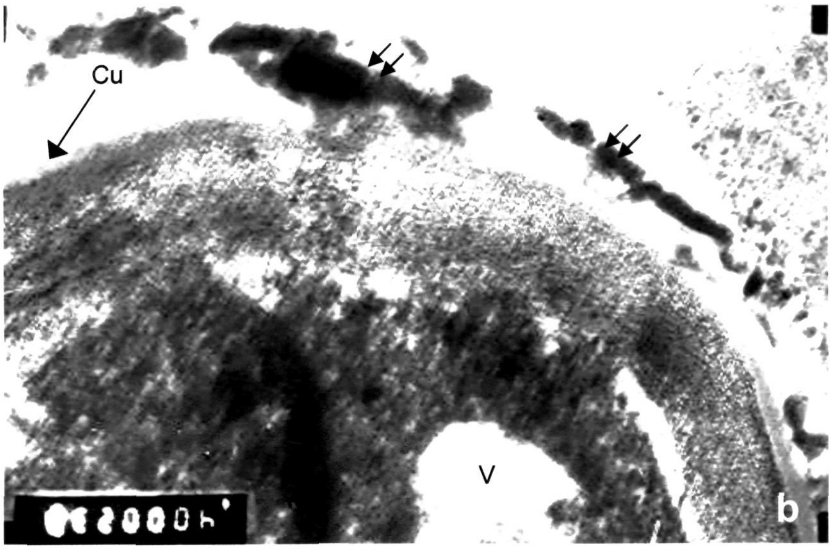
a TEM of few outer secretory cells with numerous vacuoles (V), granules or spherosomes (Sph). The cuticle (Cu) of the cell wall shows discontinuity with regular interruptions (double arrows ahead). The cytoplasm is dense.

b TEM of outer secretory cell wall. Secreted materials through the cuticular ruptures are found on the outer surface of the gland (arrow ahead). (V=vacuoles).

Plate- 5.4 Ultrastructure of internal organization of tentacle



TEM of few outer secretory cells



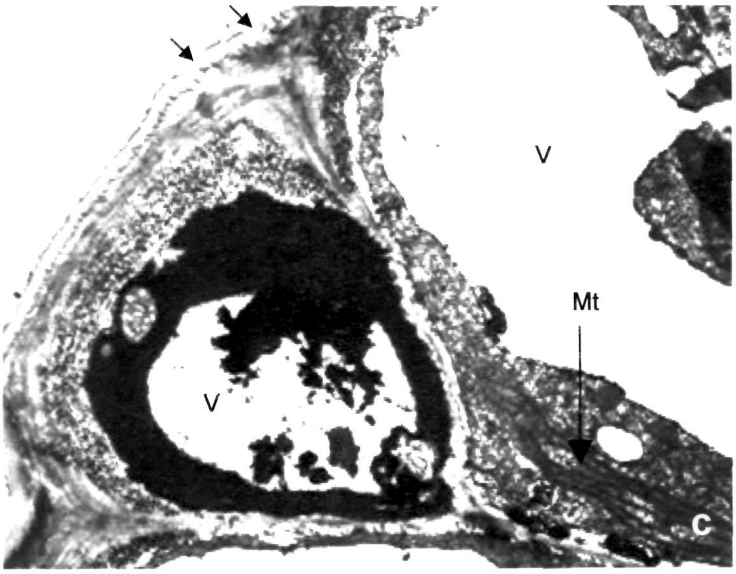
TEM of outer secretory cell wall

Plate- 5.4

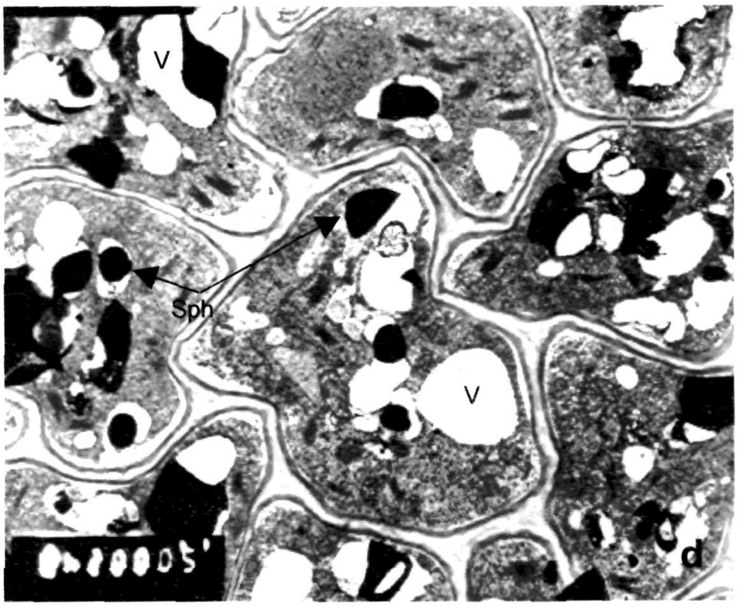
c TEM of a single secretory cell. The cell is with large vacuoles (V). The cell wall is fibrous in nature and microtubules (Mt) are found parallel to the longitudinal axis of the cell. (Arrows pointing the cuticular ruptures)

d TEM of a few middle layer cells. The cells of the communicatory layer are compact without any intercellular spaces. The cytoplasm is dense. Note the number of vacuoles and granules. The radial walls of the cells are thicker than the tangential walls.
(Sph =spherosomes; V =Vacuoles)

Plate- 5.4 Ultrastructure of internal organization of tentacle



TEM of a single secretory cell



TEM of a few middle layer cells

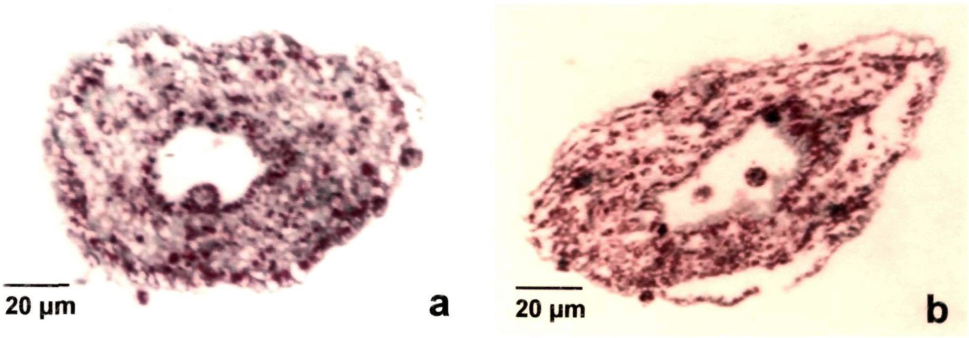
Plate- 5.5

a & b Tentacle primordiium when the leaf is at 0.3 mm. The tentacles start initiation from the epidermal and hypodermal cells of the upper leaf surface. The glands in the middle of the leaf are the primordia of the sessile glands.

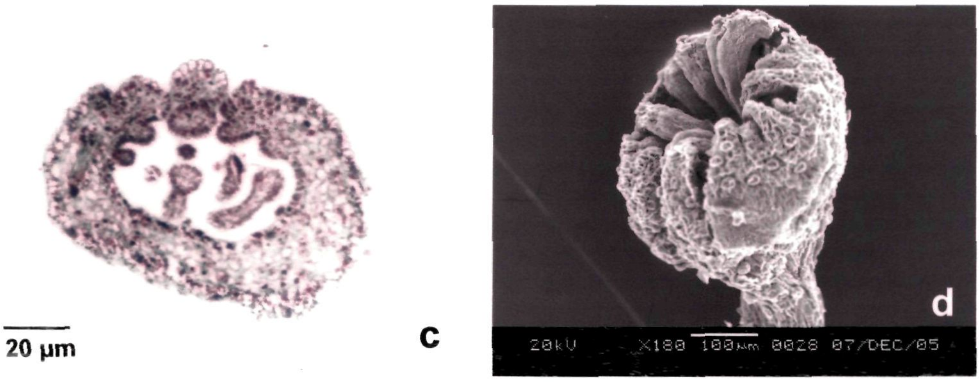
c & d Tentacle primordium when the leaf attains 1.0 mm and SEM of the same.

e & f Tentacle primordium with dense protoplasm and prominent nuclei. The cells are massive with prominent nucleus and nucleolus.

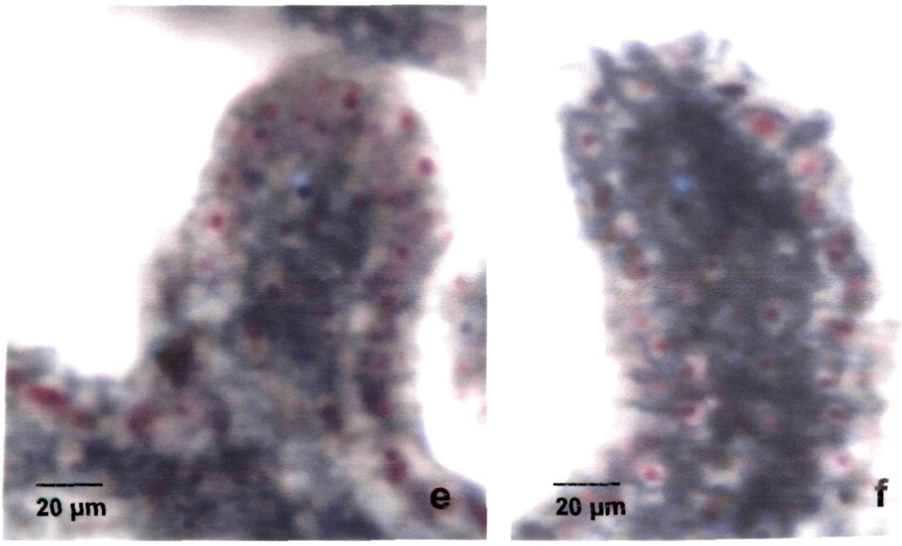
Plate- 5.5 Development of Tentacle glands



Tentacle primordium at 0.3 mm sized leaf



Tentacle primordium at 1mm sized leaf and SEM of the same



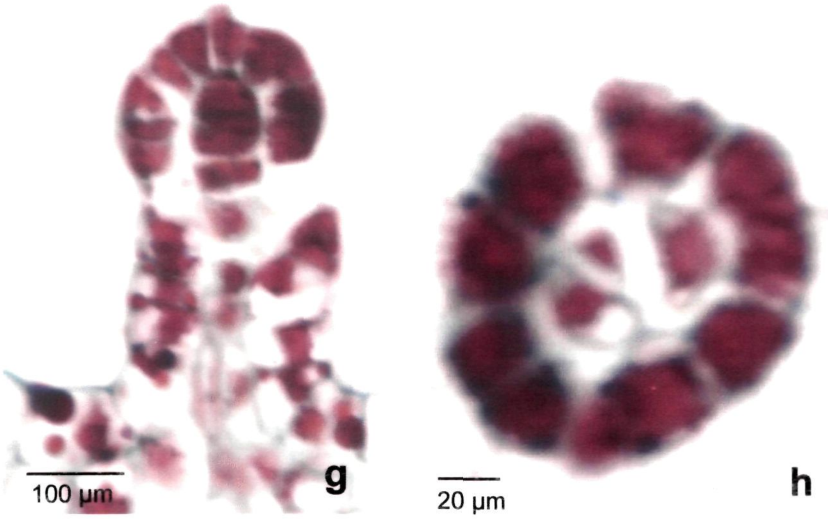
Tentacle primordium with dense protoplasm

Plate- 5.5

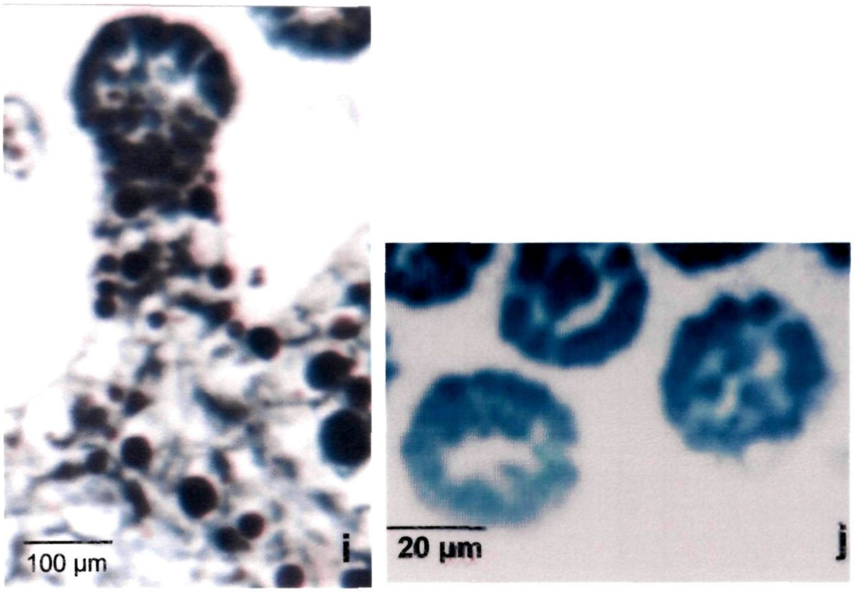
g & h Longitudinal and transverse sections of tentacle primordium. At the initial stage, the central cells divide transversely to attain 2 mm in height of the tentacle. The outer cells simultaneously divide radially (anticlinal) to compensate the growth of the central cells.

i & j Longitudinal and transverse sections of tentacle primordium with globular head at 3 mm in height. The head portion becomes globular after attaining approximately 4.4 mm in height.

Plate- 5.5 Development of Tentacle glands



L.S. and T.S. of tentacle primordium



L.S. and T.S. of tentacle primordium with globular head

Plate- 5.5

k Longitudinal section of globular head of tentacle showing numerous cells at the stage of 5 mm in height.

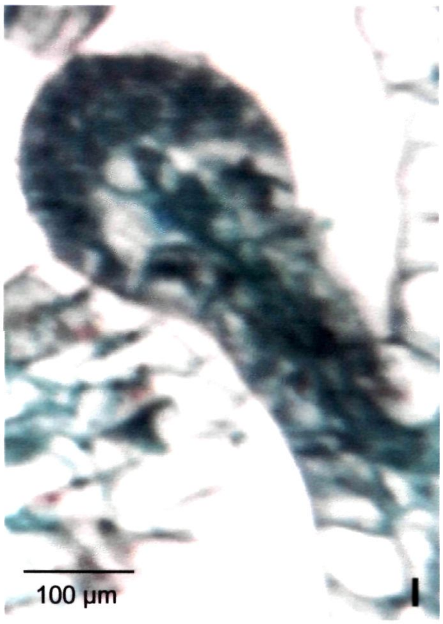
l Longitudinal section of globular head showing the initial stage of cell differentiation with the central portion where vasculature would be differentiated (arrows in the center).

m Longitudinal section of incomplete differentiation of gland cells showing the early stage of differentiated vasculature in the middle portion. Note the cells of the different layers with dense cytoplasm.

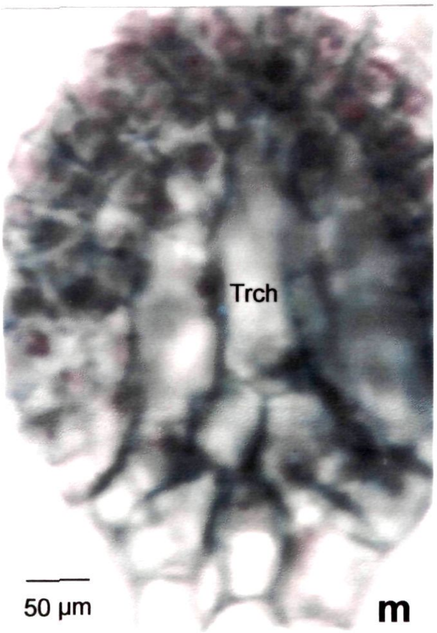
n Longitudinal section of complete differentiation of gland layers in the head portion of the tentacle. The outer cell layer forms the secretory layer, the middle cells form the communicatory layers and the inner cells form the endodermal layer. The central cells differentiate into tracheal cells. The endodermal layer demarcates the treachery cells.

Plate- 5.5 Development of Tentacle glands

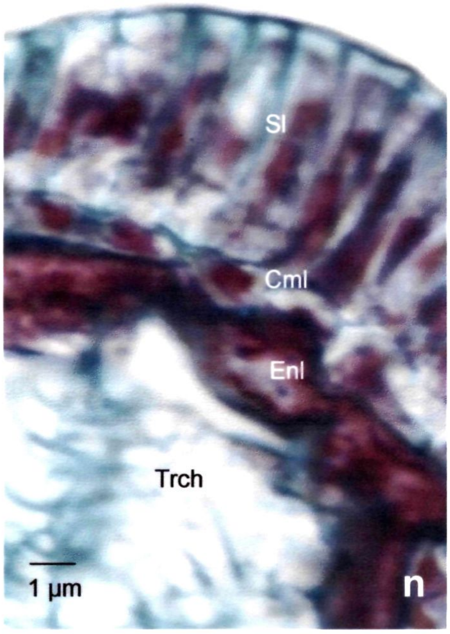
L.S. of globular head with cell division at different planes



L.S. of globular head at the initial stage of differentiation of globular head



L.S. of incomplete differentiation of gland cells



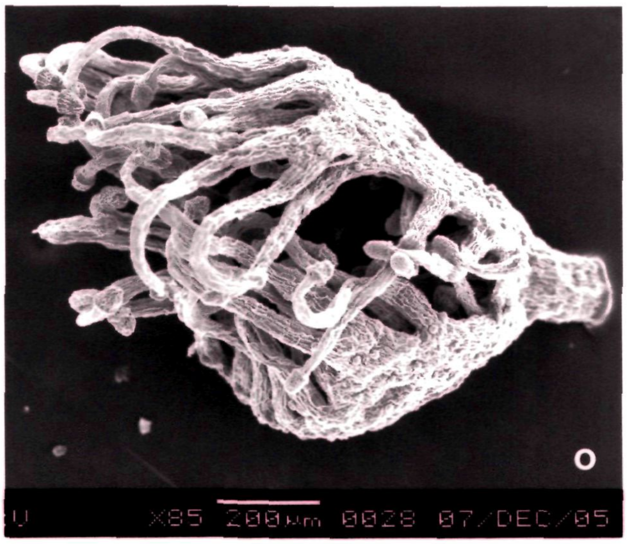
L.S. of complete differentiation of gland layers

Plate- 5.5

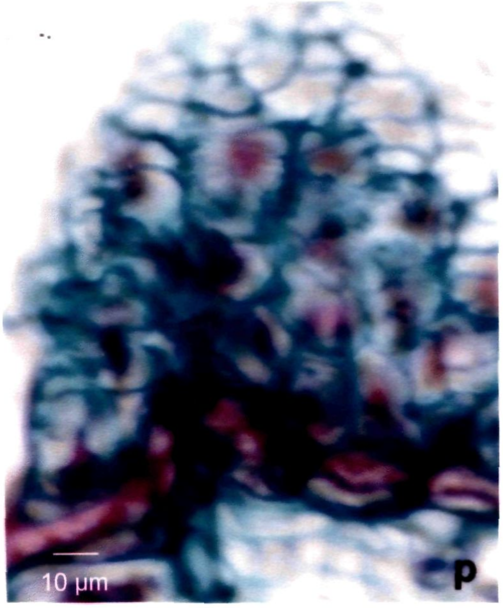
o SEM of a 3 mm sized leaf. At this stage the tentacles are almost completely differentiated with their head and stalk portions.

p An oblique L.S. of tentacle head region showing the spiral thickenings due to the deposition of cell wall materials on their tangential wall . Note the casparien wall thickenings also.

Plate- 5.5 Development of Tentacle glands

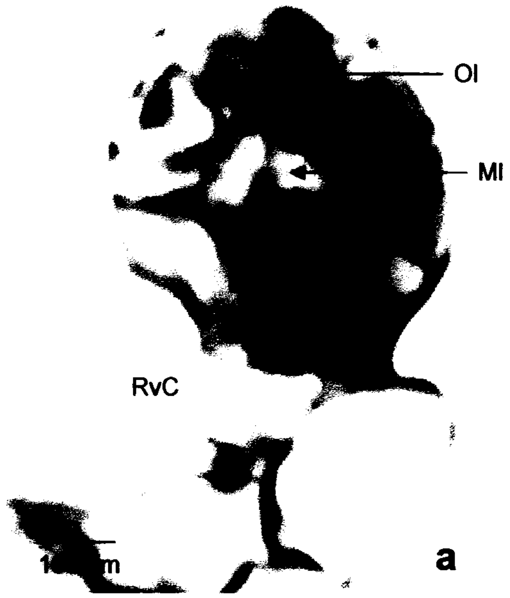


SEM of a 3 mm sized leaf

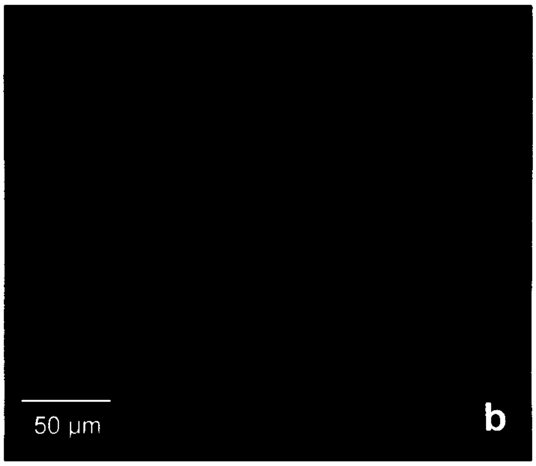


L.S. of tentacle head wall

Plate- 5.6 Internal organization of sessile gland



A matured sessile gland



Fluorescing sessile gland

a A matured sessile gland with three layers at its matured stage viz. the outer layer, four cells in two tiers forming the middle layer and the inner single foot cell.

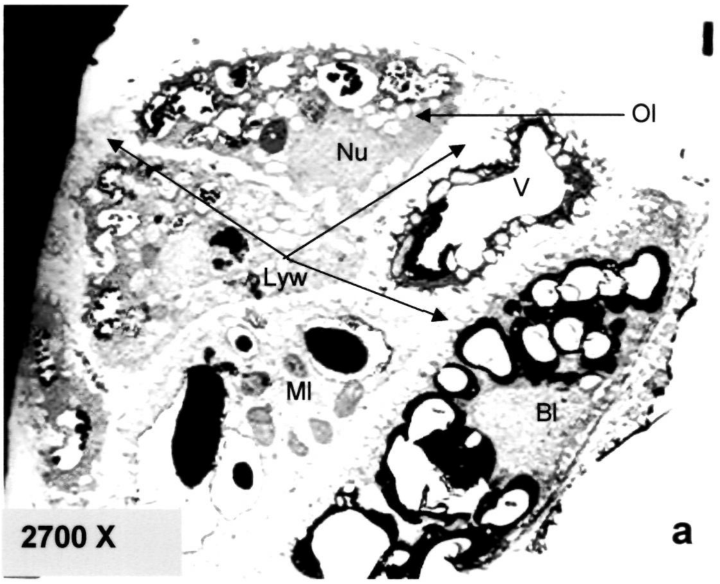
b Fluorescence microphotograph of sessile gland. The cell walls emit fluorescence with calcofluor white.

Plate- 5.7

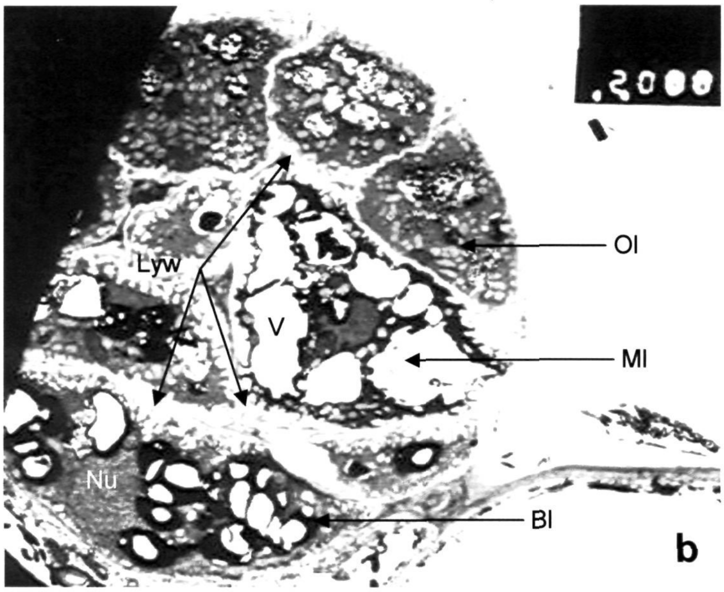
a TEM of a young sessile gland with more vacuoles of large size, mitochondria and spherosomes. Radial wall show more ramification than that of the tangential wall. The nuclei are prominent.

b TEM of a matured sessile gland. The number of cells in each layer is more with numerous mitochondria and spherosomes, and vacuoles of smaller size. Wall ramification is more in both the tangential and radial walls.

Plate- 5.7 Ultrastructure of internal organization of sessile gland



TEM of a young sessile gland



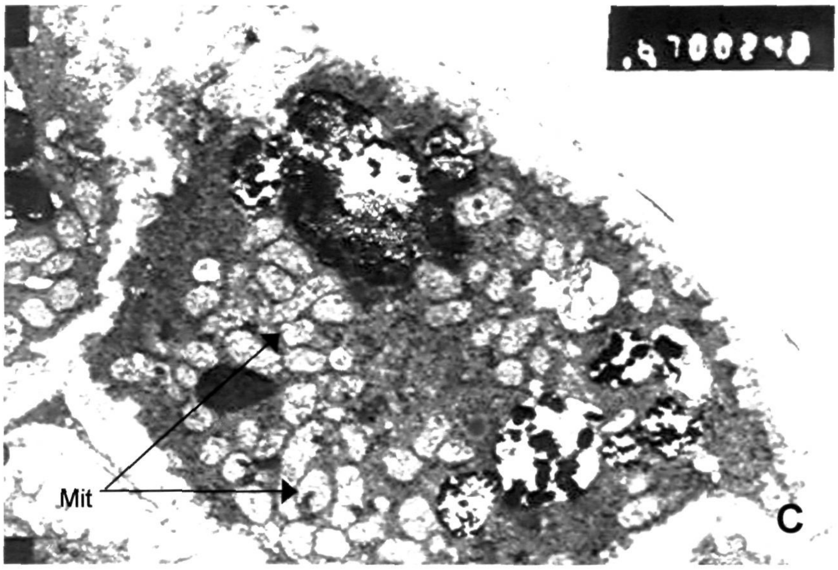
TEM of a matured sessile gland

Plate- 5.7

c TEM of single absorptive cell with numerous mitochondria and few vacuoles.

d TEM of outer cell wall. The cell wall shows several tunnels like cannels through which probably absorption might be possible.

Plate- 5.7 Ultrastructure of internal organization of sessile gland



TEM of enlarged view of outer single cell

TEM of enlarged view of outer cell wall

Plate- 5.8

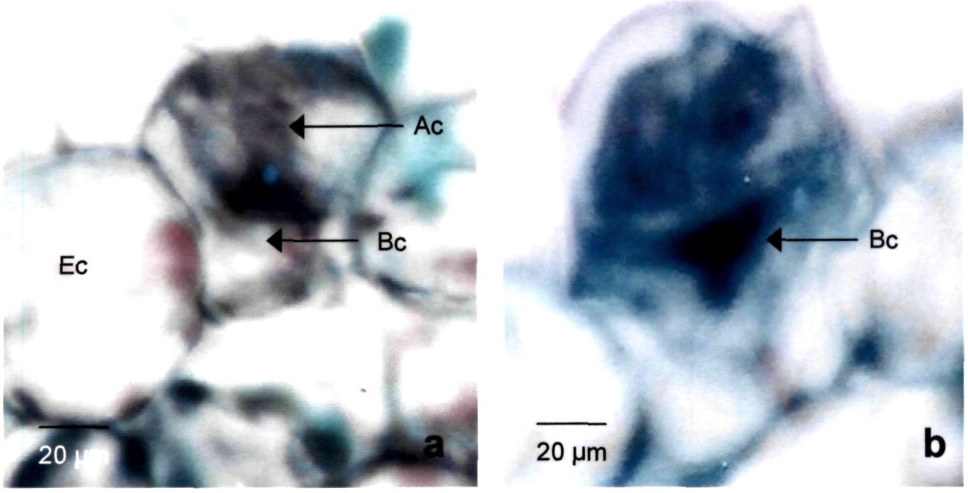
a Transverse division of epidermis. A single epidermal initial on the upper surface, divides transversely to form the upper apical and the lower basal cells.

b Radial division in the apical cell. This is the three celled stage during the development of the sessile gland.

c Four celled stages in which all the cells are in a single tier. Note four nuclei lie in the same plane. The middle tier is of two cells.

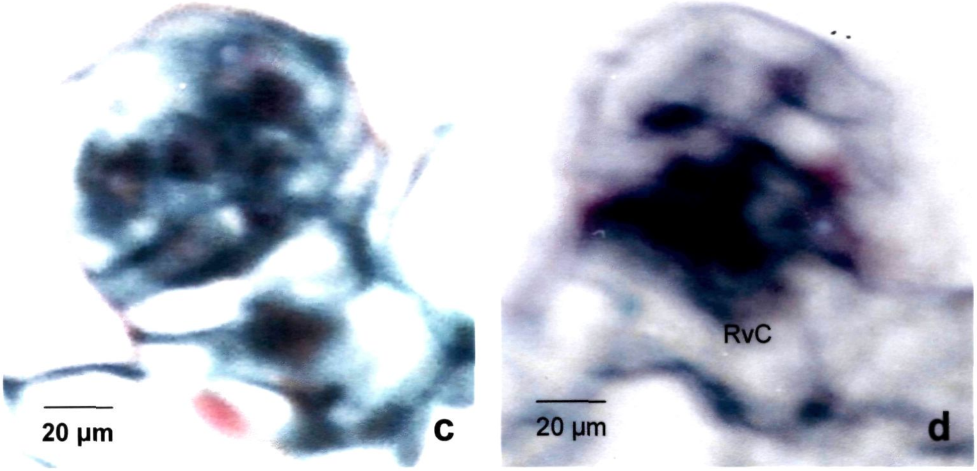
d Oblique division of the outer four cells. The outer cells divide in oblique pattern to form eight cells on the outermost tier.

Plate- 5.8 Development of sessile gland



Apical and basal cells of sessile gland initial

Radial division of the apical cell



Outer cells in single tier

Oblique division of the outer cells

CHAPTER- 6

Utricularia species

6.1 Introduction

The family Lentibulariaceae, commonly known as 'Bladderworts' accounts for *ca.* 280 species in which 210 species belong to the genus *Utricularia*. *Utricularia* is commonly distributed in tropical, subtropical and few species in temperate zones. In India, a total of 35 species of *Utricularia* are reported (Janarthanam and Henry, 1992). They are widely distributed in tropical, subtropical and a few species in temperate zones. *Utricularia bifida* Sm. and *Utricularia pubescens* Sm. which are recorded from different parts of Meghalaya are commonly distributed other parts of India, Africa, Malaysia and Sri Lanka.

The external morphology of *Utricularia* species varies one from the other depending on different substratum of the habitat. The 'Bladderworts' are annuals or perennials growing mostly in the marshy, rocky and aquatic habitats (Plate- 6.1a- c). Based on their habitats, species of *Utricularia* can be divided into aquatic, terrestrial and partially epiphytic. Aquatic species are distributed in fresh water ponds, lakes, ditches and rice fields from sea- level up to some extent of higher elevation. In case of rocky and marshy habitats, the plants are generally alive in the monsoon season and become completely dry during other seasons. The plants anchor the substratum probably for the absorption of available minerals and water, and store them to overcome the drought. They sprout immediately after the exposure to the first rain. Partially epiphytic species grow either on the tree trunk covered with the mosses or on the dripping vertical faces of rocks which are directly exposed to sunlight.

Taylor (1994) and Adlassnig et al. (2005) described four different modifications of the stem: i) horizontal stems, so called stolons which serve for the regeneration of the plant; ii) colourless shoots that grow downwards into the soil which anchor the plants to the substratum (Adler et al., 1994; Schubert and Went, 1986). These organs are easily recognizable as shoots, as they bear numerous 'Bladders' or the traps; iii) root-like formations of the stem 'rhizoids' are the specialized shoots with limited growth that emerge from the base of the flowering axis without any leaf. They also fix the plant to the masses of other floating weeds in the same habitat (Hegi, 1906); iv) in terrestrial species, the stem tubers that arise from stolons serve as storage organ to supply nutrients during drought, and also to anchor the plants to the substratum. Moreover, they contribute to the propagation of the plant, which lead to the formation of large clones (Taylor, 1994).

The generic name *Utricularia* is derived from the Latin word 'utriculus' meaning a 'small bag'. The trap functions passively in capturing prey, which later on, demonstrated by several investigators that it also responds actively. It has been suggested that the hairs inside the trap are responsible for absorbing water and digested products. The typical prey includes insect larvae (especially those of mosquitoes), aquatic worms, water ticks and other tiny swimmers sharing the same habitat.

Various works on the physiology of the trap have been carried out (Sorenson and Jackson, 1968; Sasago and Sibaoka, 1985). Kristen (1974) studied the ultrastructure and development of the external gland in *U. minor* L. In *U. neglecta,* Vintejoux (1974) studied the production of digestive enzymes by the internal glands and secretion of mucilage by the hairs on the doorway. Fineran and Lee (1974 a & b; 1980) studied the ultrastructure and organization of hairs and external glands in the traps of *U. monanthos* Hk. f. Cheema et al. (1992) studied the development and histochemical localization of the major metabolites of the bladder of *U. stellaris.* Architecture of the transfer cell wall in the secretory hairs of *U. intermedia* and the development of the wall labyrinth in the pavement epithelium hairs of *U. volubilis* Brown, *U. stygia* Thor and *U. intermedia* Hayne studied by Plachno and Jankun (2004) and Plachno et at. (2005 a, b), respectively.

6.2 Morphology of the bladder or the trap

The bladders or the traps are sac-like structure, guarded by a doorway, adapted to capture and digest small animals or animalcules. They are also known as urceoli, ampullae, vesiculae, utriculae or pitchers (Lloyd, 1942) and are attached to the submerged vegetative organs (rhizoids, stolons and foliar organs). The size of each horse-shoe shaped bladder ranges from 0.3 mm to 1mm in diameter (Plate-6.2 a-c). Each bladder has an orifice or the mouth that is closed by a valve-like door on the upper or dorsal end which is the continuation of the dorsal surface of the bladder (Plate 6.3 a). The valve functions as hinge joint, i.e. its movement is unidirectional. In the ventral surface, the orifice possesses a few layers of cells called the collar or the ridge, attached to the thick radial wall called pavement epithelium (Plate 6.3 a, b). In *U. bifida*, the antennae are bifid or forked, unbranched and slender on the upper orifice, whereas in case of *U. pubescens*, antennae are long and multicellular forming a fringe (Plate- 6.2 c & d). In both the cases, the antennae possess many bristles that spread outward near the entrance (Plate- 6.3 c). Thus, the main bladder parts concerned with the capturing of prey are the valve, the ridge or collar, unicellular-uniseriate antennae and bristles bordering the mouth forming the threshold.

The valves as well as the pavement epithelium are directed towards the bladder. The valve or the door presses against the thick pavement epithelium, and thus, it is prevented from being opened outward. In the normal condition, the valve is tightly closed against the ridge or collar. In addition, the outer surface of the bladder also bears many dome-shaped external glands. In *U. bifida* and *U. pubescens,* the inner wall of the bladder possesses bifid or quadrifid glands, respectively (Plate- 6.3 c & d).

6.3 Anatomy of the bladder or the trap

The bladder is a two-cell layered structure. The lower bulged portion i.e. two-third of the bladder wall is made up of only two cell layers, whereas the remaining one-third of the bladder is made up of three cell layers which perform the suction of animalcules. The mouth portion of the bladder is a complicated structure, in which some of the upper cells differentiate into the valve and the lower portion into a collar or the ridge with a pavement epithelium of thick radial walls. Both the structures are directed towards the bladder (Plate-6.3 a). The collar is made up of three cell layers, the outermost capital cell layer, the middle thick walled rectangular shape cells and the innermost radially elongated basal cells (Plate- 6.3 d).

The cells of the inner layer of the bladder are comparatively smaller in size with thick walls whereas, cells of the outer layer possess thin walls. Being less extensible, the inner layer acts as a resistant layer, whereas the outer layer controls the shape of the bladder. At the junctions of four to five bigger cells of the bladder wall, small angular or basal cells are present in the interior wall of the bladder. Bifid and quadrifid digestive glands are terminated from these small angular shape basal cells.

6.3.1 Gland architecture

Bifid and quadrifid glands in the inner wall of the bladder are terminated by a short stalk which is placed at right angle to the bladder wall (Plate- 6.3 g, h). The stalk cells are the basal portion of the arm or the terminal cell which is abutted from a pedestal cells. The pedestal cells are subconical in shape with circular bases (Plate- 6.3 j). In other words, pedestal cell supports the terminal or the arm cells. In surface view, the arms and the stalk of a quadrifid form an H-shaped structure, which lie parallel to the inner surface of the trap (Plate- 6.3 h). Thus, in surface view, four pedestal cells are arranged in tetrad form (Plate- 6.3 i). Simply, two terminal cells which are originated from a single pedestal cell are modified into the two arms in bifid, and four arms in quadrifid with their respective lower stalk portion. Transverse sections of the stalk show four portions of the terminal cells in quadrifids and two in bifids (Plate- 6.3 i & j).

6.4 Ultrastructure of gland

6.4.1 Pedestal cell

The pedestal cell is a transfer cell type characterized by wall ingrowths which are mostly confined to the lateral side as well as to the junction of the stalk with pad-like appearance (Plate-6.4 a). The pedestal cell walls are highly differentiated with three wall layers, the outermost thick cuticle layer which extends till the base of the terminal cell. This cuticle is absent in the transverse wall of the pedestal cell, adjoining to the basal portion of the stalk. The middle layer is highly impregnated with opaque materials and is fibrillar in nature. The innermost layer is unimpregnated with numerous variously shaped electron translucent vacuoles. Occasionally, electron dense granules are also present inside the vacuoles. The middle lamella is very thin (Plate-6.4 b, c). In the present study, the wall ingrowths of the pedestal cell are not easily recognizable due to close packing of the cell wall ingrowths and dark stain. The pedestal and basal cells are interconnected with plasmodesmeta (Plate-6.4 a).

The protoplast of the pedestal cell is highly modified due to the intrusion of the wall ingrowths. The wall partition between the pedestal and the basal portion of the stalk bears several fingers like projections occupying large surface area in the protoplast (Plate-6.4 b, c). The adjoining walls of the tetrad pedestal cells also shows wall projections (Plate-6.4 d). These wall projections might have help in the absorption of digested materials inside the bladder.

6.4.2 Terminal cell

The arms of the gland are unicellular irrespective of the bifid or quadrifid type. As in that of pedestal cell, the wall of the stalk portion is also thick with the outer cuticle, heavily opaque impregnated middle wall, and the inner unimpregnated vacuolar wall from which the wall ingrowths arises. The wall ingrowths arise only on the transverse wall at the base of the stalk, but are totally absent on the lateral side of the arm portion (Plate-6.4 e). It reflects the organization of the wall which changes gradually towards the terminal portion of the arm with less opaque materials or impregnation. At the base of the arms i.e. the capital or the stalk portion, the opaque cuticle is thick which becomes thinner towards the proximal end of the arms (Plate-6.4 e, f).

The cytoplasm of the arm is mostly concentrated towards the basal region. The nucleus is granular with prominent nucleolus located in the lower periphery of the arm cell, and mitochondria and ER are the dominant organelles (Plate-6.4 g & h). TEM of arms show the presence of blue green algae, unicellular and filamentous green algae, uni and multicellular organisms and bacterial cells at the basal portion of the arm (Plate- 6.4 h, i).

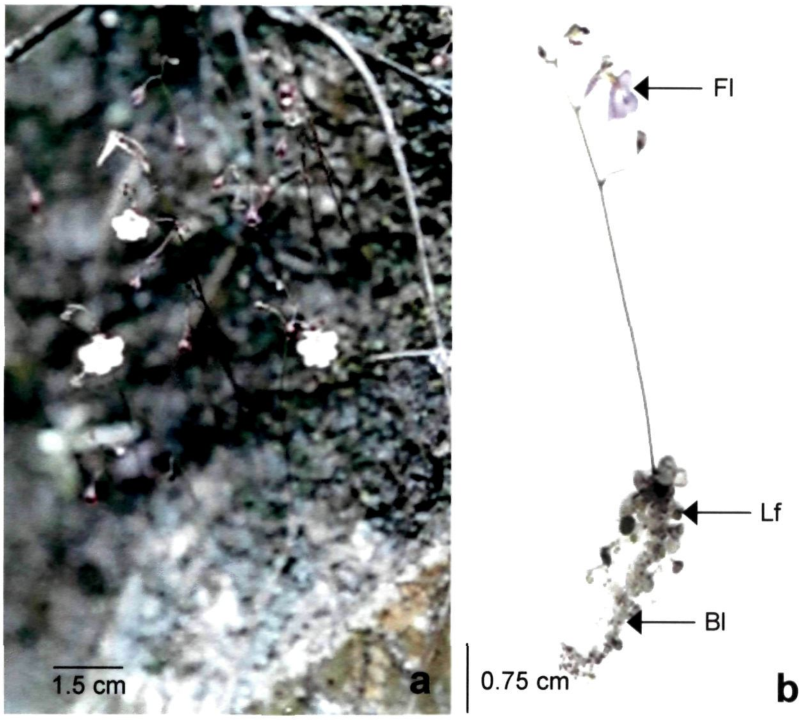
Plate- 6.1

A patch of *Utricularia pubescens* Sm. growing on the marshy and wet land slopes.

An enlarged view of a single plant of *Utricularia pubescens* Sm. Bladders (Bl) are the modified leaves (Lf) and the true root system is absent. Flowers (Fl) are purplish white.

A patch of *Utricularia bifida* Sm. population. These plants are free floating plants on marshy and wet places during rainy season. The flowers are yellow in colour.

Plate- 6.1 Habit and habitat of *Utricularia* species



A patch of *Utricularia pubescens* Sm. A single plant

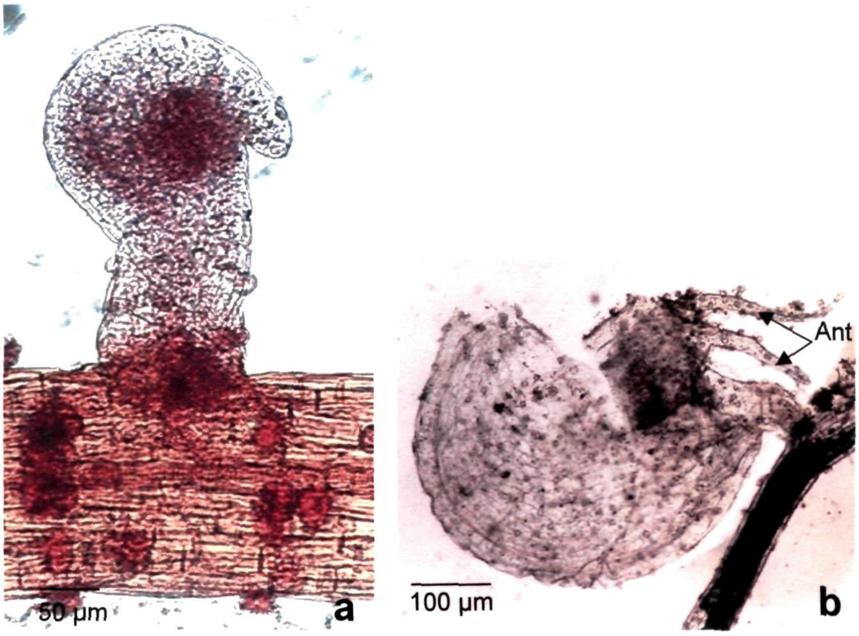
A patch of *Utricularia bifida* Sm.

Plate- 6.2

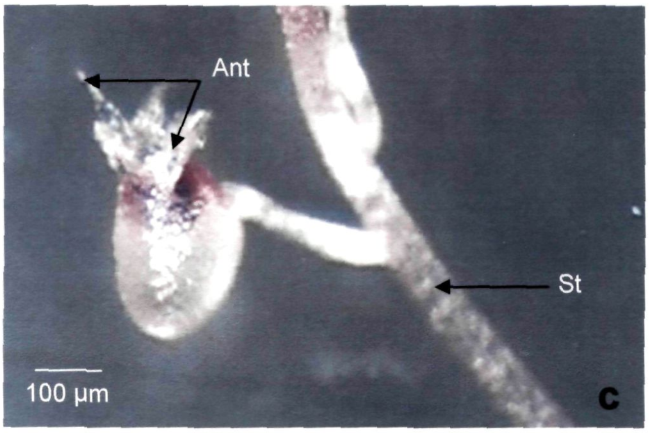
a & b Young and matured bladders of *Utricularia bifida.* At young stage the bladder develops antennae (Ant) as small protuberance. The matured bladders are horse-shoe shaped with two antennae.

c Single bladder of *Utricularia pubescens.* The bladder is with numerous unicellular and uniseriate antennae (Ant) and is attached to main stem (St).

Plate- 6.2 Morphology of bladders



Young and matured bladders of *Utricularia bifida*



A single bladder of *Utricularia pubescens*

Plate- 6.3

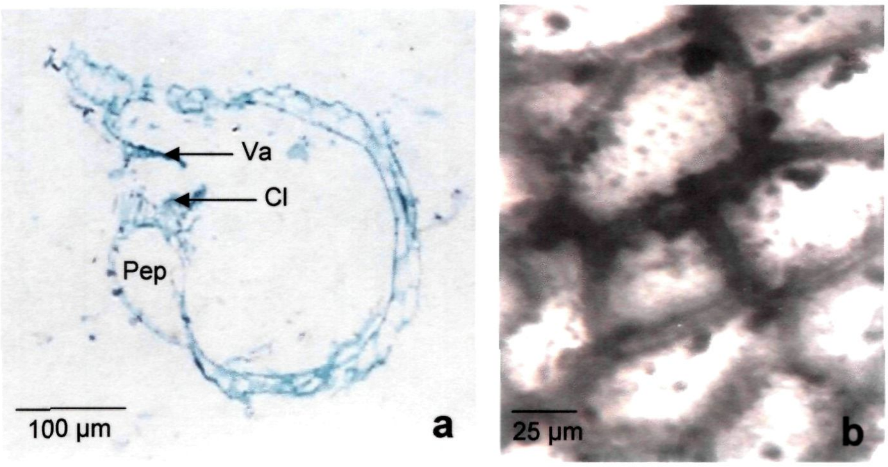
a Longitudinal section of a bladder. The mouth portion of the bladder is complicated with upper valve and lower collar or ridge with three layered cells called pavement epithelium. The valves as well as the pavement epithelium are directed inside towards the bladder.

b Whole mount of pavement epithelium. The cells of pavement epithelium are with thick radial as well as tangential walls.

c Whole mount of valve. The unicellular and uniseriate antennae or bristles on the valve portion of the mouth or orifice.

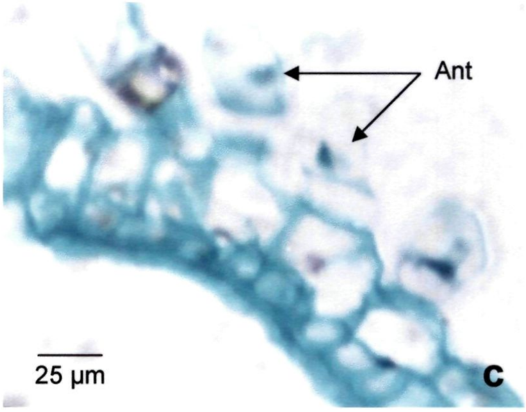
d Transverse section of pavement epithelium showing the three layers viz. the outermost capital layer and middle thick walled layer and the innermost radially elongated shape cells cell layer.

Plate- 6.3 Anatomy of the bladders and gland architecture

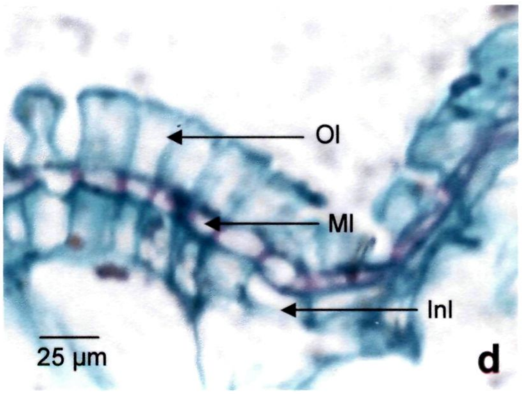


L.S. of a bladder

Whole mount of pavement epithelium



Whole mount of valve



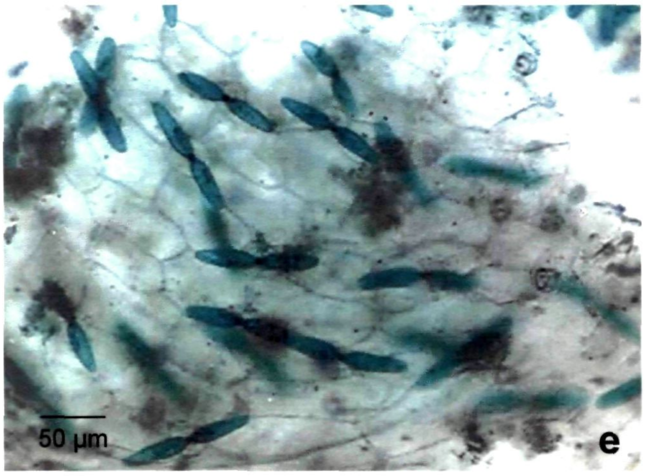
T.S. of the pavement epithelium

Plate- 6.3

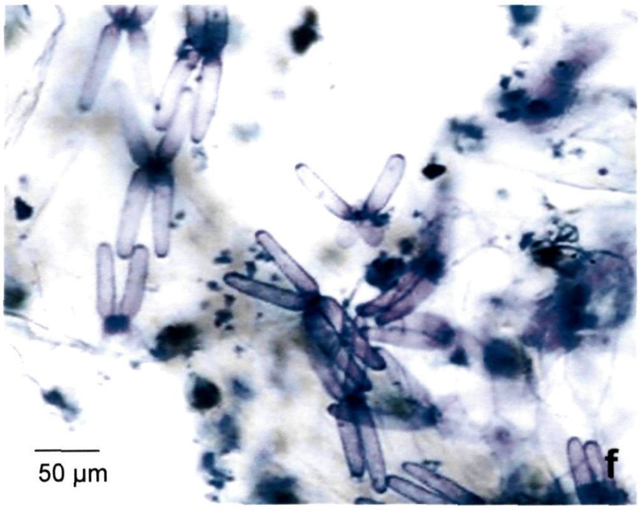
e Bifid glands of *Utricularia bifida.* The glands possess two arms or terminal cells originating from two pedestal cells arising from single basal cell.

f Quadrifid glands of *Utricularia pubescence.* The glands possess four arms or terminal cells originating from four pedestal cells arising from single basal cells.

Plate- 6.3 Anatomy of the bladders and gland architecture



Bifid glands of *Utricularia bifida*



Quadrifid glands of *Utricularia pubescens*

Plate- 6.3

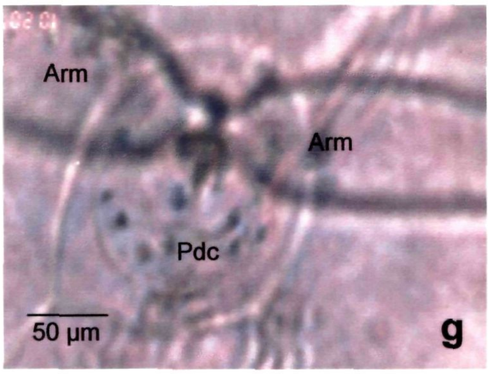
g A single gland of *Utricularia bifida* showing two arms and two pedestal cells.

h A single gland of Utricularia *pubescens* showing the four arms and four stalk portions of the arms.

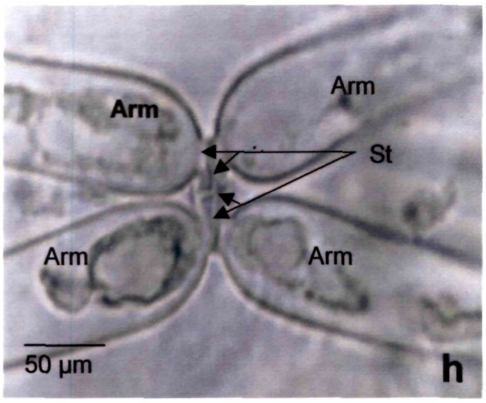
i Tetrad pedestal cells of *Utricularia pubescens* arising from a single basal cell.

j A complete quadrifid gland of *Utricularia pubescens* showing the subconical shape pedestal cell, arm and stalk cells. Note a thick 'pad' (of transfer cell type) in the pedestal cell staining with Toluidine blue O.

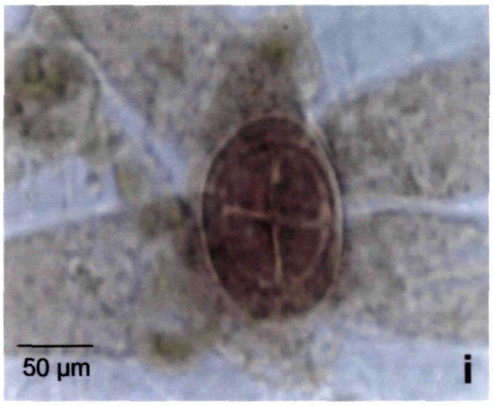
Plate- 6.3 Anatomy of the bladders and gland architecture



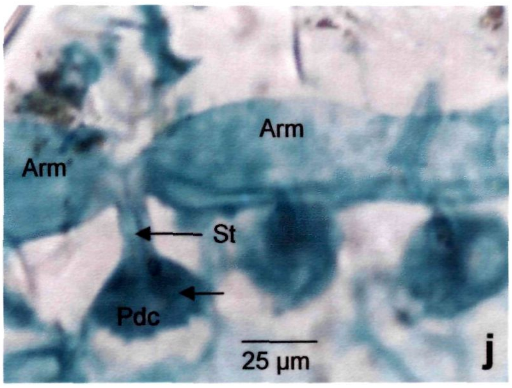
A single gland of *Utricularia bifida*



A single gland of *Utricularia pubescens*



Tetrad pedestal cells of *Utricularia pubescens*



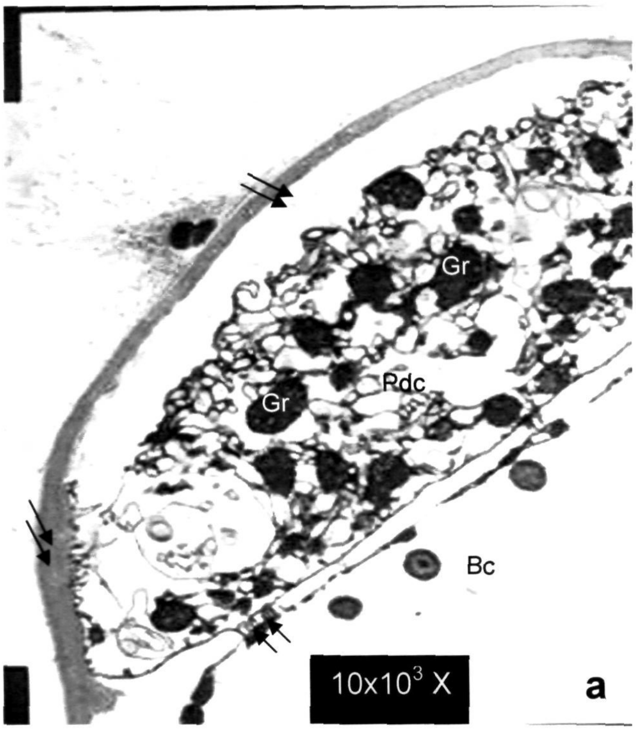
A complete quadrifid gland of *Utricularia pubescens*

Plate- 6.4

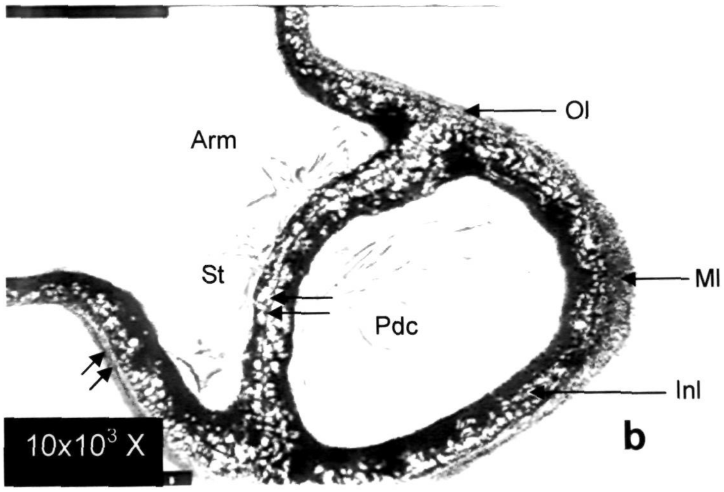
a TEM of a pedestal cell showing pad-like wall ingrowths at the lateral side and junction of stalk and pedestal cell. Numerous granules (Gr) are present. Note the pad-like wall ingrowths in the pedestal cell and plasmodesmetal connections between the basal and the pedestal cells (double arrows ahead).

b TEM of the terminal and pedestal cells showing the three wall layers: the outer cuticle (Ol), highly impregnated opaque and fibrillar middle layer (Ml)and the innermost unimpregnated vacuolar electron translucent layer (Inl). (Note double arrows showing the extension of the cuticle extended till the outer wall of the terminal cell and the thin middle lamella).

Plate- 6.4 Ultrastructure of the gland cell



TEM of a pedestal cell



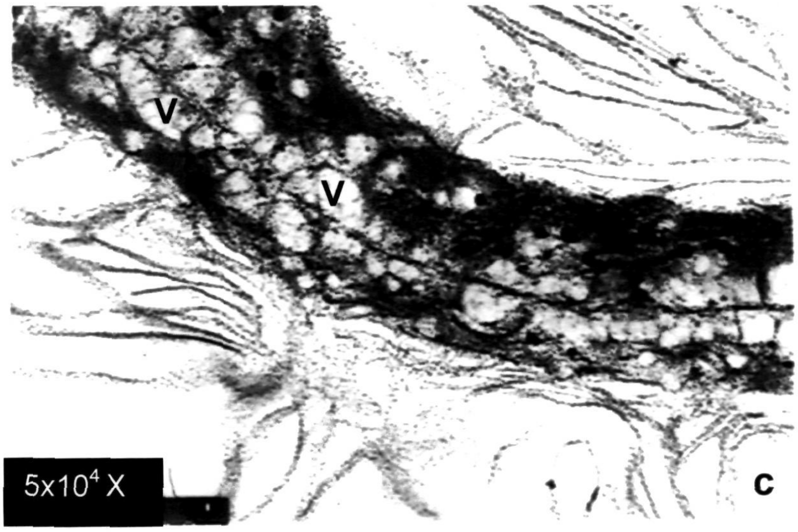
TEM of the terminal and pedestal cells

Plate- 6.4

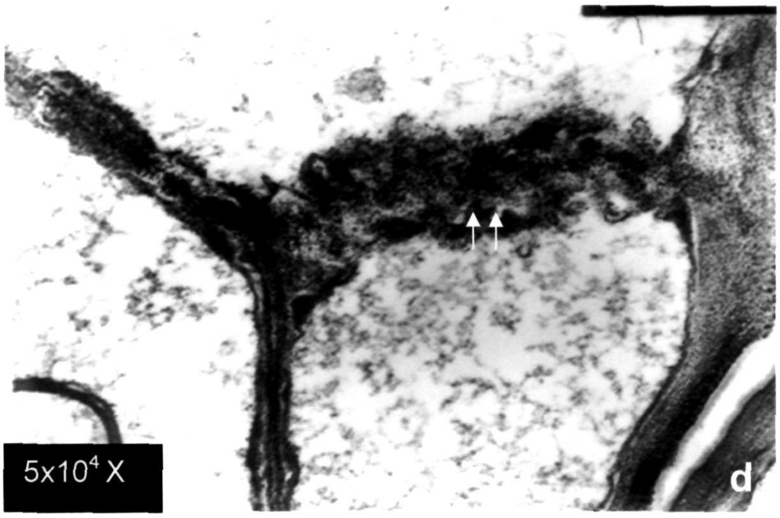
c TEM of the adjoining wall between pedestal and basal cells showing several finger like projections. (Note the vacuoles present inside the cell wall and the thin middle lamella).

d TEM of T.S. of tetrad pedestal cell. The adjoining walls of the tetrad pedestal cells shows wall projections (arrows ahead) and the cell cytoplasm is translucent.

Plate- 6.4 Ultrastructure of the gland cell



TEM of the adjoining wall between pedestal and basal cells



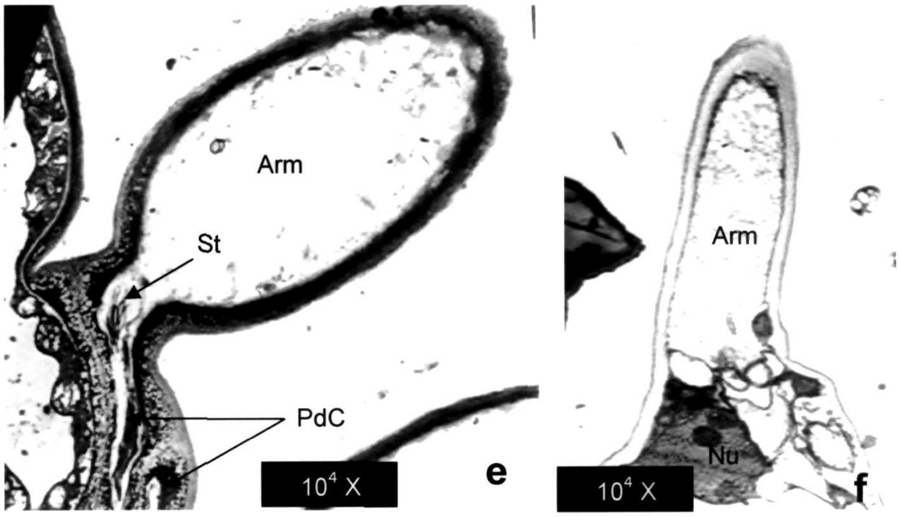
TEM of T.S. of tetrad pedestal cell

Plate- 6.4

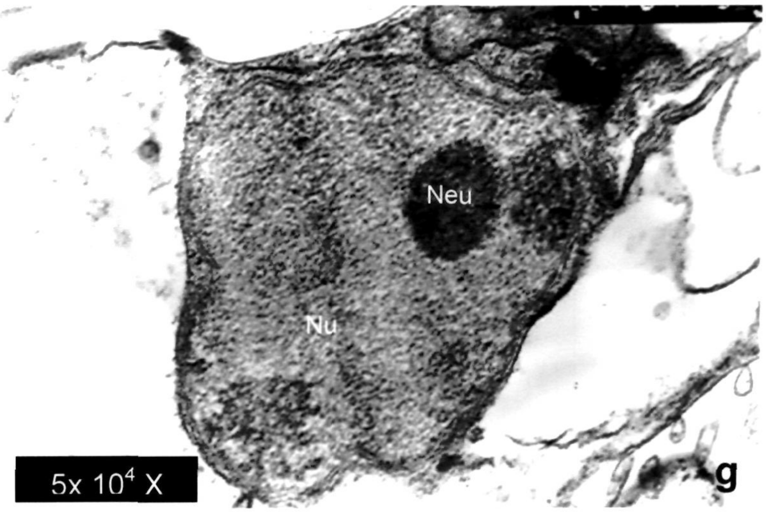
e & f TEM of terminal cells and protoplast of single arm. The arms of the gland are unicellular and cytoplasm mostly concentrated towards the basal region as a thin film.

g TEM of enlarged view of granular nucleus and nucleolus.

Plate- 6.4 Ultrastructure of the gland cell



TEM of terminal cells and protoplast of single arm

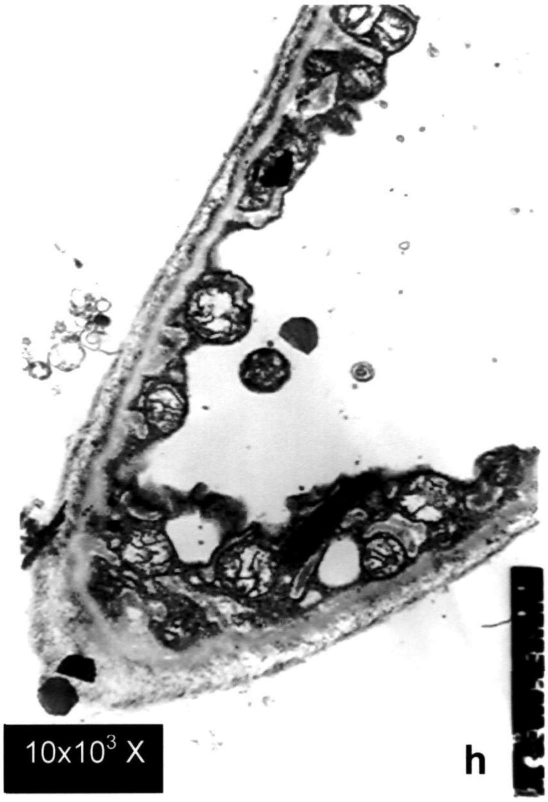


TEM of enlarged view of nucleus and nucleolus

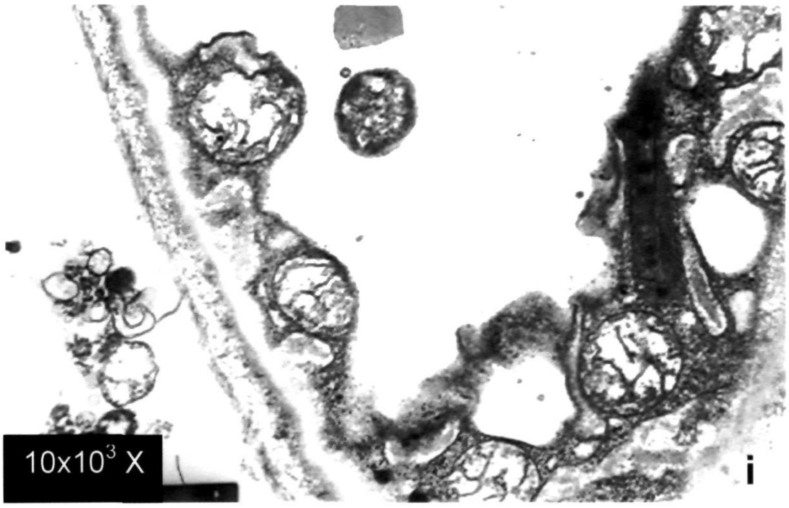
Plate- 6.4

h & i TEM of protoplast of arm and an enlarged view of arm protoplast. Mitochondria and ER are the dominant organelles. TEM of arms also contain blue green algae, unicellular and filamentous green algae, uni and multicellular organisms, and bacterial cells at the basal portion of the arms.

Plate- 6.4 Ultrastructure of the gland cell



TEM of protoplast of arm



An enlarged view of protoplast of arm

CHAPTER- 7

Histochemistry of different digestive traps of selected insectivorous plants

7.1 Introduction

Histochemical methods are very useful as they facilitate *in situ* localization of enzymes in tissues and cells. Histoenzymology is a part of histochemical methods, which provides information on the distribution and activity of enzymes present in the cells, tissue or organs. It usually involves *in situ* detection of enzymes and their localization, and the enzyme activities are determined by applying the principles of biochemistry. The activity profile of an enzyme can be studied by supplementing different concentration of substrate, pH, and incubation period. It can be expressed on fresh weight or dry weight on the basis of protein and nucleic acid. The unit of enzyme activity is expressed in µmole/min/mg protein.

Advantages of histoenzymology

i) This technique provides the opportunity to study intracellular distribution of enzymes maintaining the tissue integrity.

ii) There technique also provides a scope to determine the components of cell membrane, cell wall and leachates from cells etc.

Carnivorous plants have developed several types of specialized traps during the course of evolution (Darwin, 1875; Lloyd, 1942; Juniper et al., 1989). Animals are additional source of N, P, K and Mg for carnivorous plants in which few species can take up more than 50% of their N and P from prey (Lollar et al., 1971; Friday and Quarmby, 1994; Adamec, 1997). In addition to endoenzymes, specialized trichomes present in different traps of carnivorous plants secrete exoenzymes mainly hydrolases, e.g. acid phosphatases, esterases, proteases, ribonucleases etc. Although, very little is known about the production of hydrolytic enzymes and their mechanisms of digestion in carnivorous plants. The subcellular localization of hydrolytic enzymes has been studied in several genera (Heslop- Harrison, 1975, 1976), where detail information is available only for *Dionaea, Nepenthes* and *Pinguicula* (Juniper et al., 1989).

McNally et al. (1988) observed high activity of acid phosphatase in the protoplasm of stalk glands in *Drosera rotundifolia* after stimulation. It suggests *de novo* synthesis of acid phosphatase in the gland cells. Acid phosphatase associated with unstimulated gland walls help in mucilage (polysaccharide) transport. Heslop- Harrison (1976) studied digesting the gelatin film by the sessile glands in *Drosophyllum lusitanicum.* Knox and Heslop- Harrison (1969) also revealed the release of protease enzymes by the exine of mature pollen grains.

Several authors have studied the production of acid phosphatases by carnivorous plants (Clancy and Coffey, 1976; Robins and Juniper, 1980; Sirova et al., 2003), whereas proteases activity was absent in *Utricularia*, (Sirova et al., 2003) or *Byblis* (Hartmeyer, 1997). Plachno et al. (2006) suggested that the phosphatases are indispensable for phosphate mobilization from prey carcasses, and phosphate uptake, which is essential for many carnivorous plants. They studied acid phosphatase activity in the glandular structures of 47 carnivorous plant species, especially Lentibulariaceae, using enzyme labeled fluorescence (ELF) phosphate substrate. Positive reactions were also found in *Genlisea*, *Pinguicula*, *Aldrovenda*, *Dionaea*, *Drosera*, *Drosophyllum*, *Nepenthes*, and *Cephalotus*.

Enzymes in the traps of insectivorous plants might have also been produced by associated organisms, such as, bacteria, fungi, algae, and invertebrates, which are often detected in the trap environment of both aquatic and terrestrial insectivorous plant species (Juniper et al., 1989; Ellis and Midgley, 1996; Hartmeyer, 1997; Lowrie, 1998; Anderson and Midgley, 2002; Ellison et al., 2003; Sirova et al., 2003; Plachno et al., 2005b). The role of these enzymes in the digestion process of the associated organisms present in the traps of carnivorous plants is under debate (Richards, 2001).

7.2 Result

Localization of insoluble polysaccharides

Localization of the insoluble polysaccharide in *N. khasiana* using PAS (Periodic acid Schiff's reagent) method reveals that the cell wall of the gland, inner hypodermal cells shows purplish pink colouration with the stored starch grains in the hypodermal cells (Plate- 7.1 a & b). Starch granules are found stored in the inner tissues of the leaf not seen in the gland cells of *D. peltata* Sm. The cell walls of gland head portion and the cell walls of the tentacle shows purplish pink colouration which means that the cell wall is cellulosic in nature (Plate- 7.1c & d). Cellulosic cell wall composition has also been detected in the cell wall of the sessile gland cells using the fluorochrome calcofluor which fluoresce (Plate- 5.6 b). The principle of this process is based on the following reaction;

$$\underset{\text{(Starch)}}{\text{Glucose}} \xrightarrow[\text{Oxidation}]{KIO_3, \text{PAS}} \text{Aldehyde}$$

In this reaction, glycol group in the glucose molecules of starch are being oxidize into aldehydic group. Free aldehydes are demonstrated by staining with Schiff's reagent showing the reaction is due to aldehydes.

Localization of DNA

In *N. khasiana* localization of nuclear DNA by Feulgen method is confined more on the nuclear portion of the secretory as well as the communicatory cells of the glands (Plate- 7.1 e). In *D. peltata*, nuclei of secretory, communicatory and the endodermal cells of the gland head portion show positive reaction (Plate- 7.1 f & g). The reaction product shows pink colouration. Cytoplasmic DNA content is comparatively less which might have dissolved during the process of dehydration. This method is also based on Schiff's reaction for staining aldehyde groups a purplish pink colouration released from deoxyribose sugar, after the removal of purines at the level of purine- deoxyribose glucosidic bonds of DNA by hydrolyzing in 1N HCl. The reaction is same as that of the localization of the insoluble polysaccharides.

Localization of total proteins

In *N. khasiana*, the localization of proteins using Mercuric bromophenol blue method shows the proteins in the secretory and the communicatory cells of the gland (Plate- 7.1 h). The staining is performed at a controlled pH, which is important because the reaction will depend upon the isoelectric point of the protein. The proteins are confined in the secretory and the communicatory cells of tentacle of *D. peltata*. The stalk cells also show the same reaction. The result is found to be in the inner tissues of the leaf with the reaction product of blue colour (Plate- 7.1 i & j).

Due to the presence of free (basic) amino and (acid) carboxyl groups, protein in solution appears as amphoteric electrolytes. The basic of the method for the detection of the protein is that the dyes will dissociate in solution to form negatively or positively charged ions depending on whether they are acid or basic dyes, respectively. Below the isoelectric point of a protein, but above the isoelectric point basic dyes will combine with negatively charged proteins. Hence a tissue component will be stained by a basic dye with the pH above its isoelectric point, and by an acidic dye with the pH below this point.

Localization of lipase activity

Localization of lipase activity is confined to the innermost cells of communicatory and endodermal layers of the gland, and the some layers of the hypodermal cells of the pitcher in *N. khasiana* (Plate- 7.1 k & l). But, *D. peltata*, the localization of lipase activity is confined to the innermost cells of communicatory layer, the endodermal cell layer and the central tracheary cells of the tentacles with the reaction product showing brownish black. Some of the cells at the junction of the head and the stalk regions of the tentacle also show the reaction product (Plate- 7.1 m & n).

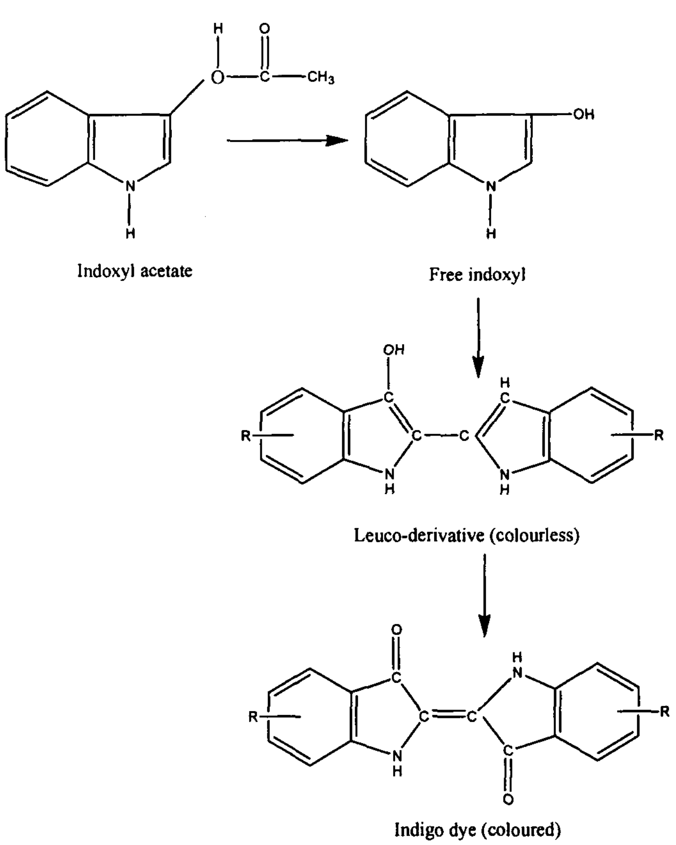
Stalk cells do not give the positive reaction. Here, hydrolysis of the long chain saturated fatty acid or lipids (Tween 60, a commercial detergent) into short chain fatty acids. These will again combine with the calcium ions to produce insoluble calcium salts. The addition of the dilute lead nitrate, Pb $(N0_3)_2$ produces lead ions, which are then treated with dilute ammonium sulphide to give a visible precipitate of lead sulphide at the site of enzyme activity.

Localization of esterase activity

Esterase activity is found in almost all the gland layers and also the hypodermal tissues of the pitcher in *N. khasiana* (Plate- 7.1 o & p). Esterase enzyme shows more activity in the entire cell layers of the head of the tentacle as well the neck and the stalk cells of *D. peltata.* with the formation of the reaction product of bluish granules in the enzyme active site (Plate- 7.1 q & r). The inner tissues of the leaf show the reaction which implies that the tentacles as well the leaves of the plant are used in the metabolism of fatty acids and other non specific esters of the plant system.

The localization of non specific esterase activity is a simultaneous coupling method with potassium ferricyanide as coupler. The esterase in the section hydrolyzes the indoxyl acetate into free indoxyl, a soluble product which is then oxidized by the potassium ferricyanide to an insoluble indigo dye of blue coloured granules. The potassium ferrocyanide in eqimolar solution with the ferricyanide prevents overoxidation of the indigo.

The formation of the product is based on the following reaction:



Localization of phosphatases activity

The acid phosphatase activity is found in all the communicatory and the endodermal layers of the gland of *N. khasiana* giving a brown dense deposit (Plate- 7.1 s & t). Acid phosphatase activity is confined to the inner cells of the communicatory layer, endodermal layer and some treachery cells of the head portion except the outer cells of the communicatory layer and the secretory layer of tentacles of *D. peltata*. All the sites of the tentacle show the activity of the particular enzyme with brown black precipitate of lead sulphide (Plate- 7.1 u- w). Activity of the enzyme is found in almost all the cytoplasm and cell wall of all the cells. Deposition of reaction product is in greater prominence towards the cytoplasm of the gland cells and as a whole towards the middle portion of the head region. Very less reaction product is found in some outer cells of the stalk region.

Localization of the acid and alkaline phosphatases activity in the gland tissue differs. The substrates used are of phosphate contents and exhibit optimum activity at pH 5.0 and pH 9.2 respectively.

Deposition of reaction product is in greater prominence towards the cytoplasm of the gland cells and as a whole towards the middle portion of the head region. Very less reaction product is found in some outer cells of the stalk region. Localization of the acid and alkaline phosphatases activity in the gland tissue differs. The substrates used are of phosphate contents and exhibit optimum activity at pH 5.0 and pH 9- 9.2 respectively. The reaction product is absent in cells incubated in control medium i.e. in the absence of the substrate. In this reaction no activator is required. The enzyme splits phosphate ions from the substrate and these from an insoluble precipitate converted to lead sulphide. The reaction of this process is of the following;

$$\text{Sodium } \beta\text{- glycerophosphate} \xrightarrow{\text{Acid phosphatase}} \text{Phosphate ions}$$

$$\underset{\text{(Lead nitrate)}}{\text{Phosphate ions + Lead ions}} \longrightarrow \underset{\text{(precipitate)}}{\text{Lead phosphatase}}$$

$$\text{Lead phosphate + Sulphide ions} \longrightarrow \underset{\text{(brown black precipitate)}}{\text{Lead sulphide}}$$

In the localization of the alkaline phosphatase activity, N, N-dimethyl formamide is used as the coupler with the substrate 5-Bromo 4-Chloro-3-indoxyl phosphate to release the napthol phosphates. The azo dye Nitro blue tetrazolium chloride (NBT) traps the napthol group and gives the blue colouration in the enzyme active sites in the tissues. In *N.*

khasiana the active site of alkaline phophatase are almost all the gland tissues (Plate- 7.1 x & y).

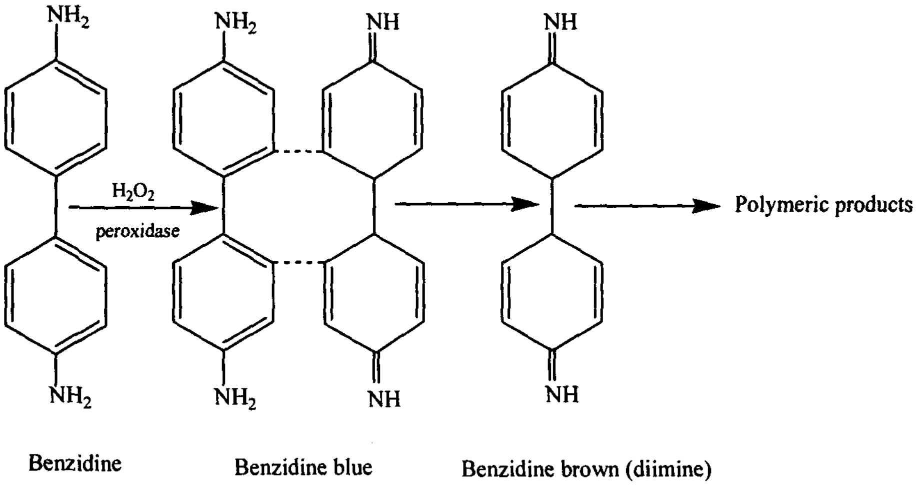
The enzyme active sites show the reaction product with blue colouration to almost all the cell layers of the head portion of the tentacle. The reaction product is found in the cytoplasm as well as the cell wall portions of the communicatory, endodermal and the central tracheae cells. But the case is different in the secretory cells where the reaction product is mainly in the cytoplasm and not in the wall side. There is a differential reaction showing in the stalk cells. More reaction product formation is found in the inner stalk cells than the outer stalk cells (Plate- 7.1 z & z_1).

Localization of peroxidase activity

The activity of peroxidase enzyme in the gland and other tissues of the pitcher in *N. khasiana* show different colour indications. The communicatory and the endodermal layers show the brown colour and blue colour in the inner tissues is due to the formation of intermediate unstable product of benzidine (Plate- 7.1 a_1 & b_1). The most common indicator method for peroxidase detection employs benzidine and hydrogen peroxide as substrates. H_2O_2 in the reaction is used as the electron and hydrogen donor giving toxicity to the cells. The peroxidase activity in the gland cells also shows that the toxicity in the cells is taken care by this enzyme. The activity is more in the cell wall region rather than the cytoplasm.

Unlike other enzyme activities, peroxidase activity is confined only to the central treachery cells of tentacles in *D. peltata* Sm. But more reaction product is found in the cells at the junction of the head and the stalk region of the tentacle, the lower treachery cells and almost all the cells of the stalk region of the tentacle. The uppermost cells of the central treachery region also show the same reaction forming the colouration (Plate- 7.1 c_1& d_1). This can also be predicted in the young stage of the tentacle, the blue intermediate product may be precipitated in the form of stable blue crystals in this region of the head region which latter on in mature stages show brown colour.

The principal of this histochemical method is based on the following reaction;



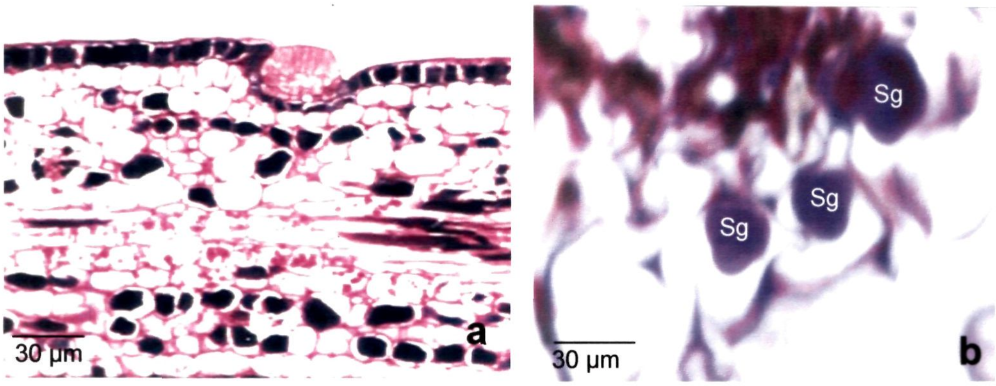
Localization of protease activity

In the substrate film method, the digestion on the exposed film shows that the gelatin surface of the film is proteinaceous and is digested by the protease enzymes present in the glandular surface of the glands in the inner surface of the pitcher of *N. khasiana*. The digestion of the exposed gelatin layer is seen after the incubation in the reaction medium for almost 1 hr. In the late stage i.e. treatment for almost 3 hrs. in the reaction medium show more digestion of the gelatin layer by the surface of the gland (Plate- 7.1e_1 & f_1).

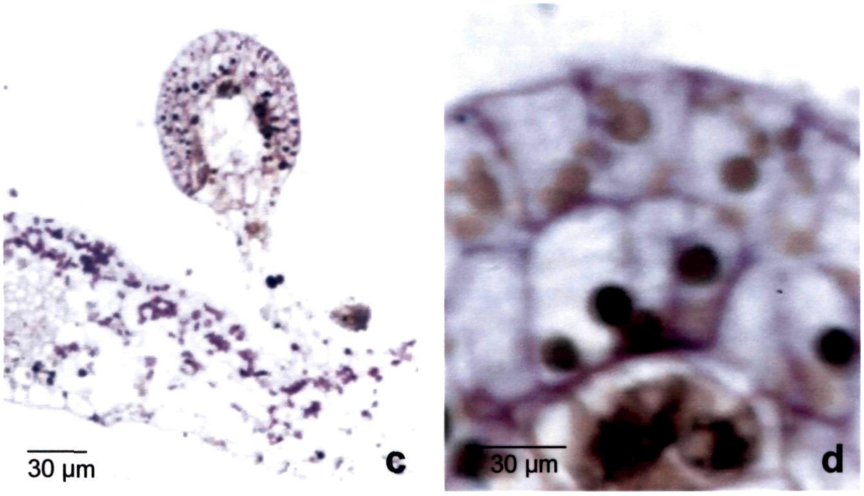
Localization has been performed only on the esterase activity in the bladders using indigogenic method and found active in all the surface of the bladder, the basal cells of the gland, and also to the antennae of the bladder (Plate- 7.1 g_1 – i_1). Due the interference of the various organisms to the bladders and the glands cells, localization of other enzymes could not be performed.

In all the methods adopted for the histochemical localizations in the gland tissues and other traps show the activity of enzymes. Tissues without the activity of enzymes, incubated in the reaction medium without the specific substrates proves that these chemicals particularly in the glands might have helped in the adaptation of the plant for in the digestion of the prey.

Plate- 7.1 Histochemical localization of insoluble polysaccharide



Nepenthes khasiana

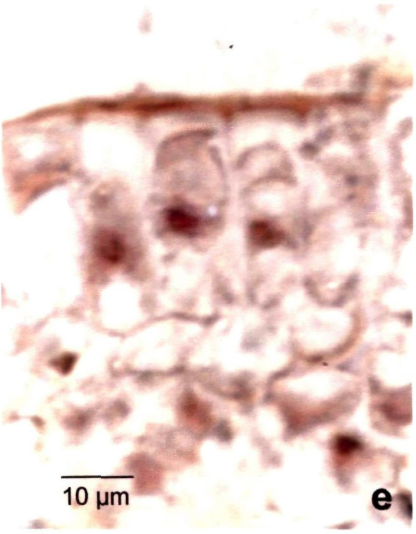


Drosera peltata

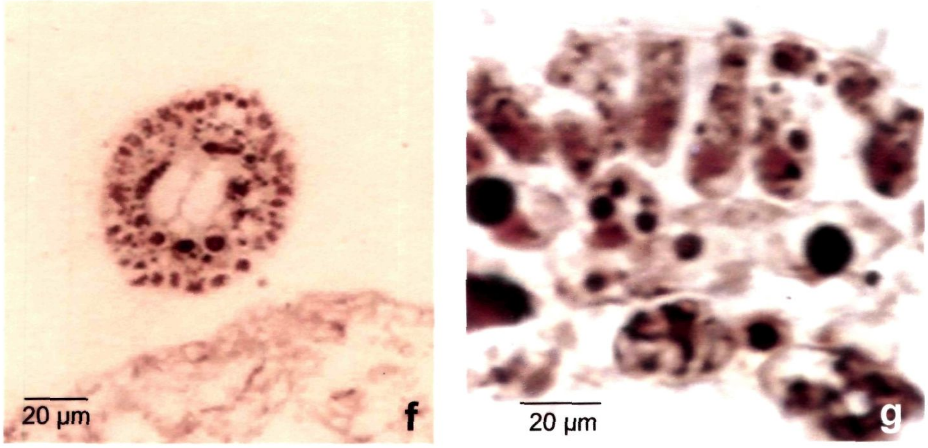
a & b Localization of insoluble polysaccharide in *Nepenthes khasiana* and a few enlarged starch grains. The cell wall of the gland, inner hypodermal cells shows pink colouration with the stored starch grains in the hypodermal cells with PAS (Periodic acid Schiff's reagent).

c & d Localization of insoluble polysaccharide in *Drosera peltata* and it's enlarge view. The cell wall of the gland head portion shows the pink colouration and the hypodermal cells with starch gains. Starch grains were not seen in the cells of the gland with PAS.

Plate- 7.1 Histochemical localization of nuclear DNA



Nepenthes khasiana

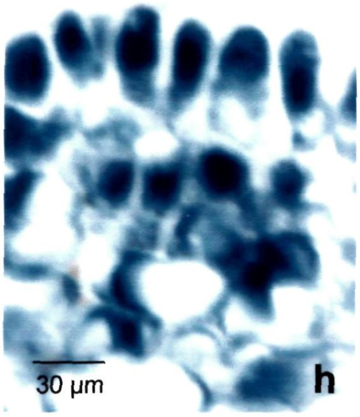


Drosera peltata

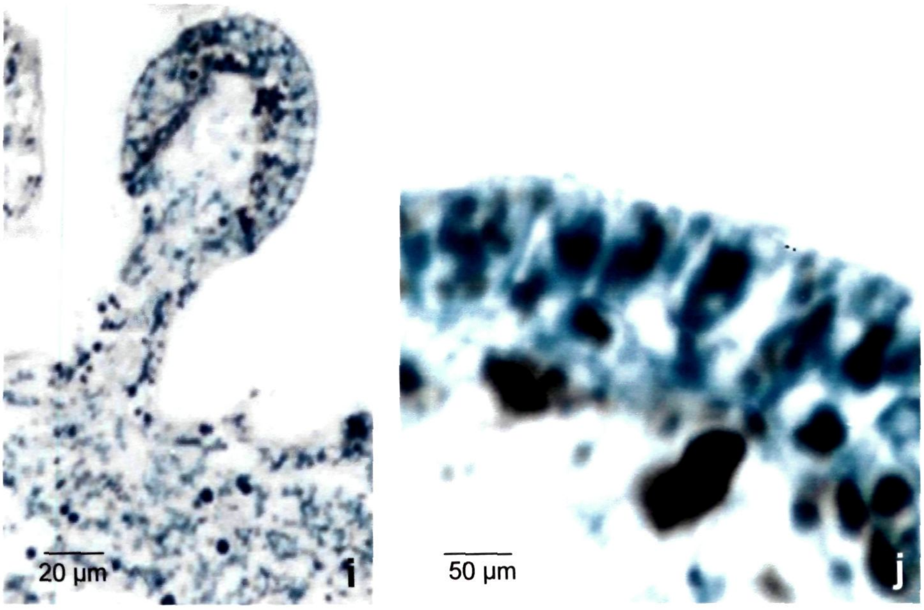
e Localization of nuclear DNA in the gland of *Nepenthes khasiana.* Nuclear DNA is localized in the secretory cells of the gland using Feulgen stain.

f & g Localization of nuclear DNA in the gland of *Drosera peltata* and it's enlarge view. Nuclear DNA is localized in the secretory and outer cells of communicatory layer of the gland head portion using Feulgen stain.

Plate- 7.1 Histochemical localization of total protein



Nepenthes khasiana

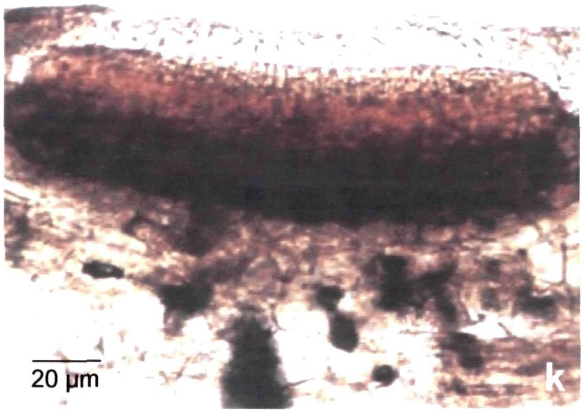


Drosera peltata

h Localization of total protein in the gland of *Nepenthes khasiana*. The protein is found confined in the outer secretory cells and some cells of the communicatory layer of the gland.

i & j Localization of total protein in the gland of *Drosera peltata* and it's enlarge view. The localization is mainly in the tentacle's head region and epidermal as well as hypodermal regions of the leaf and some of the stalk cells.

Plate- 7.1 Histochemical localization of Lipase activity in *Nepenthes khasiana* (Tween method)



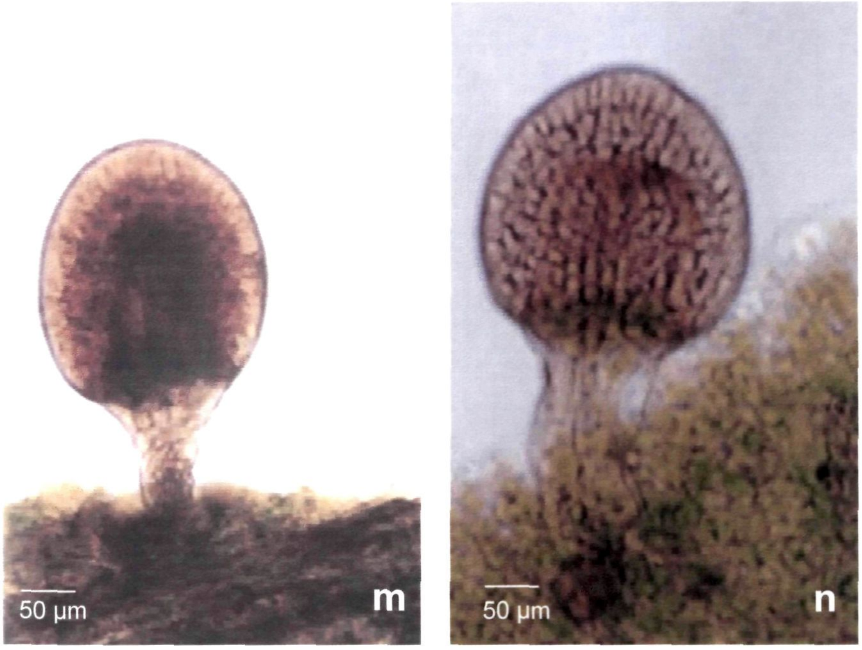
Treated

Control

k Lipase activity in *Nepenthes khasiana.* Lipase activity is localized in the inner cells of the communicatory layers, the endodermal layer and some of the cells of vascular tissues.

l Control test of Lipase activity *Nepenthes khasiana.* Without the substrate, activity is not localized in the tissues of the gland.

Plate- 7.1 Histochemical localization of Lipase activity in *Drosera peltata* (Tween method)



Treated

Control

m Localization of Lipase activity in *Drosera peltata.* The activity is found in the inner communicatory, the tracheal cells and the endodermal layer.

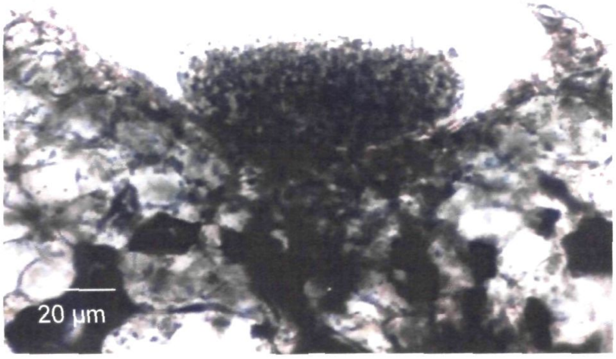
n Control test of lipase activity in *Drosera peltata*. Without the substrate, activity is not localized in the tissues of the gland.

Plate- 7.1

o & p Esterase activity in *Nepenthes khasiana* in treated and control samples. The activity is confined in almost all the gland layers except the outer wall of the secretory layer. In control test, activity is not localized in the tissues of the gland.

q & r Localization of Esterase activity in *Drosera peltata* in both treated and control samples. The activity is confined in almost all the gland layers except the outer wall of the secretory layer. Activity is also shows in the stalk cells and inner tissues of the leaf. In the control test, the colour indication gives positive results. This may be due to involvement of different biomolecules.

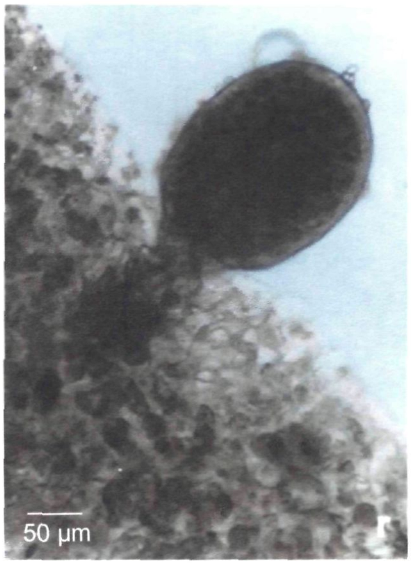
Plate- 7.1 Histochemical localization Esterase activity (Indigogenic method)



Nepenthes khasiana (Treated)

Nepenthes khasiana (Control)

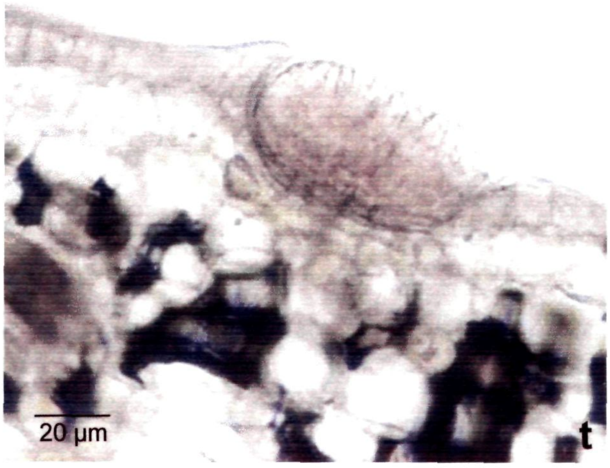
Drosera peltata (Treated)



Drosera peltata (Control)

Plate- 7.1 Histochemical localization of Acid phosphatase activity in *Nepenthes khasiana* (Metal salt method)

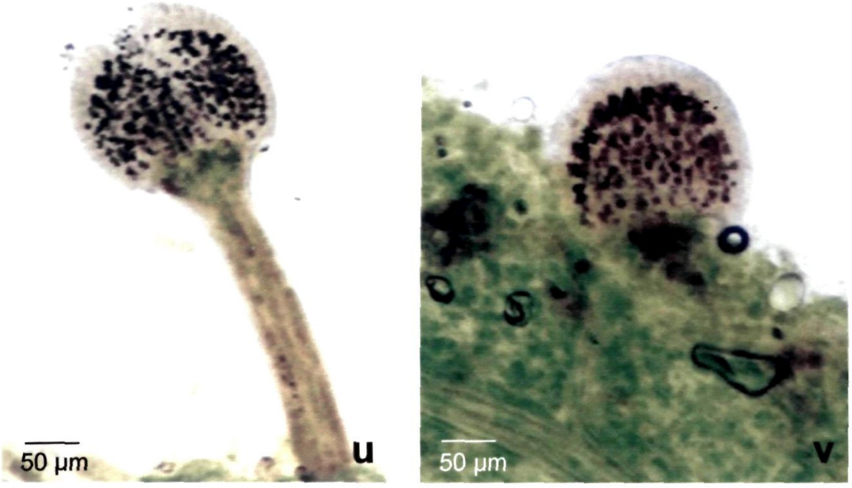
Treated



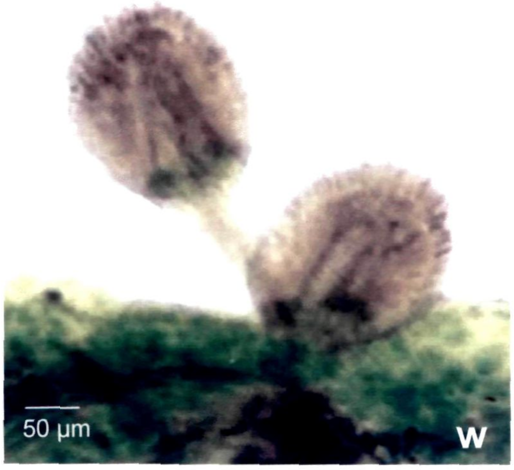
Control

s & t Localization of Acid phosphatase activity in *Nepenthes khasiana.* Activity is mostly found in the inner communicatory cells and the endodermal layer in the treated, while it is absent in the control.

Plate- 7.1 Histochemical localization of Acid phosphatase activity in *Drosera peltata* (Metal salt method)



Treated



Control

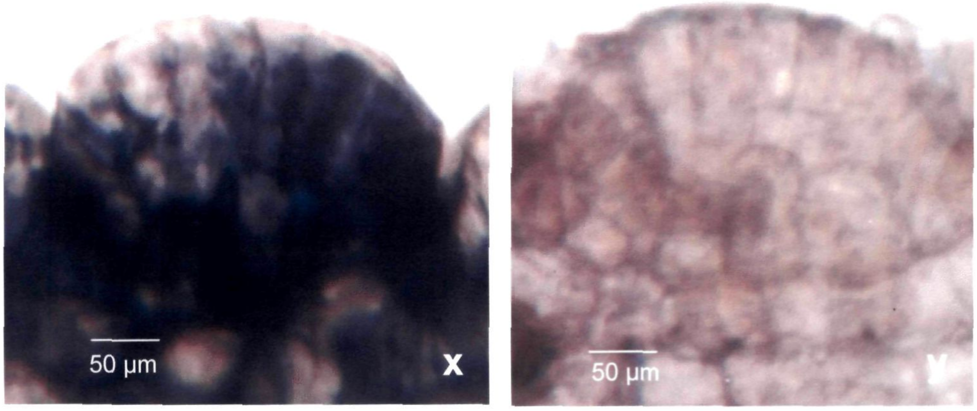
u- w Localization of Acid phosphatase activity in *Drosera peltata.* The activity is found mainly in all the cells of the inner communicatory layer, endodermal layer and the central tracheal cells. Activity is also seen in some of the stalk cells. In control activity is not localized in the tissues of the gland.

Plate- 7.1

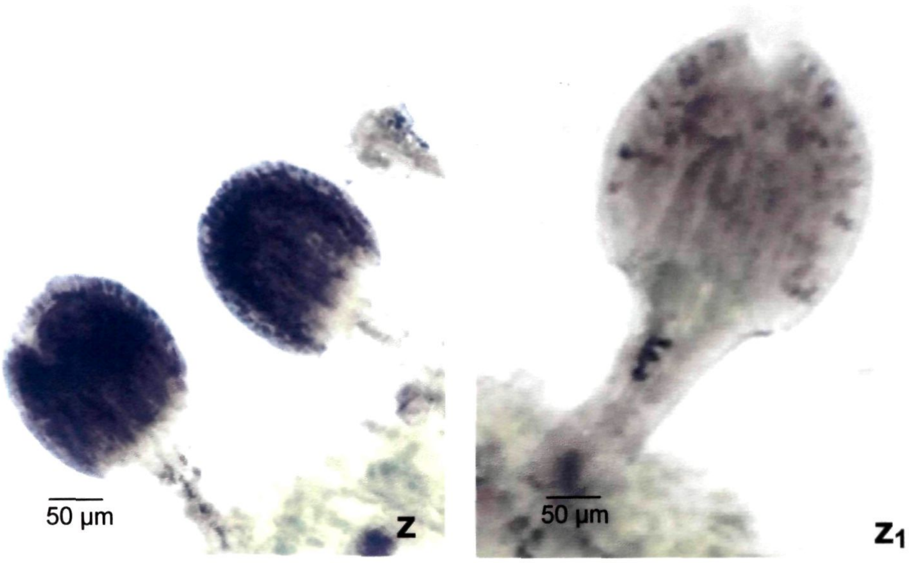
x & y Localization of Alkaline phosphatase activity in *Nepenthes khasiana* and *Drosera peltata* samples respectively. The activity is diffused throughout the cytoplasm as well as the cell wall of each layer. Cell cytoplasm and cell wall of secretory layer do not show positive result. Hypodermal cells also show positive reaction to the substrate in *Nepenthes khasiana*.

z & z_1 Localization of Alkaline phosphatase activity is found in all the cell cytoplasm and cell wall of in all the layers except the cell walls of secretory layer in *Drosera peltata*

Plate- 7.1 Histochemical localization of Alkaline phosphatase activity (Tetrazolium method)



Nepenthes khasiana (Treated) *Nepenthes khasiana* (Control)



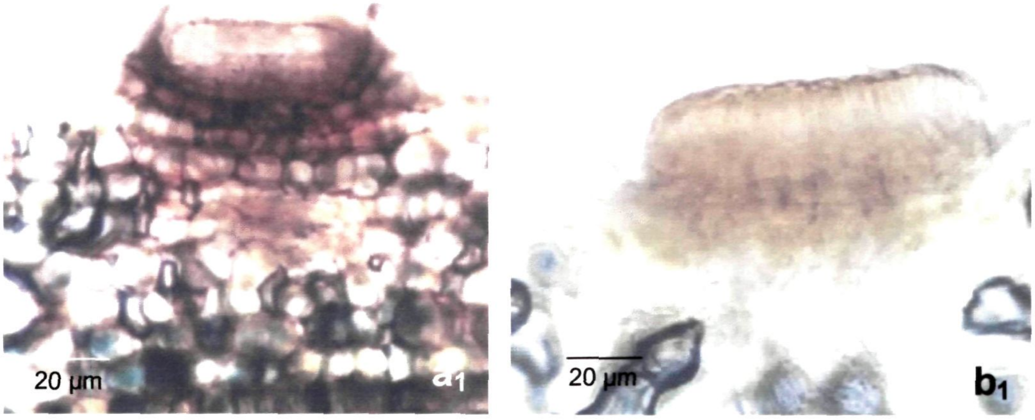
Drosera peltata (Treated) *Drosera peltata* (Control)

Plate- 7.1

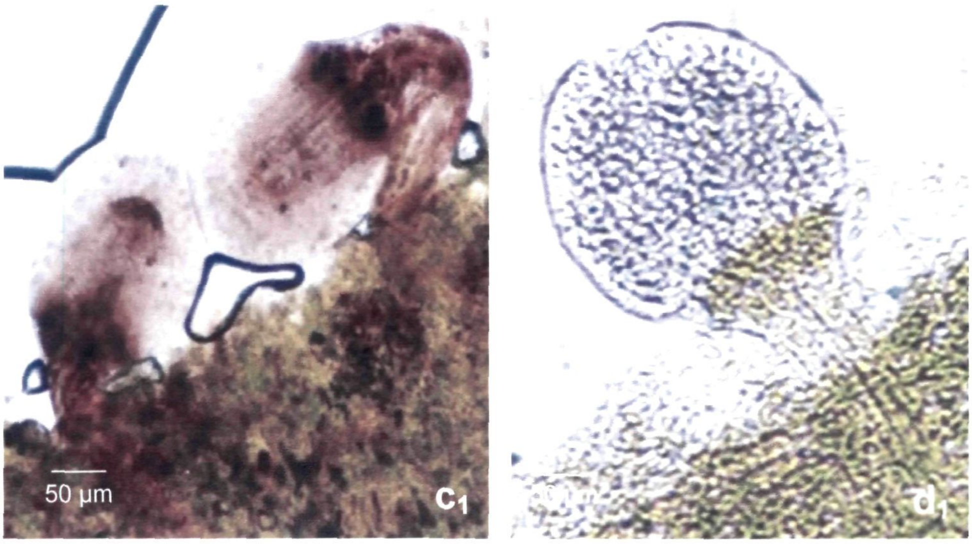
a_1 & b_1 Localization of Peroxidase activity in *Nepenthes khasiana* and *Drosera peltata* in treated and control samples respectively. Activity is mostly found active in the inner communicatory cells and the endodermal layer. The cell wall of inner hypodermal cells also shows the activity in *Nepenthes khasiana*.

c_1 & d_1 Localization of Peroxidase activity in active in the cell cytoplasm and cell wall of the central tracheae cells and the endodermal cells in *Drosera peltata* while in control test, peroxidase activity is not localized in the tissues of the gland.

Plate- 7.1 Histochemical localization of Peroxidase activity (Benzidine method)



Nepenthes khasiana (Treated) *Nepenthes khasiana* (Control)



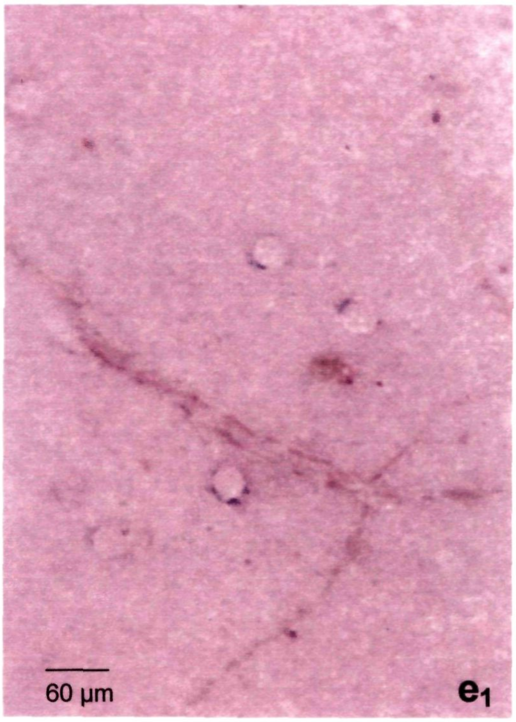
Drosera peltata (Treated) *Drosera peltata* (Control)

Plate- 7.1

e_1 Initial stage of Gelatin layer digestion in *Nepenthes khasiana.* The surface of the glands shows initial stage of digestion of gelatin layer of exposed film.

f_1 Initial stage of Gelatin layer digestion in *Nepenthes khasiana.* Prolonged incubation in the reaction medium show more digestion of the gelatin layer by the surface of the gland.

Plate- 7.1 Histochemical localization of Protease activity in *Nepenthes khasiana* (Substrate film method)



Initial stage of Gelatin layer digestion in *Nepenthes khasiana*

Initial stage of Gelatin layer digestion in *Nepenthes khasiana*

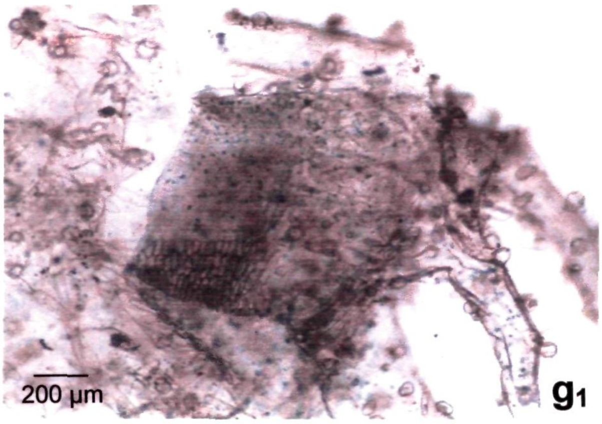
Plate- 7.1

g_1 Localization of Esterase activity in the bladder of *Utricularia bifida.* Cells on the bladder wall show the activity which is in the form of blue spots.

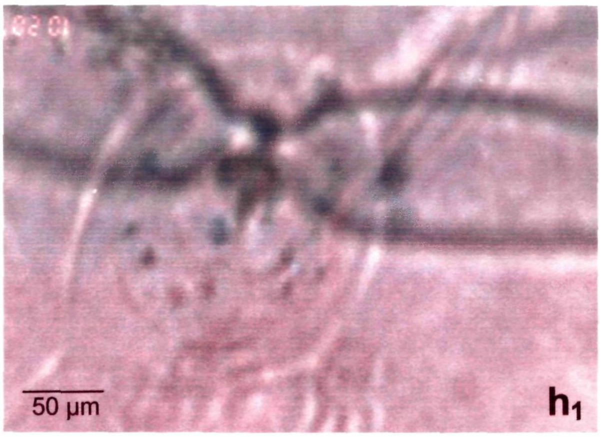
h_1 Localization of Esterase activity in the pedestal cell of *Utricularia bifida.* Pedestal cell only show the reaction, the blue spots.

i_1 Control test of Esterase activity in *Utricularia bifida.* Without the substrate, activity is not localized in the tissues of the bladder wall and the gland.

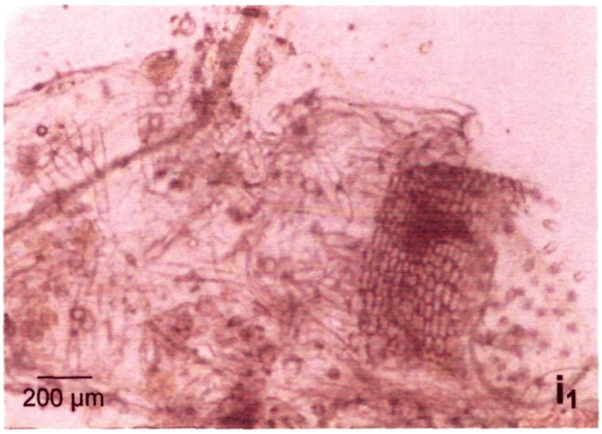
Plate- 7.1 Histochemical localization of Esterase activity in *Utricularia bifida*



Treated



Treated



Control

CHAPTER – 8

General discussion

8.1 *Nepenthes khasiana* Hk. f.

The digestive glands and their secretions in pitcher plant (*Nepenthes*) are involved in the digestion and absorption of nutrients from the prey (Darwin, 1875; Francis Darwin, 1878; Busgen, 1883; Heslop-Harrison, 1976; Dixon et al., 1980). However, studies on the morphology, development and histochemical localization of the enzymes in the glands of insectivorous plants are very meager. Therefore, this study has been carried out to understand the structure and development of glands in *N. khasiana* as their secretions are important from pharmaceutical point of view.

The number of glands and their distribution pattern in the genus *Nepenthes* varies from species to species. The average number of glands in *N. khasiana* ranged from 100 to 200 per sq. cm, which is similar to that of *N. gracillima* with ca. 100 glands per sq. cm (Danser, 1928). However, in case of *N. rajah* and *N. stenophylla,* the number of glands was reported to be *ca*. 300- 800 glands per sq. cm and *ca*. 6000 glands per sq. cm, respectively (Danser, 1928; Phillips, 1988; Steiner, 2002). In the present study, the occurrence of evenly distributed glands in the depressions of the pitcher with epidermal flaps was observed, which is supported by the findings of many authors in other species of *Nepenthes* (Pant and Bhatnagar, 1977; Adams and Smith, 1977 and Owen and Lennon, 1999). The formation of epidermal flap and groove was due to the overgrowth of the epidermis and differentiations of digestive glands which made it conspicuous. These flaps might have played a key role in preventing insects to escape from the pitcher (Lloyd, 1942; Adams and Smith, 1977).

Detailed studies on the formation of pore in the cuticle of the outer secretory layer and the groove or depression surrounding the gland has not been carried out so far in other species of *Nepenthes*. SEM studies on different developmental stages of glands indicated that, the pores were formed by the radial as well as tangential expansion of the secretory cell walls, coupled with cell divisions of the adjacent glands. The actual function of this porous nature of external surface of the gland has not been studied in other species of *Nepenthes*. The pores may allow certain molecules to be absorbed or it may increase the external absorptive area of each gland.

The three-layered structure of glands present in *N. khasiana* is a common feature present in other species such as *N. alata, Nepenthes rafflesiana, Drosera*, *Drosophyllum* (Owen and Lennon, 1999; Heslop-Harrison, 1975). Each gland is in direct contact with vessel elements which are interconnected with the venation of the leaf. This observation was reported in *N. alata* by Anderson (1994) and Owen and Lennon (1999). The columnar secretory head cells are single-layered. In *N. khasiana*, the columnar cells were composed of 100- 150 cells. It may vary from one species to other, e.g., in *N. alata*, there are 40 -60 cells (Owen and Lennon, 1999). Each secretory cell is characterized by possessing wall ingrowths towards the cytoplasm, which indicated that these wall labyrinths structure was concerned with transfer cell type. The occurrence of endodermal layer is a common phenomenon in all the *Nepenthes* species including *N. khasiana*, as well as in other insectivorous and non-insectivorous plants (Heslop-Harrison, 1976; Fahn, 1979; Owen and Lennon, 1999). However, the number of communicatory layers may vary in different species.

Ultrastructure of glands has been studied by Owen et al. (1999) during the nutrient transport in the pitchers of *N. alata*. Faraday and Thomson (1986) indicated that, transport may rely on mitochondrial respiration rather than photosynthesis. However, in *N. alata*, the number of ER and Golgi body were very less, and were not strikingly abundant, which implied that they might not have involved in the secretion and synthesis of enzymes. On the contrary, in *N. khasiana*, vesicles derived from the ER, Golgi bodies and multivesiculate bodies fuses with the plasmalemma, and discharge its content externally through apoplastic movement. The present observation is also supported by the findings of Owen et al. (1999), where lanthanum and carboxyfluorescein tracers were used. The presence of radially elongated microtubules (200 Å in diameter) in the secretory cells provided cell integrity to maintain the cell shape and size, as well as regulate the movement of vesicles towards the plasmalemma. There is no report on presence of microtubules in the secretory cells in other insectivorous plants.

The endodermal layer is characterized by casperian thickenings on their radial walls. According to Heslop-Harrison (1976), the thickening of the radial wall layer is of spongy wall type. The continuum of the endodermal layer with cuticle of epidermis was observed in *N. khasiana*. The same result was given by Owen and Lennon (1999) in *N. alata*. The plasmodesmatal connections present in the thin tangential walls of endodermal layer revealed that, this layer might have played key role in secretion and absorption of enzymes, and reabsorption of digested nutrients. The communicatory layer bridged the secretory and endodermal layers. The ultrastructure of cell organelles was more or less similar in all the cells in *N. khasiana*. According to Heslop-Harrison (1976), the communicatory layers sometimes act as reservoir cells with intermediate links.

The predominant presence of vacuoles in all the three layers of the gland indicated that, the absorbed food materials might have stored in these vacuoles, and were translocated from cell to cell through symplastic pathway.

The cuticle of the epidermis of inner surface of pitcher was continuous through out the external surface of the gland. The thickness of cuticle was more or less uniform in the young stage. In matured glands of *Drosophyllum* (Schnepf, 1965b) and *Drosera* ((Williams and Pickard, 1969), the cuticle layer breaks down forming the cuticular pores. Owen and Lennon (1999) reported that the occurrence of cuticle on the glandular surface of *N. alata* was in the form of discontinuous cuticular droplets. However, no such finding was observed in *N. khasiana*.

The development of glands in *N. khasiana* was more or less similar to that of *N. alata* as reported by Owen and Lennon (1999), where the glands were originated from the inner epidermis of the inner surface of the pitcher. The epidermal origin of the gland was also supported by the works of Oudemans (1864); Macfarlane (1908) and Lloyd (1942). The sub-epidermal origin of the digestive gland has also been proposed by Meyen (1837) and Kothals (1839). In *N. khasiana*, almost seven to ten epidermal cells were involved in the initiation of the gland, whereas four epidermal cells were found to initiate in the formation of digestive gland in *N. alata* (Owen and Lennon, 1999). The formation of bilayered stage during the gland development when the pitcher reaches a height of 2 cm is also reported in *N. alata* by Owen and Lennon (1999). The formation of bilayered gland in *N. khasiana* due to the radial elongation followed by transverse (periclinal) division of the initials was observed to be different in the case of *N. alata*, where Owen and Lennon (1999) reported the formation of bilayer was due to the periclinal and anticlinal divisions of the initial cells.

The differentiation of various cell layers in the digestive gland took place when the primordium reaches the four layered stage. The middle layer divides transversely, so that, it turns two-tiered arrangement. Further, enlargement of the gland was brought out by typical longitudinal divisions in the secretory head cells, both longitudinal and oblique divisions in the middle layers to form the communicatory layer. The innermost single layer was differentiated into the endodermal foot layer with casperian strips (Plate- 4.7 k - l). A similar type of organization was noticed in *N. alata* (Owen and Lennon, 1999). All these patterns of cell divisions are regular and synchronous. Both in *N. alata* and *N. khasiana*, the endodermal layer was in continuation with the epidermal cells of the inner surface of the pitcher and each gland was interconnected by the inner vasculature. Almost 300- 400 cells have constituted the complete discoid digestive gland structure in *N. khasiana,* whereas only around 60 cells were found in the four different layers in the digestive gland of *N. alata*. In *N. khasiana*, the growth of digestive glands was concomitant with increase in size of the pitcher which has not been reported in other species of pitcher plant.

8.2 *Drosera peltata* Sm.

The species possesses hibernating tubers which is a common feature to other tuberous species like *D. gigantea, D. macrantha, D. stolonifera*, *D. ramellosa, D. macrophylla* and *D. whittakeri* (Nash, 1973). The leaf trap with tentacles on its upper surface of *Drosera peltata* Sm. is common to all the species of Droseraceae family. The marginal tentacles on the leaf are longer and inclined, whereas those on the central part of the leaf are short and stand upward. In *D. peltata* the sessile glands are dispersed on the leaf, main stem and also on the stalk of the tentacles as in *D. capensis* (Lloyd, 1942; Fahn, 1979). The tentacles of *D. peltata* are radially symmetrical irrerespective of its location. But in *Drosera capensis* the marginal tentacles are bilaterally symmetrical while those occurring on the leaf surface are radially symmetrical (Fahn, 1979). The tentacles secrete both mucilage and enzymes and are involved in the function of absorption of digested products (Heslop- Harrison, 1975; Fahn, 1979; Matusikova et al. 2005). There are about 120 to 160 tentacles on the upper surface of the leaf in *D. peltata*. But the number of tentacles is nearly double (approximately 260) in the case of *D. rotundifolia* (Darwin, 1875).

The overall internal configuration of the tentacles in *D. peltata* observed similar with other species of the genus of *Drosera*, consisting of three layer of cells viz. the outer secretory single layer with columnar shaped cells; two to four middle layers or communicatory layers and the innermost single endodermal layer (Heslop- Harrison, 1975; Fahn, 1979; Ragetli et al., 1972). The secretory layer was double layered structure in *D. capensis* (Heslop- Harrison, 1976) while in *D. peltata* it was single layered structure. The secretory mantle is enclosed by cuticle with pores. The same feature has been reported in *D. capensis* (Heslop- Harrison, 1975; William and Pickard, 1969, 1974 and Chafe and Wardrop, 1973).

Juniper and Gilchrist (1976) proposed the possible pathway for the digested substances through these cuticular pores into the apoplast of the outer secretory cells in *Drosera*. Schnepf (1961a, b) also have the same view that the presence of wall protuberances on the cell walls of the secretory layer. Cytoplasm of the young secretory cells have numerous spherosomes, golgi bodies, proplastids, mitochondria etc which indicate that the cells are metabolically active and so, the mucilage secreted is being synthesized and condensed in the Golgi bodies (Schnepf, 1961a, b,1963a; Dexheimer, 1972, 1976). The outer tangential walls of the secretory cells have annular or spiral thickenings. Ultrastructure of secretory cells reveals that microtubules of 60-70Å in thickness are present running parallel to the outer tangential walls of young secretory cells.

In *D. peltata*, the single endodermal layer was found inverted cup shaped structure having cutinized radial walls with casperian strips which was also reported in *D. intermedia* (Fenner, 1904; Llyod, 1942). The communicatory layer connects the secretory layer and the central core of tracheas through the tangential walls of the endodermal layer. The endodermal layer is distinct externally by a clear demarcation at the junction of the head of the gland and the stalk region. The same feature has been reported in the glands of many insectivorous plants such as *Drosophyllum lusitanicum, Dionaea muscipula, Drosera capensis, Nepenthes rafflesiana* and *Pinguicula grandiflora* (Heslop-Harrison, 1976; Fahn, 1979). Poly or multivesiculate bodies with single membrane bound granules observed in all the gland layers might be the site for synthesis and storage of enzymes. The contents of these polyvesiculate bodies as well as dictyosomes derived vesicles fused with the plasmalemma and release its contents in the periplasmatic space through apoplastic movement.

The development of the tentacles in *Drosera intermedia* was studied by Fenner (1904) and Homes (1928). According to them, the development of tentacle is epidermal in origin, wherein, there is a swelling on the epidermis: the inner part of this swelling consists of a row of subepidermal cells; the uppermost cell of this row develops into the inner layer of the 'secreting mantle'. In the lower part of the swelling, the cells undergo more rapid divisions and enlargement. In the central region a strand of tracheids is connected with vascular bundle. Fahn (1979) studied the gland structures in *D. capensis* giving more emphasis on the cell layers of the glands but not on the developmental aspects of the glands.

In the present study the tentacles are of subepidermal or hypodermal in origin. There were two types of origin of tentacles i.e. formation of massive subepidermal layer or formation of a single row of cells. The dorsal and marginal tentacles are initiated simultaneously when the leaf primordium attains 1 mm in diameter. The differentiation of the four different layers i.e. secretory, communicatory, endodermal and vasculature occurs concomitantly in the head portion. In the fully matured stage of tentacles, the endodermal layer becomes inverted cup shape because of overgrowth in the secretory layer coupled with enlargement.

Very few studies have been carried out on the sessile gland of *Drosera* species. Fahn (1979) described the four different forms of sessile gland on the upper leaf surface of *Drosera capensis.* But the detail information of development of the sessile glands was not studied in *Drosera peltata.* In general, the sessile glands of *Drosera* consist of four to six outermost cells (Schmucker and Linnemann, 1959; Lloyd, 1942). But in *D. peltata*, in the surface view of mature sessile glands have eight cells like the secretory head portion of sessile gland is *Pinguicula grandifolia* (Heslop- Harrison, 1976). A matured sessile gland consist of 14 cells; the outermost eight cells, central four cells and two celled foot or basal layer (see Plate- 5.6 a). Heslop-Harrison and Heslop-Harrison (1981) used the terminology head, endodermal and the reservoir cells for the three layers of the sessile gland of *Pinguincula* species.

The ultrastructure of sessile gland cells in *D. peltata* show different structural variations during the different developmental stages of the gland as in *Pinguincula* species (Heslop-Harrison and Heslop-Harrison, 1981). The primary wall of the outer cells shows a microfibrilar structure. The outer cells are cutinized similar to that of *P. grandiflora, P. vulgaris,* and *P. ionantha,* reported by Heslop-Harrison and Heslop-Harrison (1981). There is continuity of the outer cuticle layer with the epidermis of the leaf surface. The cuticle is discontinuous with pores like that of *Drosophyllum* and *Drosera capensis*, which are not found in *Pinguicula* species (Heslop- Harrison, 1975, 1976). In *D. peltata* the radial and tangential walls of all the cells of the gland layer clearly show ramification of the transfer cell type. This is also a common feature of leaf traps in the family Droseraceae (*Dionaea, Drosera*), *Drophyllum lusitanicum* (Schnepf, 1963a, b & c, 1974), in *Genlisea* (Heslop- Harrison, 1976) and in *Pinguincula* (Heslop-Harrison and Heslop- Harrison, 1981) and among the members of Lentibulariaceae.

Periodic acid Schiff's reagent (PAS) and calcofluor white stain the primary cell wall heavily and fluoresces strongly, suggest that the cellulosic nature of the cell wall, which is also a distinct feature of sessile head cells of gland of *P. grandiflora* (Heslop-Harrison and Heslop-Harrison, 1981). The cells of the outer layer of young sessile gland of *D. peltata* possess numerous granular bodies with vesicles. Mitochondria are less with well defined cristae, more plastids without starch and large and less number of vacuoles as in the case of *P. bakerina* (Schnepf, 1961a, b, 1963a, b & c), and *P. grandiflora, P. vulgaris* and *P. ionantha* (Heslop-Harrison and Heslop-Harrison, 1981). Numerous small vacuoles are present in the mature glands. These features suggest the cells to be metabolically active.

In the young gland the cell wall ramification is abundant towards the tangential side, whereas in the case of mature gland, ramification is more on the radial wall. The microfibrilar components of the cell wall retained throughout the different developmental stages of the gland. The cell wall ramification indicates the absorptive function of the sessile gland. According to Heslop-Harrison and Heslop-Harrison (1981) capturing tentacles of *Drosera* also secrete the digestive enzymes, while in *P. grandiflora* and *Dionaea* capture is effected by the stalked glands and digestion principally by the sessile glands only when the leaves are stimulated.

There is a temporal relationship between the differentiation of tentacle and sessile glands. The development of sessile gland in *D. peltata* is more or less similar to that of *P. grandiflora, P. vulgaris,* and

P. ionantha in which the whole gland is developed from a single epidermal cell (Heslop-Harrison and Heslop- Harrison, 1981). The epidermal cell elongates and divides transversely into two daughter cells. In the three species of *Pinguicula*, the upper cell divides in paradermal plane to endodermal cell, followed by the anticlinal division of the upper cell to form eight cells on the outer layer (Heslop-Harrison and Heslop- Harrison, 1981). But, in case of *D. peltata,* the upper cell divides twice in radial plane at right angle to each other to form four cells in a single tier, followed by transverse division in oblique pattern to form four outer and four inner cells. The outer four cells again divide vertically to form eight cells. In *Pinguicula* a single endodermal cell represents the four central cells with a distinct casperian strips and a single basal or reservoir cell (Juniper and Martin, 1970 and Heslop-Harrison and Heslop- Harrison, 1981) whereas, in case of *D. peltata*, the basal layer is two celled due to vertical division of the single basal cell. The formation of casperian strips on the radial wall is absent in the sessile glands of *D. peltata*.

8.3 *Utricularia* species

The horse shoe shape bladder with the valve, collar and the unicellular, uniseriate antennae bordering the mouth to capture prey in U. *bifida* and *U. pubescens* is a common feature of the genus *Utricularia* (Drawin, 1875; Fineran and Lee, 1975*)*. The uniform bilayered wall of the bladder is consistent to *U. vulgaris* (Friday, 1991) and *U. stellaris* (Cheema et al., 1992). The observation of two and four arms in *U. bifida* and *U. pubescens,* respectively differs from that of *U.*

monanthos bearing both the types of arms in the same bladder (Fineran and Lee, 1975). In both the species, the terminal cells unite at their proximal end (basal portion) to form a common stalk, which latter on, separated into their respective arm portions. The main two wall layers in the pedestal and the base of the stalk portion are also a common feature of *U. monanthos* (Fineran and Lee, 1975).

The differentiation of a single terminal cell into an arm and a stalk without any wall partition is highly a specialized structure, in which the arm is meant for both absorption of nutrients and secretion of enzymes and the stalk both for support and conduction. This feature of continuity does not occur to other plant trichomes, where the conduction from the terminal cell is usually carried out by a separate stalk cell (Fahn, 1979). The wall protuberances in the pedestal and the stalk portion also show functional differentiation of the cells particularly increasing the surface area for absorption of nutrients. These structural features in the cell wall of *U. bifida* and *U. pubescens* were similar to the findings of Fineran and Lee (1974 a) in *U. monanthos.* The more extension of the wall ingrowths on the base of the arm also resembles to the epidermal transfer cells of certain aquatic plants as reported by Pate and Gunning (1972).

The vacuolated cell wall of the pedestal, proximal cell wall portions of the arm and the stalk, and the cell wall partition between the pedestal and the stalk portion is the peculiar characteristic in *U. bifida* and *U. pubescens* which have not been reported previously. The finger like projections between the pedestal and the stalk portion might have enhanced in the absorption and transportation mechanisms of nutrients. The confinement of the cell organelles especially mitochondria and nucleus at the base of the arm also suggested an advantage in adaptation of the whole plant system by controlling the metabolic activities of the cell e.g. in providing necessary energy for active uptake of materials or in transporting metabolites to the pedestal cells (Fineran and Lee, 1975).

The absorption and transportation of nutrients are carried out through the apoplast and symplast pathways. The direct absorption of the materials absorbed from the lumen of the bladder is probably through the thin fibrillar cuticle of the terminal arm portion, base of the stalk and the pedestal cell which are apoplastic in nature. The products are transported to the pedestal cell after digestion in the arm portion probably by the finger like projections present in the cell wall partition between the stalk and the pedestal cell. During this process, part of the digestion takes place in the vacuolated cell wall between the two cells. The products are again transferred to the adjoining basal cells through the plasmodesmetal connections on the tangential cell wall through symplastic movement. Although, Fineran and Lee (1975) have the opinion that, the opaque impregnated wall of the pedestal and base of the stalk portion would not probably allow substances to pass directly to the wall labyrinth into the cells. Bonnett (1968) and Robards et al. (1973) have also compared the impregnated zone in the cell wall with the casparian strip of endodermal cells.

Through the present study it can be assumed that, the enzymes present in the vacuoles of the cell wall might have helped in the digestion of the absorbed prey through the impregnated zone of the cell wall which does not allow substances to pass directly into the cells from the lumen of the bladder. Thus, the vacuolar nature of the cell wall is a peculiar feature present in this plant species which might be an adaptational feature of carnivory. The digested products are then absorbed by the transfer cell wall.

8.4 Histochemical localization

Localization of insoluble polysaccharides in the cell walls and starch granules in the cytoplasm of the inner tissues of the pitcher in *N. khasiana* in the modified leaf of *D. peltata* revealed the functional similarity between the two species. The starch granules are stored in the chloroplast. There is a correlation between the behavior of dictyosome and functions of the gland. This can be evident or explained by the ultrastructure of the gland cells. The presence of numerous active dictyosomes and their vesicles in the cytosol of the secretory cells in both the species shows that these gland cells might have involved in the secretion process. This finding is supported by the report of Dexheimer (1972, 1976), in which the presence of numerous dictyosomes present in the cytoplasm of the secretory cells were reported during the production of the mucilage droplet on the gland head of *Drosera*. The similar phenomenon was also reported in the stalked glands in *Pinguicula* and *Drosophyllum* (Schnepf, 1961a, b, 1963a, b & c).

High protein content observed in the gland cells, stalk cells and inner leaf tissues of *D. peltata* is similar to that of *D. capensis* as reported by Heslop-Harrison (1976). The intense stainable nature of nuclear DNA which localizes in the outer secretory layer of the glands in *N. khasiana* and *D. peltata* revealed the importance of nucleic acids in protein synthesis during the developmental stage of the glands.

Studies on localization of lipase activity in insectivorous plants are very meager. The present study revealed that lipase activity is mostly confined to the cytoplasm of the innermost cells of the communicatory, endodermal layer and the inner hypodermal cells of the discoid glands of *N. khasiana* and tentacles of *D. peltata*. In *D. peltata*, lipase enzyme activity is also observed in the treachery cells.

The presence of esterase activity in almost all the gland layers and also in some of the hypodermal tissues in both *N. khasiana* and *D. peltata* is supported by the findings of Heslop-Harrison (1971, 1976) who worked in *N. rafflesiana*, *D. capensis*, *Drosophyllum, Dionaea* and the reports of Heslop-Harrison and Heslop-Harrison (1981) in *Pinguicula*. They also reported high esterase activity in the spongy wall of the outer layer of sessile gland in *D. capensis*. This observation has also supported higher enzyme activity in the growing stage of plant parts, where the cells are metabolically active. It is also supported by the observations of the cytoplasm of matured root cap cells, and the cell walls of *Vicia faba* (McLean and Gahan, 1970). Similar enzyme activity was also reported in the stigmatic cells of angiosperms during the peak receptive period (Bhattracharya et al., 2004), and apical portion of hyphae which are associated with many orchids (Williamson, 1973). The esterase enzyme present in the gland cells might have involved in breaking down the pectic substances of the middle lamella, which helps in loosening the cells by breaking down the intercellular component especially pectic substances.

In *N. khasiana*, was localized in the communicatory cells and in the endodermal layers of glands, whereas in *D. peltata*, except the outer communicatory layer and the secretory layer, the inner communicatory layer, endodermal layer and some tracheid cells of tentacle showed acid phosphatase activity. Similar finding was also reported by Dexheimer (1978a, b) and Heslop-Harrison (1975) while working with *Drosera capensis.*

While analyzing the enzyme activities, it has been observed that, the deposition of the reaction product is significantly prominent in the cytoplasm of the gland cells. Detailed study revealed that, the reaction product was accumulated in the lumen of the nuclear membrane, ER, dictyosome and their associated vesicles. The result of this study also shows that, the secreted materials especially the enzymes were transported from the synthesizing sites (mainly the communicatory and the endodermal layer) to the outer secretory cell layer through the apoplastic pathway. This observation is supported by the findings of Heslop-Harrison (1976) and Plachno et al. (2006), who observed high activity of acid phosphatase in the digestive gland cell walls of *N. rafflesiana.* Parkes (1980) also reported similar finding on acid phosphatase activity in the cytoplasm of gland cells of *Nepenthes* *khasiana*, *N. rufescens* and *N. maxima*, and glucose 6- phosphatase activity in the cell walls of the gland cells. Highest deposition of acid phosphatase in the spongy radial walls of the sessile head cells not in the cell cytoplasm of *D. grandiflora* was also reported (Heslop-Harrison, 1976).

Localization of the alkaline phosphatase activity has not been reported so far in any insectivorous plants. But, in the present study, the activity of alkaline phosphatase activity in *N. khasiana* using Tetrazolium method (Lojda et al., 1979) showed positive reaction in almost all the gland tissues.

The distribution pattern of acid phosphatase and esterase enzymes in the gland cells are similar, where the reaction product is distributed throughout the cytoplasm of the gland layers. This may be explained by the ultrastructure of the young gland cells, which bear numerous spherosomes with vesicles in which the enzyme are stored. During the young stage, the cells are metabolically more active with the production of enzymes in the GERL (Golgi endoplasmic reticulum lysosome) system. In the differentiating cells, the enzymes appeared to be passed from the rough ER to the smooth ER, which may lie in close proximity to the cell wall. The smooth ER then, budded off vesicles containing the esterase enzyme which passes to the plasmalemma, where the enzyme is translocated to the cell wall and secreted extra cellularly through exocytosis. In other words, it may be considered that the enzyme activity in the gland cells of *N. khasiana* and *D. peltata* are both particulate as well as diffuse in nature. It can also be concluded that there is a correlation between the production of enzymes and the growth of the pitchers, which is a common feature found in the shoot and root growth of other angiosperms. Similar incidence of production of more acid phosphatase activity are reported in the phloem cells (DeJong, 1965; McLean and Gahan, 1970; Braun and Sauter, 1964a, b; Lester and Evert, 1965; Benes and Opatrna, 1964), in the root cap region, protoderm, epidermis and cortical layer of root apices (Sheikh and Roberts (1974), and in the lateral root differentiation of many other plant species (Ashford and McCully, 1970).

High peroxidase activity was observed in the cytoplasm and cell walls of communicatory and endodermal cell layers of gland in *N. khasiana* and *D. peltata*. This enzyme activity has not been reported in other species of insectivorous plants except in *Drosophyllum* (Heslop-Harrison, 1976).

Localization of protease activity in *N. khasiana* through the substrate film method (Adams and Tuqan, 1961; Fratello, 1968) revealed high protease activity in the glandular surface of the glands. It has indicated that, the protease activity was highly concentrated on the outer secretory layer of the gland. The same result was observed by Heslop-Harrison (1976), who found the digestion of the gelatin film by the sessile glands in *D. lusitanicum*. Knox and Heslop-Harrison (1970) also reported release of protease enzymes from the exine of mature pollen grains. Heslop-Harrison (1976) also reported the secretion of protease enzymes in *Dionaea*, an insectivorous plant.

In *Utricularia* and *Genlisea*, little is known about the origin of digestive enzymes in the traps. There are few *in situ* studies on the secretory glands of *Utricularia* spp. (Vintejoux, 1974; Heslop- Harrison, 1975, 1976; Parkes, 1980; Sirova et al., 2003). In *Utricularia*, the activity of phosphatase was detected in internal glands of 27 species from both primitive and advanced sections (Plachno et al., 2006). The phosphatase activity was localized in the glands using ELF (enzyme labeled fluorescence), and also found that the secretion of enzymes is independent of stimulation by preys. But, in the present study, most of the enzymes except esterase could not be localized due to interference of several micro-organisms.

This study has revealed the ultrastructure and histochemical localization of different enzymes in the glands of *N. khasiana*, *D. peltata*, *U. bifida* and *U. pubescens* which are ecologically as well economically important. *N. khasiana*, an endangered plant species which is endemic to Meghalaya is an important ornamental and medicinal plant. Hence, its large scale exploitation from the natural habitat may lead towards the verge of extinction. Understanding the ornamental as well as medicinal values, conservational strategies of this particular plant species may be developed. Similarly, this study has also revealed the secretion of different digestive enzymes in insectivorous plants which are biologically active in nature, might be having valuable properties in curing many diseases. These enzymes have been confirmed to be secreted by the secretory glands in traps which are the modified organs of the insectivorous plants. The use of accumulated fluid in the pitcher of *Nepenthes* as well as their different plant parts in traditional aliments provides the opportunity in conserving the insectivorous plants at social and cultural level. These ethno-medicinal importance and application has also given the clue for the presence of some important metabolites in these plant groups. Although, there is no such evidence or proof in support to the perfect cure of particular diseases by the application of such insectivorous plants which were traditionally used.

In continuation to this work, further studies may be carried out on qualitative as well quantitative assessment of enzymes and metabolites which are present in insectivorous plants. Studies of these plants at molecular level may result in identifying important genes which are responsible for the secretion of those enzymes and metabolites of pharmaceutical importance. These genes may further be introduced to some other potential organisms (plants, animals and microbes) for the large scale production without exploiting the insectivorous plant itself.

Identification and quantification of bio-active compounds which may be present in those plant species may be helpful in drug designing and curing different critical diseases of human being and animal origin.

CHAPTER- 9

Summary

- The insectivorous plants such as *Drosera peltata* var *lunata*, *Nepenthes khasiana* Hk. f grow on rocky and sandy soils that are moist and acidic, whereas *Utricularia bifida* Sm. and *Utricularia pubescens* Sm. grow in fresh water waterlogged areas.
- *D. peltata* has been reported from Jowai, Jarain, Pynursla, Shillong, Raliang of Meghalaya (Joseph and Joseph, 1986). The tubers of *D. peltata* Sm. are found in association with mychorrhiza (Venugopal et al., 2007).
- *N. khasiana*, the unigeneric species of the family Nepentheceae is an endemic and endangered insectivorous plant growing in Khasi, Jaintia and Garo hills of Meghalaya state of North Eastern India (Hooker, 1886; Jain and Sastri, 1980; Joseph and Mani, 1982). Few population of this species was rediscovered from certain other parts of Meghalaya such as Jarain, Sutnga, Magheshkola and Lawbah (Haridasan and Rao, 1985; Rogers and Gupta, 1989; Choudhury, 2000; Raseshowri and Venugopal, 2006).
- *Utricularia bifida* Sm. and *Utricularia pubescens* Sm. which were reported from different parts of Meghalaya are also commonly distributed in other parts of India, Africa, Malaysia and Sri Lanka.
- Matured pitchers of *N. khasiana* bear two distinct portions; the upper half with waxy inner surface and the lower bulbous portion

which contains numerous glands on the inner surface of the pitcher which can be discernible externally by a slightly elevated circular line. These glands secrete several types of enzymes. The digestive glands appeared as glomerulus, sessile discoid or spherical structures, attached by disc-shaped depressions and partially covered by epidermal flap or outgrowth. The average number of glands per sq. cm ranged from 100 – 120 and the average size ranges from 140-150 µm in the matured digestive gland.

- The digestive glands in *N. khasiana* are multicellular bodies consisting of three different cell layers viz. the outermost single columnar layer, secretory head portion which secretes the digestive enzymes; two to four communicatory or the middle layers consisting of rectangular shaped cells and a single layered endodermal or foot layer with casparian like wall thickenings in their radial walls.
- In *D. peltata*, the leaves are reniform with broader base. Approximately 40 to 50 tentacles were located on the margins and around 60 to 80 tentacles with short stalk were present on the dorsal surface. The globular sessile glands were approximately 120 to 160 per leaf. The marginal tentacles were longer than the dorsal ones. Each tentacle consisted of a thin, straight, hair-like stalk, carrying a globular head on its summit, demarcated by a distinct endodermal collar. The sessile glands were also dispersed evenly on the stalk of the tentacles and the main stem as well as in the axis of leaves of the plant.

- The tentacles are multicellular bodies consisting of three different cell layers which were similar to that of *N. khasiana*; (1) the outermost columnar single layered secretory head portion which secretes the digestive enzymes; (2) two to four communicatory or the middle layers consisting of rectangular shaped cells and (3) single layered endodermal or foot layer.
- The matured sessile glands was having three-tiered arrangement (1) the outer eight celled layer was meant for absorption of digested food materials enclosing (2) the middle layer with four cells and (3) the basal layer with two cells.
- In *Utricularia* species, the horse- shoe shaped bladder ranged from 0.3 mm to 0.1mm in diameter. In *U. bifida*, the antennae were bifid or forked, unbranched and slender, whereas in *U. pubescens* the antennae were long, multicellular forming a fringe. The bladder wall was made up of two layered cell structure. The glands situated in the inner wall of the bladder had two terminal cells with its respective arms originated from a single subconical broad pedestal cell in *U. bifida* and four arms in *U. pubescens* with their respective lower stalk and pedestal cells.
- In *N. khasiana* and *D. peltata* the glands were connected with vasculature of the pitcher and leaf, respectively. The endodermal layers with casparian thickenings on their radial walls of the glands were invariably present in these two plants, but absent in the two *Utricularia* species. The endodermal thickenings (casparian wall like thickenings) controlled the flow of secretion and absorption

mechanisms. But in *U. bifida* and *U. pubescens*, the bladders did not have vascular connection. Only through the bladder wall the absorption and translocation of digested food materials were taken place.

- In *N. khasiana,* the porous nature of the secretory head cells of glands has been reported for the first time, which may enhance the absorptive surface. The direct connection between the protoplast and the pores could not be observed.
- In *D. peltata,* the labyrinth cell wall structure and plasmalemma indicated the absorptive nature of the sessile glands. In the mature stage, the ingrowth of the walls enlarged and ultimately pushes the plasmalemma towards the cell cytoplasm i.e. more wall ramification which showed more absorptive and active in translocation of the digested substances.
- The development of the sessile gland of *D. peltata* and *N. khasiana* were epidermal in origin, whereas the tentacles of *D. peltata* were subepidermal.
- The cell wall of glands of *Utricularia* spp. had three layers viz. opaque, impregnated and unimpregnated vacuolar wall from which the wall ingrowths arises.
- The wall partition between the pedestal and the basal portion of the stalk bears several finger-like projections which resulted in the development of a large surface area of plasmalemma relative to the volume of the protoplast.

- The thick partition wall with vacuoles between the stalk cell and the pedestal cell was the site of translocation.
- In *N. khasiana* and *D. peltata,* spherosomes (granules) with single membrane bound vesicles were present invariably in the gland cells, which are presumed as the carriers of digestive enzymes.
- There was a correlation between the abundance of dictyosome and the gland functions. The presence of numerous active dictyosomes and its vesicles in the cytosol of the secretory cells in *N. khasiana* and *D. peltata* showed that, these cells were involved in the secretion process.
- Enzymes such as lipase, esterase, acid phosphatase, alkaline phosphatase, peroxidase and protease have been localized in the digestive glands of *N. khasiana* and *D. peltata.*
- The synthesis of enzymes could include *de novo* release of compartmentalized enzyme or an activation of pre-existing forms of enzyme i.e. *in vivo* synthesis.
- The secreted enzymes were transported to the exterior of the gland through apoplastic pathway.

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2. N. Venugopal, Ksh. Raseshowri Devi and C.S. Rao (2007) An interesting observation on the mycorrhizal symbiosis in the insectivorous plants, *Drosera peltata* Sm., in Meghalaya, North- East India. *Carnivorous Plant Newsletter*, The International Carnivorous Plant Society, 36(1): 9- 13.
3. N. Venugopal, H. Lalruatsanga and Ksh. Raseshowri Devi (2006) Diversity of some members of *Podostemaceae* in Meghalaya. North East India in *"Ecology", Diversity and Conservation of Plants and Ecosystems in India"* (Eds. Pandey, H.N. & Barik, S.K.) Regency Publications- New Delhi, pp. 267-273.

Declaration:

I hereby declare that all the statements made in this C.V. are complete, true and correct to the best of my knowledge and belief.

16/7/07

Place: Shillong, Meghalaya

(Ksh. Raseshowri Devi)

AN INTERESTING OBSERVATION ON THE MYCORRHIZAL SYMBIOSIS IN THE INSECTIVOROUS PLANT, *DROSERA PELTATA* SM., IN MEGHALAYA, NORTH-EAST INDIA

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Keywords: Ecology: *Drosera peltata, Suillus luteus* — physiology: *Drosera peltata*.

Abstract

The work deals with new observations of a mycorrhizal symbiotic association in the corms and subterranean lateral foliar organs of *Drosera peltata* Sm. North-East, India. The fungal species is *Suillus luteus*, an indigenous common mycobiont in this region. This mycobiont enhances the efficiency of mineral uptake, particularly phosphorous. Calcofluor fluorochrome does not fluoresce at the subepidermal region of the corm, due to a mycorrhizal hartig net. This is an additional report of mycorrhizal symbiosis in the carnivorous plant *Drosera peltata* after Fuchs & Haselwandter (2004) in *Drosera intermedia*.

Introduction

In angiosperms about 645 species have evolved a carnivorous habit to capture prey (Rice 2006, pers. comm.), secreting digestive enzymes, reabsorbing the digested products and benefiting from the supplementary nutrition to the usual nutrition of plants (Fahn 1979; Heslop-Harrison 1976b). The carnivorous habit is most important in supplementing nitrogen supply (Heslop-Harrison 1976a). However, the experiments conducted by Pringsheim & Pringsheim (1962, 1967) and Harder (1963) on *Utricularia*, and Harder & Zemlin (1967, 1968) on *Pinguicula* showed that carnivory may contribute to the supply of other mineral elements.

The association of fungi with roots or underground plant organs is the chief organ of nutrient uptake by many land plants (Smith & Read 1994). Harley & Smith (1983) reported that 118 angiosperm genera in 35 families and 10 gymnosperm genera have mycorrhizal associations. Recent research work of Fuchs & Haselwandter (2004) showed the occurrence of vesicular arbuscular mycorrhiza in *Drosera intermedia*, Hayne an endangered carnivorous plant in Salzburg, Austria. There is some evidence of mycorrhizal association in the roots of *Nepenthes* (Moran, in Clarke, 2001). During the course of study on the developmental aspects of secretory glands in the carnivorous plants of Meghalaya, the authors came across an interesting observation of the mycorrhizal symbiosis in *Drosera peltata*. The underground organs of *Drosera peltata* consist of a corm, vertical shoot, and numerous lateral foliar organs produced exogenously, that while appear rootlike, lack a root cap and true root hairs. Goebel (1923) described them as "dubious roots."

Drosera peltata is a seasonal perennial plant that commences above-ground growth in the month of July and completes its life cycle at the end of October. Globally, *Drosera peltata* occurs throughout much of southeast Asia, Australia, and New Zealand. *Drosera peltata* grows in certain areas of Meghalaya such as Jarain, Sohrarim and Mawsynram where the soil has a low nutrient content. During the dry, hot winter these plants survive as underground corms. The corms and poorly developed lateral foliar organs are sheathed by a mycorrhizal mantle. *Drosera peltata* perennates by these corms, which are filled with abundant starch grains.

Materials and Methods

The corms and lateral foliar organs of *Drosera peltata* were fixed in FAA and sections were taken 8-10 mm thickness after dehydration and paraffinization. The sections were stained with

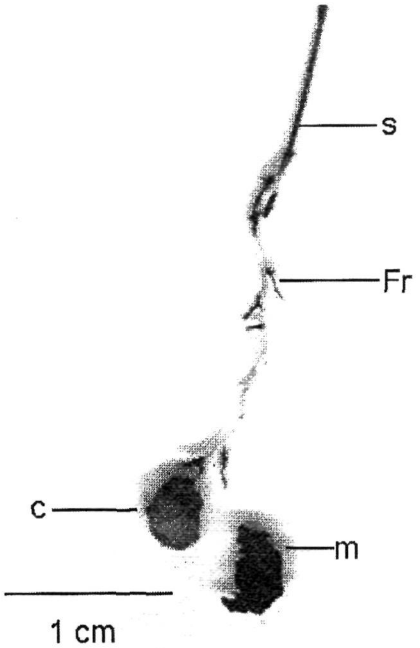

Figure 1: An enlarged portion of the *Drosera peltata* root system showing the corm (c) with detached mycorrhizal mantle (m), dark lateral foliar "roots" (Fr) covered with mycorrhiza and the main stem (s). Scale bar = 1 cm.

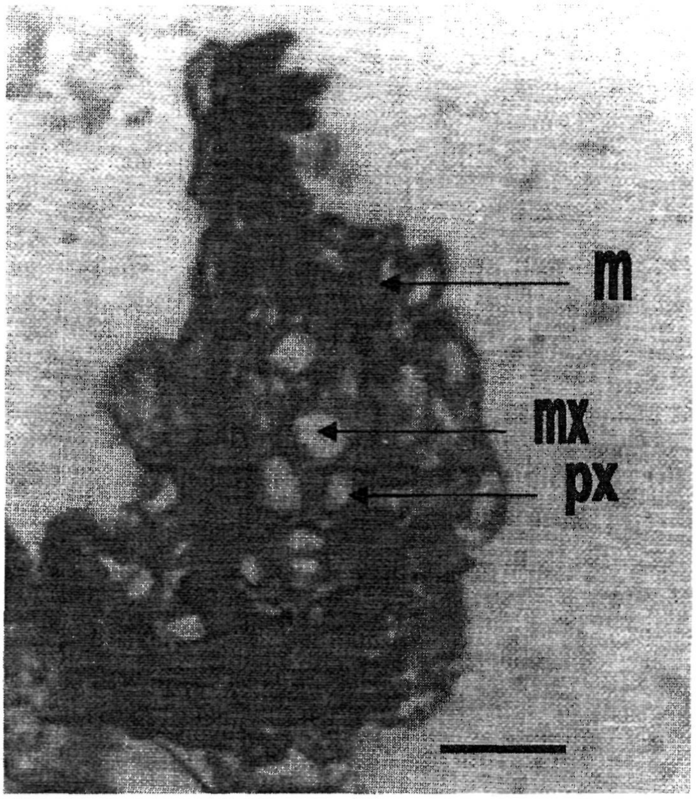

Figure 2: Transverse section of a lateral foliar "root" showing mantle (m), the disorganised vasculature of the protoxylem (px) and metaxylem (mx). Scale bar = 60μm.

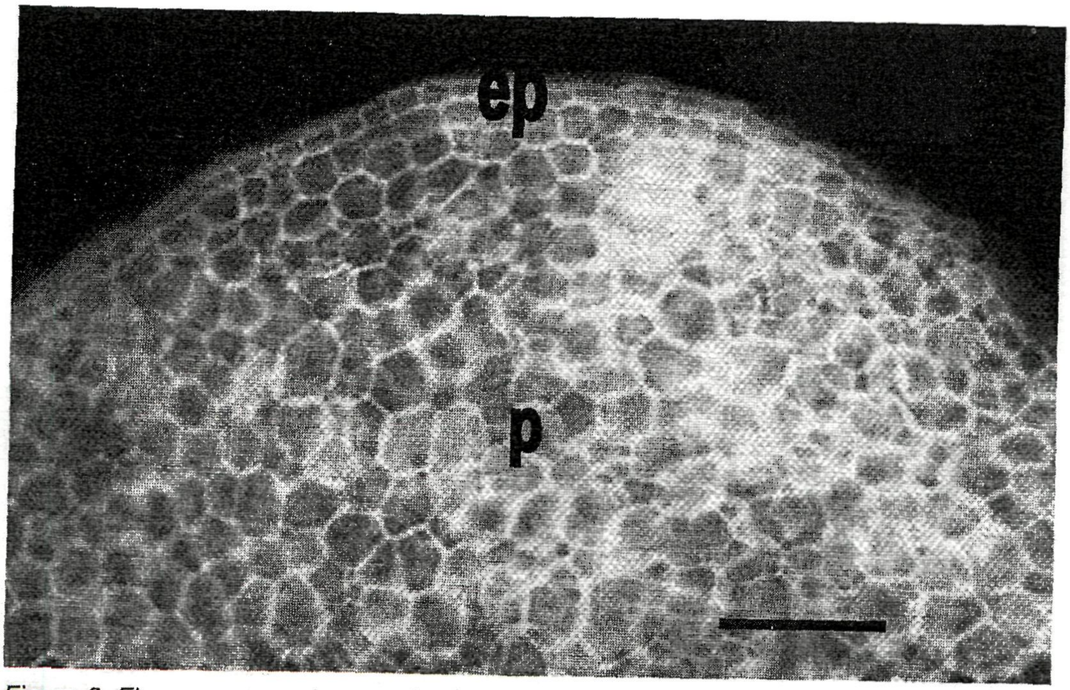

Figure 3: Fluorescence micrograph of transverse section of *Drosera peltata* corm illustrating the difference between the non-mycorrhizal and mycorrhizal zones. The hartig net is absent below the epidermis. Note the fluorescing epidermal (ep) and peridermal (p) layers. Scale bar = 240 μm.

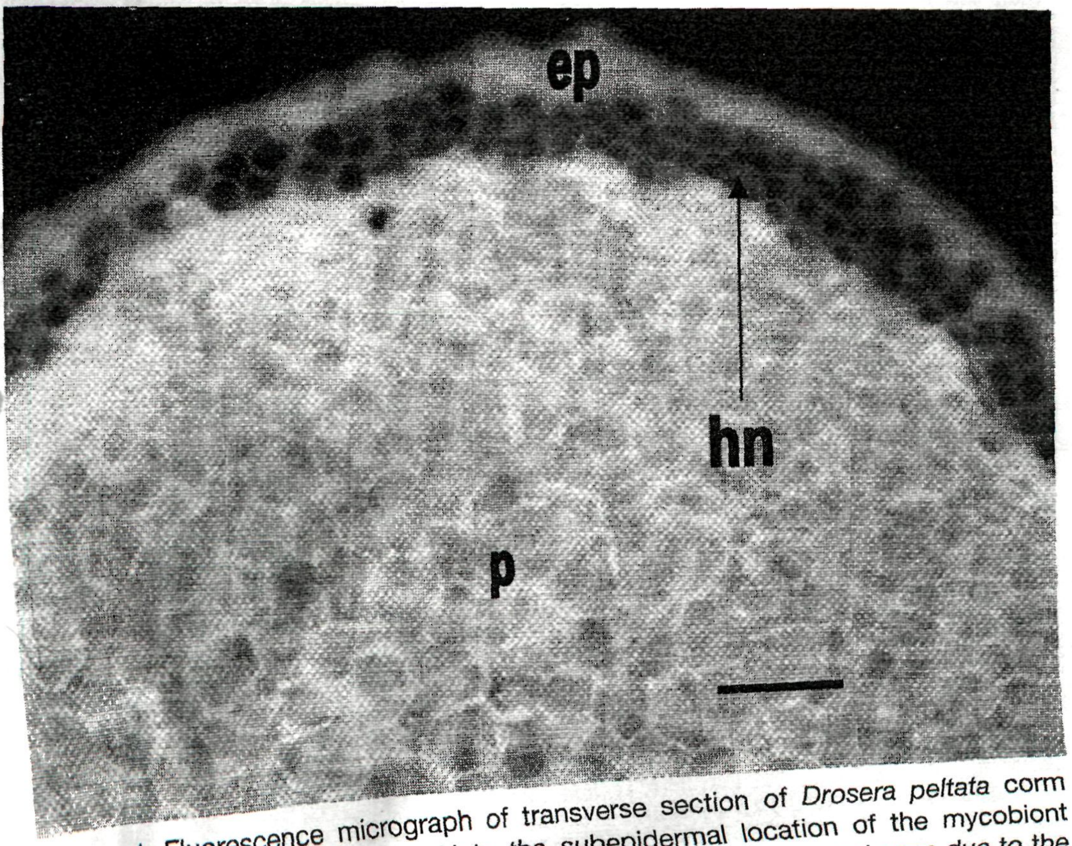

Figure 4: Fluorescence micrograph of transverse section of *Drosera peltata* corm showing the mycorrhizal zone. Note the subepidermal location of the mycobiont *Suillius luteus* and the the absence of fluoresence in the mycorrhizal zone due to the presence of hartig net (hn), and the fluorescing epidermal (ep) and peridermal (p) layers. Scale bar = 240 μm.

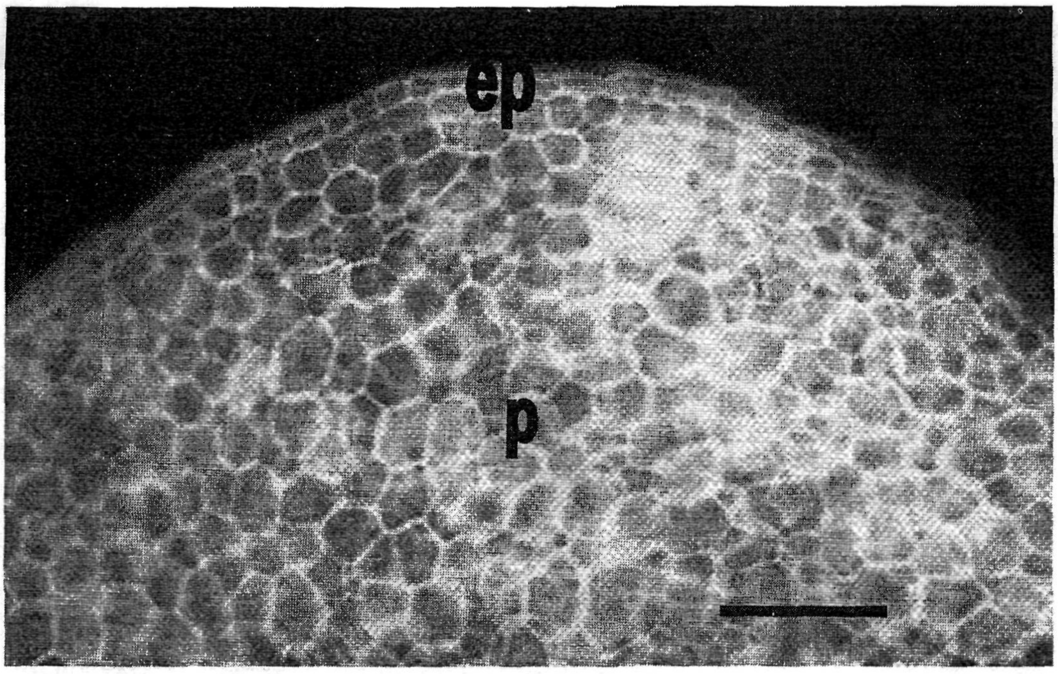

Figure 3: Fluorescence micrograph of transverse section of *Drosera peltata* corm illustrating the difference between the non-mycorrhizal and mycorrhizal zones. The hartig net is absent below the epidermis. Note the fluorescing epidermal (ep) and peridermal (p) layers. Scale bar = 240 μm.

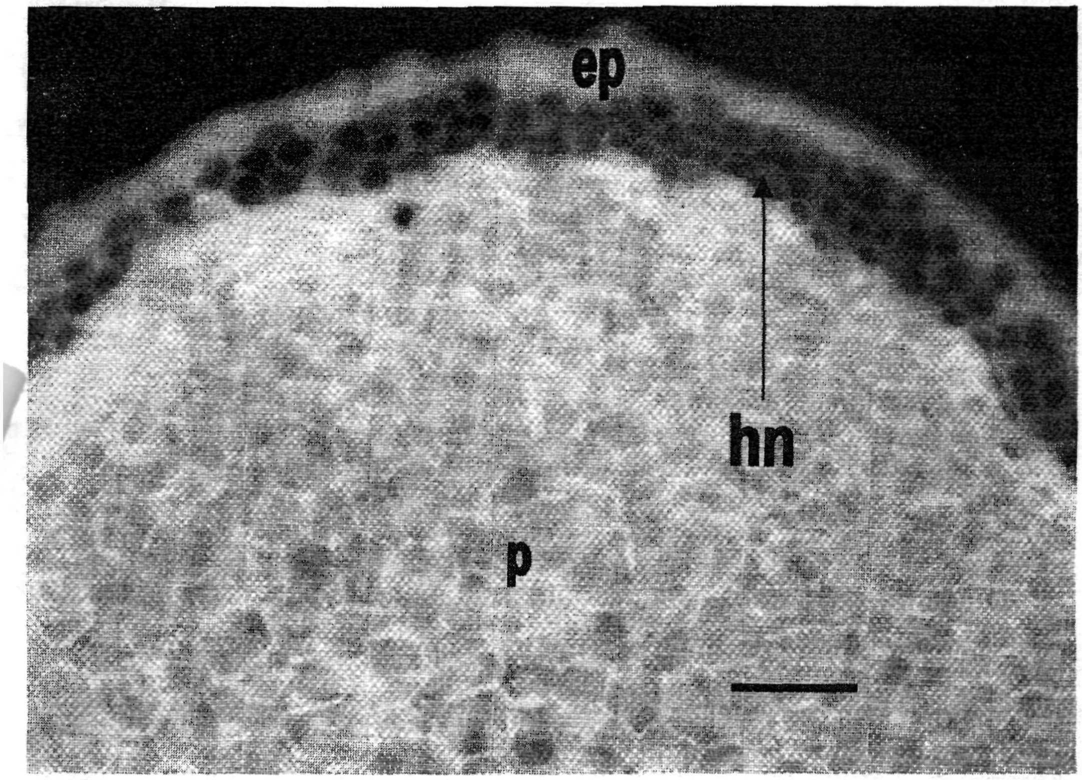

Figure 4: Fluorescence micrograph of transverse section of *Drosera peltata* corm showing the mycorrhizal zone. Note the subepidermal location of the mycobiont *Suillius luteus* and the the absence of fluoresence in the mycorrhizal zone due to the presence of hartig net (hn), and the fluorescing epidermal (ep) and peridermal (p) layers. Scale bar = 240 μm.

Aniline blue and Cotton blue (Johansen 1940; O'Brien & McCully 1981). Mycorrhizal spores were extracted from the 50 g of soil surrounding the corm. This extraction was performed using the sieving method (Daniels & Skipper 1982; Brundrett *et al.* 1996) and centrifugation using a 60% sucrose gradient. Spores were extracted from the supernatant by pipette and mounted in polyvinyl alcohol-lacto-glycerol (PVLG) without staining. The spores were observed under Nikon E 600 microscope. The spores are smooth, hyaline, broadly ellipsoid to sub-globose, ochraceous or buff coloured, and measured 3-4x4-7 μm. Identification of the fungus as *Suillus luteus* was based on spore wall structure and followed published descriptions (Schenck & Perez 1990). Moreover, *Drosera peltata* grows in the understory of *Pinus kesiya* Royle ex Gorden, which is also associated with *Suillus luteus*. Fluorescence microphotographs were taken by using Leitz Biomed Fluorescence microscope. Phosphatase activity was measured using the method described in Dodd *et al.* (1987). The mantle portion of mycorrhiza was removed and washed with double distilled water. The total phosphorus content in the root and corm was estimated after an acid wet oxidation in $HNO_3 + H_2 SO_4 + HClO_4$; analyses were performed for phosphorus as suggested by Allen (1974). Translocation percent of phosphorus to the shoot was calculated as described by Theodorou & Bowen (1993): %P (translocated)=100% × shoot P (mg)/total P (mg).

Result and discussion

In *Drosera peltata the* "root" system (i.e. the lateral foliar organs described in the Introduction) is frail, weak and thin. The lateral "roots" are 5-10mm in length and 1-2 mm in diameter completely covered by dark colored mycorrhizal sheath. In transverse section the "roots" have an outer most epidermis in which the cells are radially stretched. The epidermal layer does not produce any root hairs. The cortex consists of 1-2 layers of isodiametric cells with starch grains. The xylem of the "roots" is either di- or tri-arch. The corm is devoid of any lateral true roots. In addition to the modified "lateral roots", at the junction of the corm and the main stem arise small horizontal shoots that during the growing season can form the new shoot, or during the end of the season or winter can produce the resting corm (Adlassnig *et al.* 2005; Slack 2000). In transverse section, the Hartig net is 3-5 cells thick in the peripheral portion of the corms. This region does not fluoresce with Calcofluor.

The primary mycobiont was identified as being *Suillus luteus*. Phosphatase activity (375.0±29.50 μg P- nitrophenol g^{-1} dry wt.$^{-1}$h) in the underground organs was greater in plants associated with the mycobiont than in plants without the mycorrhizal association (190.0±12.40 μg P-nitrophenol g^{-1} dry wt.$^{-1}$h). Similarly, phosphorus content of the shoot and corm was greater in mycobiont-associated plants (86.0±6.75 μm/gm) than in plants without mycorrhizal associations (45.0±1.90 μm/gm). Translocation efficiency of phosphorus (52%) from soil to the shoot by plants was higher in *Suillus luteus* infected plants. Plants without *Suillus luteus* had lower translocation efficiency (39%). The results depict that mycorrhizal infection and production was better in plants associated with *Suillus luteus* as compared to plants without the symbiont.

Drosera peltata frequently grows with grasses and other vegetation, and as such the soil is likely have enhanced amounts of organic material. Mycorrhizal fungi can degrade these composts and make them available to the associated plants (Schisler & Linderman 1989).

The improved phosphorus uptake in mycorrhizal associated plants, as we observed in *Drosera* peltata is supported by other authors (Stribley *et al.* 1980; Lodge *et al.* 1994; Robinson 1994). Higher nutrient uptake by mycorrhizal plants is due to improved hyphal growth and improved exploitation of the soil volume by *S. luteus*. Higher phosphate uptake by plants is correlated to higher rate of phosphatase activity in mycorrhizal than non- mycorrhizal ones (Tarafdar & Marschner 1994).

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