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Ultrastructural Analysis of the Digestive Gland Secretory and Absorptive Processes in *Nepenthes glandulifera*

A Thesis submitted in partial satisfaction of the
Requirements for the degree of
Bachelor of Arts
With honors in Biochemistry, Cellular & Molecular Biology

By

Shannon Manuel

May 1, 2019

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Abstract

Nepenthes glandulifera is a carnivorous pitcher plant native to tropical environments, of which the majority live on the islands in the Malay Archipelago where sunlight and water are abundant. The pitcher trap style contains a modified pitfall trap that attracts insects and other small invertebrates through various attractive mechanisms. These plants also contain digestive glands that secrete digestive fluid to break down prey into nutrients for the plant. The digestive fluid, with remarkably unique properties, has been analyzed in relation to multiple areas of study including the development of an enzyme-supplementation strategy for the treatment of celiac disease and the synthesis of gold nanoparticles for tumor imaging and targeting. Although the composition of the digestive fluid has been studied, there is limited data on the ultrastructural components of the digestive glands. In this study, electron and light microscopy were used to analyze the digestive glands in relation to the secretory and absorptive processes in *Nepenthes glandulifera*. Electron and light microscopy data suggest that *Nepenthes* use limited symplastic and apoplastic transport, requiring transmembrane transporters for nutrient uptake. A novel structure in the nucleus of multiple cells suggests an evolved mechanism for the continuous acidification of pitcher fluid and intracellular communication for rapid and efficient nutrient uptake.

Introduction

Carnivorous plants have been of interest to scientists for centuries, as these remarkable plants have adapted to survive in nutrient-limited conditions, capturing insects and other small invertebrates to gain inorganic nutrients, especially nitrogen and phosphorus (Adlassnig et al., 2011). These plants exhibit stunning characteristics including fluid within their pitcher shaped leaves to attract, capture, and digest arthropod prey (Takeuchi et al., 2015) which are then metabolized and absorbed for plant growth and reproduction. Depending on the environment and the type of trap employed, carnivorous plants are able to obtain between 10% and 80% of their total nitrogen from insects (Behie et al., 2013). Some pitchers of carnivorous species including *Nepenthes* are so large, that frogs sporadically enter the pitcher for shelter and to feed on the plant's prey (Adlassnig et al., 2011), then benefit by collecting the frog fecal matter, rich in nutrients.

Charles Darwin was the first to show that carnivorous plants secrete digestive fluid, in response to nitrogenous substances (Darwin, 1875; Ellison and Gotelli, 2008), laying the groundwork for current research on carnivorous plants. To date, there are more than 650 known species of carnivorous plants, in 8 families and 18 genera, and the majority of these plants thrive in ecosystems where sunlight and water are abundant including bogs, marshes and rocky outcrops (Behie et al., 2013; Clarke and Moran, 2016; Ellison and Adamec, 2018). Carnivorous plants utilize many of the structures and compounds used by non-carnivorous plants to capture and digest prey, including the evolution of structures that are associated with defense against pathogens (Thorogood et al., 2017).

Carnivorous plants have evolved complex and sophisticated trap styles like venus flytraps, sundews, suction traps, corkscrew traps, and pitchers (Yilamujiang et al., 2017). One

genus that Darwin never worked with was the tropical pitcher plant, *Nepenthes,* whose name originates from an anti-sorrow drug taken by Helen in Homer's The Odyssey (Eilenberg et al., 2006). Pitcher plants have evolved specialized pitfall traps to maximize prey capture depending on the environment, ecological niche, and feeding habit (Ravee et al., 2018). For example, *Nepenthes hemsleyana* maintains a slender pitcher, capturing few insects and instead, providing a roosting site for bats (Thorogood et al., 2017).

There are 160 species of *Nepenthes* identified to date (Ellison and Adamec, 2018). *Nepenthes* are distributed from Northern Australia throughout Southeast Asia to China, of which the majority grow in tropical conditions on the islands in the Malay Archipelago (Ellison and Adamec, 2018, Renner and Specht, 2013). There is also an existence of outlying species in India, and on the islands of Madagascar, the Seychelles, and Sri Lanka. According to phylogenetic data, *Nepenthes* may have evolved from a *Drosera*-like progenitor (Meimberg, et al., 2000; Thorogood et al., 2017).

Multiple mechanisms may contribute to prey attraction in *Nepenthes.* Possible mechanisms include extrafloral nectar bribes, peristome and/or flower fragrance, and UV light absorption patterns near the pitcher opening that attract insects to the plant (Di Giusto et al., 2008; Bohn et al., 2004). Another possible theory is the presence of a CO_2 gradient that is dispersed around the pitcher when it opens to attract and capture prey (Baby et al., 2017). In many species, like *N. rafflesiana,* the pitcher fluid has viscoelastic and retentive properties to ensure optimal prey capture and retention during heavy rain or flooding (Gaume and Forterre, 2007). *Nepenthes* capture a broad spectrum of arthropods, most commonly ants (Ellison and Adamec, 2018).

Sir Joseph Hooker's studies of protease activity in the trap fluid of *Nepenthes* was one of the earliest investigations of digestive enzymes involved in plant carnivory (Hooker, 1875; Renner and Specht, 2013). Since this time, there has been a large body of research published on the fluid composition in *Nepenthes.* The pitcher fluid is generally acidic, with a pH ranging from two to six (Takeuchi et al., 2015), to facilitate the enzymatic breakdown of prey. The digestive glands secrete a wide array of enzymes including proteases, chitinases, RNAses, and phosphatases (Eilenberg et al., 2006; Amagase, 1972).

Most of the proteins that have been characterized in *Nepenthes* function in the digestion of prey (Rottloff et al., 2016). Other proteins that are not directly involved in prey degradation (Rottloff et al., 2016) may be involved in trap maturation including a β-d-xylosidase found in *N. alata* secretions (Hatano and Hamada, 2008). Additional proteins identified in *Nepenthes* are committed to plant defense against pathogens (Hatano and Hamada, 2008; Rottloff et al., 2011), including nepenthesins I and II, homologues of what researchers term, "pathogenesis-related proteins" (Hatano and Hamada, 2008), functioning to inhibit bacterial growth in the pitcher fluid and provide rapid prey decomposition for efficient nutrient use (Takeuchi et al., 2015; Eilenberg et al., 2006; Hatano and Hamada, 2012; Buch et al., 2013).

There is controversy over the origin of enzymes found in the digestive fluid, whether the enzymes are secreted by the plant and/or derived from the microbial community within the digestive fluid. Takeuchi et al. (2015) suggest the fluid in *Nepenthes* pitchers may originate from the phyllosphere and be transmitted from the leaf surface into the pitcher. Once broken down by digestive enzymes, the insect-derived nitrogenous compounds will be transported through the vascular tissue to support plant growth and reproduction.

Carnivorous plants frequently contain enzymes with novel properties as a result of adaptation to harsh and nitrogen-poor environments with limited nutrients. The aspartic protease, nepenthesin, has been of interest to scientists as this protease is more stable and works best at even higher acidity than the mammalian digestive protease pepsin (Kadek et al., 2014). A novel class of prolyl endopeptidases was also recently discovered in *Nepenthes*, neprosin 1 and neprosin 2. Schräder et al. (2017) investigated the use of neprosin for whole proteomic profiling and histone mapping, instead of the commonly-used enzyme trypsin that hydrolyzes proteins in the vertebrate digestive system. To understand the processes that drive disease states in cancer which includes histone-tail regulated epigenetic mechanisms, neprosin has the potential to be utilized for whole proteomic profiling and histone mapping due to its low molecular weight and high activity at a low concentration and pH, and its ability to effectively degrade proteins of any size (Schräder et al., 2017). Neprosin has also been found to provide an alternative to aspartic proteases, like pepsin in hydrogen/deuterium exchange experiments, or in the analysis of native disulfide bridges (Schräder et al., 2017).

According to Rey et al. (2016), the combined action of a neprosin and nepenthesin from *Nepenthes* pitcher fluid demonstrates a potential application for effective gluten detoxification. Mice treated with low doses of proteolytic components from *Nepenthes*, including nepenthesin and neprosin, showed enhanced gliadin solubilization rates leading to elimination of intestinal inflammation compared to mice that were treated with pepsin only (Rey et al., 2016). Through the development of an enzyme supplementation strategy, the use of naturally occurring compounds found in plants expands the possibilities of treating celiac disease. Eilenberg et al. (2006) also found that chitin induction stimulated N-acetylglucosamine (GlcNAc) oligomers in *Nepenthes khasiana,* resulting in a large amount of soluble endochitinases, enzymes that digest

the trapped insect's inner and outer skeleton. GlcNac oligomers have been implicated in various medical treatments, like wound healing, and represent a great biotechnical advantage because of the ability to selectively synthesize them in closed non-induced pitchers (Eilenberg et al., 2006; Cole et al., 1999; Schwaitzberg et al., 2004).

Ethnomedicinal properties of *Nepenthes* have also been reported, including the use of pitcher fluid as an eyewash and a cure for headaches, asthma and burns in traditional Indonesian and Madagassean medicine (Adlassnig et al., 2011). In Malaysian tribes, *N. ampullaria and N. gracilis* boiled roots are used to cure stomach ache while infused parts of the stem are used for fever (Miguel et al., 2018). Beyond these traditional uses, Dhamecha et al. (2016) found that aqueous extract of *N. khasiana* was a successful reducer in the synthesis of gold nanoparticles (GNP) from gold salts and may be used as a tool in nanomedicine for drug diagnosis and treatment, including tumor targeting and imaging (Dhamecha et al., 2016). This method illustrates the successful use of plant extracts as an alternative to the synthetic method for the production of GNPs, thereby reducing the use of toxic chemicals and hazardous byproducts (Dhamecha et al., 2016).

Additional applications include the use of carnivorous plants as a supplement for pest control in agriculture, reducing the use of harmful and toxic pesticides. Miguel et al. (2018) also suggest, the root and stem of carnivorous plants exhibit tough and elastic properties, that may have the potential to be used as building materials in housing construction, in place of rattan.

The expression of digestive enzymes in pitcher plants may be the result of developmental regulation and a signal transduction mechanism. In the pitcher plant, *Sarracenia purpurea,* the expression of hydrolytic enzymes needed for digestion is developmentally regulated in young traps while later replaced by a signal transduction mechanism (Gallie and Chang, 1997). Clancy

and Coffey (1977) found that feeding *Drosera rotundifolia,* gelatin, stimulated the maximal secretion of acid phosphatase and protease enzymes within four days of feeding. Possible interpretations of observed increases in hydrolytic activity after feeding include *de novo* synthesis, activation of pre-existing forms of enzyme, or the induced enzymes may be microbial in origin (Clancy and Coffey, 1997; Chandler and Anderson, 1976). *Nepenthes* may also have a mechanism in response to prey capture, by which fluid pH decreases to the optimum pH of the digestive proteases (An et al., 2001).

Pitcher plants have evolved cellular structures that reduce the amount of photosynthesis that takes place in the plant. This includes the replacement of chlorophyll-containing cells with digestive glands, an advantageous evolutionary selection to survive in nutrient-poor habitats (Pavlovič et al., 2007). *Nepenthes* have become of interest for cost-benefit and mineral nutrition studies because these plants contain the lowest Rubisco content and corresponding photosynthetic capacity among all pitcher-plant genera (Pavlovič et al., 2007; Pavlovič and Saganová, 2015)**.** Insect prey contributes to 54-68% of total foliar N in *Nepenthes* (Ellison and Adamec, 2018).

The abundance of multicellular digestive glands located at the inner bottom of the *Nepenthes* pitfall-traps are involved in multiple processes including the perception of chemical stimuli, secretion of digestive enzymes, and the absorption of nutrients (Schulze et al., 1999). Secretion precedes absorption slightly in *Nepenthes* (Robins and Juniper, 1980) and both processes occur simultaneously during several-day-long digestion (Owen et al., 1999). Morphological studies demonstrate the digestive gland surface is rough in surface (Gorb al., 2004). There are more than 6,000 digestive cellular glands per cm^2 (Eilenberg et al., 2006) in

Nepenthes and the glands are anatomically similar across carnivorous sundews including *Drosera* and *Drosophyllum* (Thorogood et al., 2017).

An abundance of energy is required to fuel active processes in the digestive glands that will contribute to the successful and efficient breakdown of prey. An et al. (2001) found that a plasma membrane H+ -ATPase proton pump may be responsible for the acidification of pitcher fluid in *Nepenthes*. The digestive glands secrete a diverse group of molecules, including proteolytic enzymes synthesized in the rough endoplasmic reticulum, organic acids, Cl^- and Na^+ ions, and water (Owen and Lennon, 1999), while concurrently regulating the secretion of various digestive enzymes during and after pitcher development (Thornhill et al., 2008). The symplastic pathway, mediated by plasmodesmatal connections between adjacent cells, is often observed in secretory cells. The plasmodesmata are preferentially located near the endodermis, as the plasmodesmata control nutrient distribution and systemic responses around the plant.

After the secretory phase, digestive glands will absorb dissolved nutrients from the pitcher fluid including ions and low molecular mass solutes (Owen et al., 1999). During the absorptive phase, an increased number of vacuoles can be seen, functioning to store the absorbed material and nutrients (Robins and Juniper, 1980). At the end of the absorptive phase, the cell walls are rebuilt and the glands return to their state, as they were in before the signal transduction pathway that stimulated them (Robins and Juniper, 1980). Insect-derived nitrogenous compounds must be imported from the pitcher fluid and transported through vascular tissue to support plant growth and survival. The absorption of prey is thought to occur through carriers in the plasma membrane of glandular cells (Adlassnig et al., 2012). Adlassnig et al. (2012) provides evidence that endocytosis forms an additional uptake mechanism of absorption. Bradshaw and Creelman (1984) also found that passive diffusion may contribute to N uptake in *Sarracenia purpurea* L.

In order to ensure the pitcher does not digest itself due to the acidity within the digestive fluid, the digestive glands contain a cuticular coat that functions in secretion and ensuring the protection of the inside traps from self-digestion and microbial infection (Joel et al., 1983). Uptake into the apoplast is performed via pores in the cuticle (Joel et al., 1983). The cuticle surface is covered in a cuticular wax, a hydrophobic layer of predominantly long chain aliphatic molecules (Shepherd and Wynne, 2006).

The endomembrane system plays a critical role in the secretory and absorptive processes of carnivorous plants, contributing to the secretion of digestive enzymes by exocytosis and the absorption of nutrients by endocytosis. The endomembrane system includes the endoplasmic reticulum (ER), nuclear envelope, the Golgi apparatus, vacuoles/lysosomes, endosomes, the plasma membrane, and vesicles. The endomembrane system also contributes to a network of membrane trafficking, that is essential for the transport and exchange of materials like proteins and lipids, including the trans-Golgi network (TGN). The TGN functions in the sorting and transporting of proteins to various locations (Morita and Chimada, 2014).

A suberized, endodermal-like layer analogous to the Casparian strip in roots is often seen next to the digestive glands, suggesting a mode of nutrient transport. Casparian strips, blocking the free diffusion of water and nutrients present in the apoplastic space, generate a paracellular barrier that is thought to be particularly important in selective nutrient uptake and the exclusion of pathogens (Naseer et al., 2012). Elements of vascular bundles are also often in close proximity to the digestive glands, typically located one or two layers beneath the base of the gland (Owen et al., 1999; Schulze et al., 1999).

The digestive gland consists of three layers and the shape and size of the glands typically correlate with the location and level of the digestive zone (Gorb et al., 2004). Glands of the upper

digestive zone are the smallest while glands at the bottom are much larger (Gorb et al., 2004). The first layer of the gland consists of columnar cells that vary in size with few cells that have cytoplasm, instead containing secretion inclusions that may contain three-quarters of the cell volume (Gorb et al., 2004). The second layer consists of cells that vary more in shape and contain thin cell walls with the presence of cytoplasm. The third layer consists of large, laterally elongated cells characterized by thin walls and the presence of cytoplasm (Gorb et al., 2004).

Although the overall structure of *Nepenthes* has been studied and various applications have been generated, morphological data on the digestive glands and the function that these glands have in secretion and absorption is limited. Most of the literature surrounds the chemical function of enzymes that are secreted from the glands. Thus, the focus of this study was to analyze the cellular ultrastructure of the digestive glands in *Nepenthes glandulifera* using light and electron microscopy and to investigate the secretory and absorptive mechanisms of this carnivorous plant in relation to the metabolic pathways that have been previously studied.

Materials and Methods

Nepenthes glandulifera plants were obtained from a commercial source and grown in the Connecticut College greenhouse misting bench provided with deionized water. An opened pitcher, 6.7 cm in length from the peristome to the base, was collected, then cut into sections 3.5 cm from the peristome (constituting the digestive zone). For transmission electron microscopy (TEM), the samples were fixed in 1.25% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky, 1965) **i**n 0.1M sodium phosphate buffer (pH 7.2) for 3 hours at room temperature, then washed twice in buffer, 10 minutes each. The samples were post-fixed with 1% OsO_4 in buffer overnight at 4 °C. The samples were then dehydrated with increasing concentrations of acetone (30% - 100%) in increments of 30 minutes, then dehydrated twice with 100% acetone, each for 30 minutes. Spurr's (1969) plastic was slowly infiltrated over 12 hours with an increasing rate after 50% plastic. The sample was put into 100% plastic and polymerized in molds at 60 degrees °C for 20 hours.

Polymerized samples were trimmed with a razor blade to get past the cut layer of tissue, then thick sectioned with a dry glass knife. Sections (0.5 - 1 μm) were placed on a drop of water dried by heat, then stained with Toluidine Blue and examined by light microscopy. Using an ultramicrotome and a diamond blade, thin sections (60 - 80 nm) were prepared. Samples were then transferred to grids.

Grids were placed on droplets of uranyl acetate (2% aqueous), staining for approximately 20 minutes, washed by dipping into pure water, then lead citrate (Reynold's formula) for 90 seconds in a CO_2-free chamber made with NaOH and washed as above. Grids were dried and stored, and examined with an FEI TEM at 80KV. Digital images were obtained with an AMT 4K

camera system, saved as TIFF files. Contrast was adjusted uniformly in Photoshop using the "Levels" command; slight sharpening was applied through an unsharp mask filter.

Results

The digestive glands of *Nepenthes glandulifera* were analyzed using light and electron microscopy. The multicellular glands sat in a depression of the epidermis (Fig. 2A). Electron microscopy results illustrated an abundance of mitochondria (Figs. 1A-C; Figs. 3A) visible in all of the cells and the presence of some Golgi (Figs. 1A-C) that exhibit visible cisternae or folds in the membrane, and plastids (Figs. 1C and 3B). The plasma membrane (Fig. 1A) is characterized by an uneven border, suggesting some exocytotic events. There are limited endocytic events (Fig. 1B) and clathrin-coated vesicles (Fig. 1B).

Light microscopy data illustrates that one face of the digestive gland faces the pitcher fluid (Fig. 2A). The cuticle (Fig. 2A) borders the digestive gland, forming a barrier between the gland and the fluid. Bounded to the digestive gland on the inner side of the gland opposite the digestive fluid is the presence of suberin and a Casparian-like strip (Fig. 2A). Few plasmodesmata (Fig. 3B) are in close proximity to the endodermis (Fig. 3B). The presence of xylem connects to the gland, while a vascular bundle is in close proximity to the gland (Fig. 2A).

There were no protrusions in the cell wall, but the cell wall exhibits an unusual staining pattern (Fig. 3A). An unusual structure, preferentially located near the nucleolus was observed in the nucleus of multiple cells (Figs. 1C and 3B).

Discussion

The digestive zone of *Nepenthes* consist of large multicellular glands that develop from single epidermal cells (Owen et al., 1999). There are one to four (most typical) and up to eight secretory cells that combine to form two or more layers within the glands. The endoplasmic reticulum (ER) is the first organelle of the secretory pathway where proteins, like digestive enzymes, are synthesized and assembled for export to the Golgi apparatus (Langhans et al. 2012). The Golgi (Figs. 1A-C) will collect membranes and lumenal content from the ER for packaging. Further processing takes place in the trans Golgi network (TGN), vacuoles, and the plasma membrane. The presence of Golgi activity may be associated with the replenishment of digestive material for the next digestive cycle (Schwab et al., 1969).

The gland's outer cell wall exhibits an unusual staining pattern (Fig. 3A) that may contain microchannels and increased surface area for enhanced secretion and intercellular communication, including the regulation of molecular movement and responses to local and long-range elicitors like hormones, sugars, proteins, and RNAs (Houston et al., 2016). In contrast to the nectary glands of *Nepenthes*, that typically have cell wall protuberances (Bolt et al., 2018), there were no cell wall protuberances observed in the digestive gland. This is an unusual feature because the digestive glands of many insectivorous plants exhibit complex cell wall protuberances to enlarge the effective area of the plasma membrane (Fig. 1A) and amplify the symplast/apoplast interface (Heslop-Harrison and Heslop-Harrison, 1981).

An abundance of mitochondria (Figs. 1A-C; Figs. 3A) is a characteristic of plant secretory cells (Fahn, 1979), including the secretory cells of *Dionaea, Drosera, and Pinguicula* (Heslop-Harrison and Heslop-Harrison, 1981). Active processes include the acidification of pitcher fluid through a proton transporter in the plasma membrane (An et al., 2000). The xylem,

which is in direct connection to the gland that was studied (Fig. 2A) may function in supplying water for the pitcher fluid before the pitcher opens (Owen et al., 1999). Other active processes include enzyme storage and release through the plasma membrane, which may occur through exocytosis (Fig. 1A), either eccrine or granulocrine secretion (Fahn, 1987).

The presence of suberin (Fig. 2A) indicates a Casparian-like strip, functioning as an endodermis in the gland. The preferential location of the Casparian strip (Fig. 2A) bounded to the endodermal cell, is a mechanism that has likely evolved over time to promote efficient water and nutrient transport (Heslop-Harrison and Heslop-Harrison, 1981). The paracellular barrier, blocking the free diffusion of water and nutrients present in the apoplastic space, has been implicated in selective nutrient uptake and the exclusion of pathogens (Naseer et al., 2012). High levels of suberin (Fig. 2A) also indicate this plant's ability to cope with nutrient availabilities and modulate uptake or retention in the vasculature.

Although the presence of a Casparian-like strip and suberin suggest symplastic transport, limited plasmodesmata (Fig. 3B) were observed near the endodermis (Fig. 3B), suggesting there are additional mechanisms for water and nutrient uptake. This includes large molecule uptake into the vascular tissue via endocytosis (Fig. 1B). Another likely mechanism of absorption in *Nepenthes* is clathrin-mediated endocytosis (Fig. 1B). Adlassnig et al. (2012) found that the application of the clathrin inhibitor chlorpromazine significantly reduced uptake of nitrogenous compounds by endocytosis in *Nepenthes* and *Drosera.*

The epidermal cuticular coat (Fig. 1A), ensuring the protection of the inside traps from self-digestion and microbial infection (Joel et al., 1983), has been implicated in the formation of a barrier to apoplastic transport. Gorb et al. (2004) finding suggests that the presence of cutin in thick external cell walls in the epidermis and glandular head cells of *Nepenthes* digestive glands

contributes to the formation of a barrier to apoplastic transport and also promotes increased mechanical strength of the glandular surface (Owen and Lennon, 1999). In order to prevent insects from chewing through the pitcher plant and escaping from the trap, plastids (Figs. 1C and 3B) can also synthesize secondary products for defensive compounds.

Consistent with the lack of cell wall protuberances, plasmodesmata, exocytotic/endocytic events, and abundance of mitochondria throughout the gland, ultrastructural data suggest that *Nepenthes* require transmembrane transporters to transport nutrients through the vascular tissue. Schulze et al. (1999) found an amino acid transporter encoded by NaAAP1 in bundle sheath cells in *Nepenthes,* promoting the uptake of amino acids (intermediate products of digestion), from the cortex of the vascular tissue into bundle sheath cells (Schulze et al., 1999; Thornhill et al., 2008). After the prey has been broken down by the digestive enzymes into small, soluble proteins, peptides, amino acids or NH_4^+, these molecules are likely imported through transport proteins in the plasma membrane. Protons are used for the co-transport of molecules, which would also be consistent with the low pH of the pitcher fluid (Fig. 2A).

There were no chloroplasts observed in the digestive gland, illustrating the low photosynthetic capacity in *Nepenthes* (Pavlovič et al., 2007; Pavlovič and Saganová, 2015). Chia et al. (2004) found that there are increased numbers of chloroplasts beneath the digestive glands in *Nepenthes,* resulting in the conduction of solutes into the pitcher that will generate oxygen free radicals by pitcher fluids. Gel electrophoresis data showed that myosin, an abundant protein component of insect muscle tissue, was rapidly broken down by free radicals. Thus, oxygen free radicals likely aid in the digestion of insect prey (Chia et al., 2004).

The presence of a vascular bundle is in close proximity to the digestive gland that was studied (Fig. 2A). The vascular bundle, containing both phloem and xylem, promotes water,

sugar, and amino acid uptake through the roots and supplies photosynthesis in these plants. Gorb et al. (2004) found that vascular bundles are typically located one or two layers beneath the base of the gland in the digestive glands of *Nepenthes,* functioning to transport nutrients back into the plant after prey digestion (Owen and Lennon, 1999).

A notable structure that was observed in the nucleus of multiple cells is the presence of an unusual nuclear extension, spanning half of the nucleus (Figs. 1C and 3B). Plachno et al. (2017) found a similar plant nuclear tubular extension/projection in the placental nuclei of *U. nelumbifolia*. Marked irregularities in the large nuclei of onion epidermal cells have also been observed (Collings et al., 2000). The term "chromatubules," or chromatin-filled tubules, was suggested by Plachno et al. (2017) for this unusual structure in the nucleus of some plant cells. The grooves and invaginations in the chromatubules, possibly containing actin bundles, may substantially increase the nuclear surface area and provide a mechanism to increase the rates and efficiency of nucleocytoplasmic transport (Fricker et al., 1997). Chromatubules may also function to anchor and stabilize the nucleus within the cell.

The chromatubule structure is preferentially distributed near the nucleolus (Figs. 1C and 3B). As a result of the increase in nuclear volume and the close proximity of this structure to the nucleolus, the chromatubules may promote efficient trafficking of RNA out of the nucleus and of proteins into the nucleus. It is likely that the unusual structure functions in the facilitation of ribosome export to the cytoplasm, for protein synthesis, which is a significant function in *Nepenthes,* to maintain an acidic environment in the pitcher for the rapid breakdown of prey and absorption of nutrients.

Another hypothesis for this unusual structure, is that the nuclear invaginations which provide increased nuclear surface area, contribute to the transmission of calcium signals from the

cytoplasm to the nucleus (Lui et al., 1998 a, b). To date, most literature that investigates calcium signaling in plants only relates calcium signals in the cytosol. Studies of nuclear calcium signaling are gaining traction, as each cell compartment has the capacity to use its own calcium signatures to drive downstream signaling events, including the modification of gene transcription. Hardingham et al. (2012) analyzed gene transcription in hippocampal neurons and found that nuclear calcium signals activate gene transcription by a mechanism that is distinct from gene regulation driven by cytoplasmic calcium signals. Thus, it seems likely that this process may occur in plants. Although microscopy studies suggest this role, further experiments are required to determine whether this process occurs in plants. In order to determine the functional role of this structure, Plachno et al. (2017) suggests the use of an *in vivo* experimental system and green fluorescent-marked molecules to identify intracellular and cell-cell transport.

In this study, the cellular ultrastructure of the digestive glands in *Nepenthes glandulifera* was studied in relation to the secretory and absorptive functions of this plant. Microscopy analysis suggest that this plant uses transmembrane transporters, with limited symplastic and apoplastic transport for nutrient absorption. A novel structure was found in the nucleus of multiple cells. Possible functions for this unusual structure includes the continuous acidification of pitcher fluid, the transmission of calcium signals from the cytosol to the nucleus, and the trafficking of macromolecules between cells and tissues.

The special morphology of the digestive glands represents the evolution of an ancient plant with multiple functions. This research may also inspire the application of this plant in the pharmaceutical, agricultural, and industrial industries.

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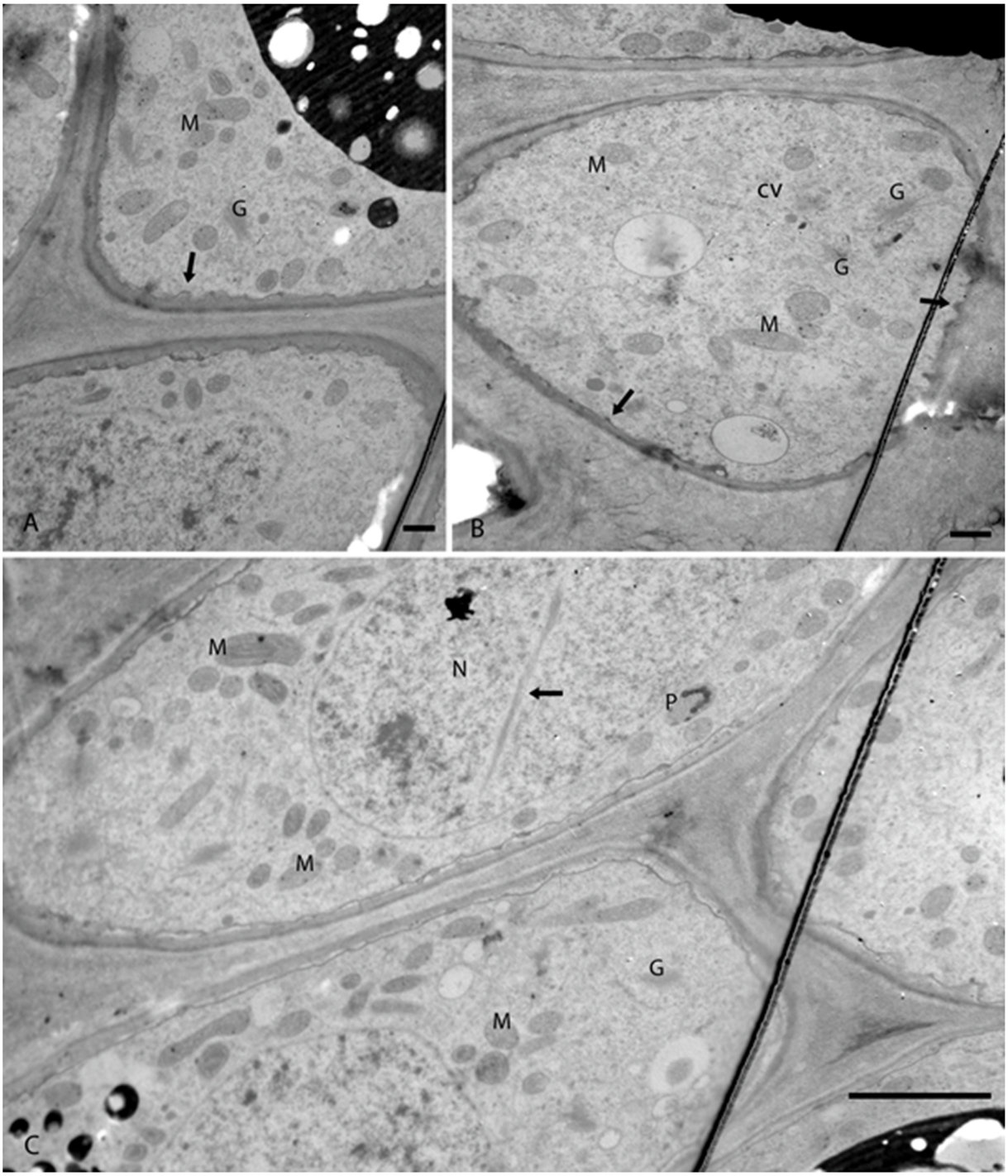
Figure 1. A-C: Mitochondria (M) and some Golgi apparatus (G) are observed throughout the gland. A: The gland is undergoing exocytosis (arrow). Bar= 500 nm. B: The gland is also undergoing endocytosis (arrow) and clathrin-coated vesicle endocytosis (CV). Bar = 500 nm. C: Nucleus (N) with an unusual "chromatubule" structure (arrow). Plastid is observed (P). Bar = 2 microns.


M
G
A
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CV
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B
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P
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G
M
C

Figure 2. A: Overview of digestive gland location using light microscopy. The gland is peripheral to the the pitcher fluid (PF) surrounded by a cuticular (C) surface. The gland is next to a vascular bundle (VB) and in direct connection with xylem (X). There is suberin and a Casparian-like strip bounded to the outside of the gland (arrow).

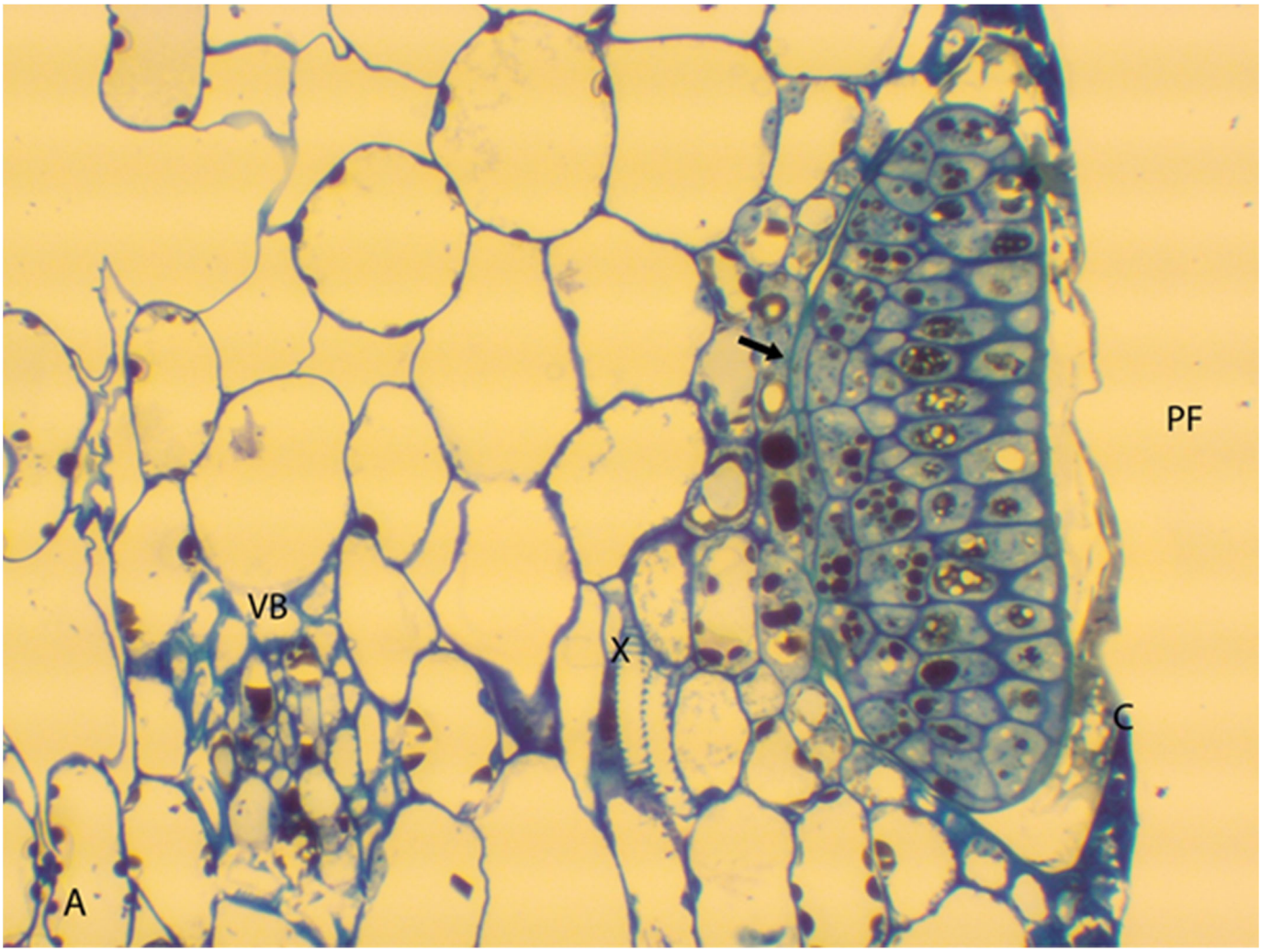
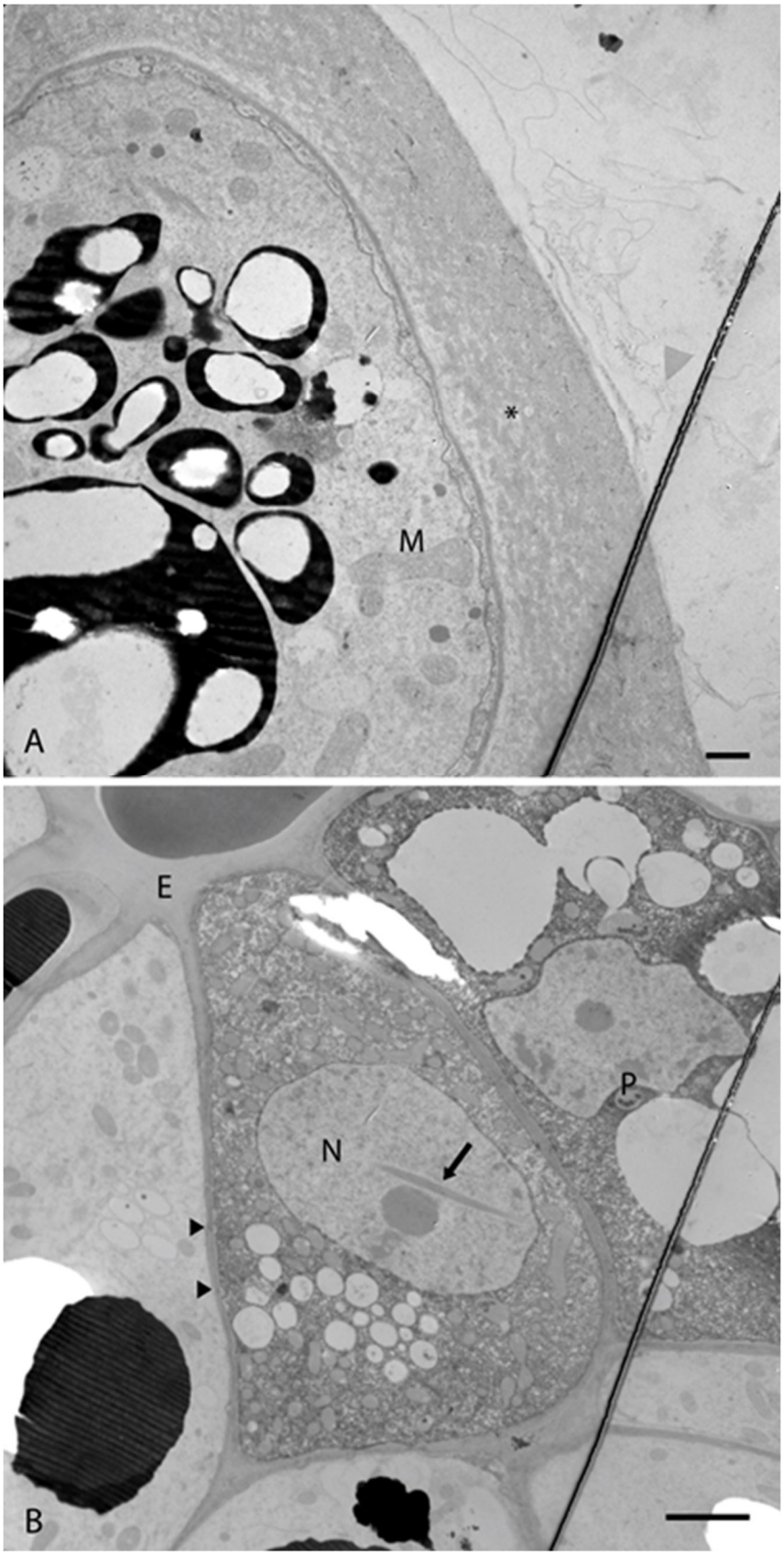

PF
VB
X
C
A

Figure 3. A: Outer component of digestive gland with an unusual staining pattern in cell wall (*). There is also mitochondria (M) near the plasma membrane. Bar= 500 nm. B: Nucleus (N) with “chromatubule” structure (arrow). Few plasmodesmata (arrowheads) near the endodermis (E) are observed. Bar= 2 microns.


M
A
E
P
N
B