Protease production and *in vitro* growth of *Sarracenia purpurea*

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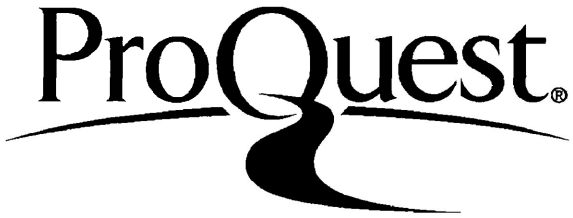

1.0 Abstract

Prey digestion in the insectivorous plant *Sarracenia purpuera* was investigated with consideration of three sources of proteolytic enzymes self-digestion by the prey, bacteria in the plant pitcher, and enzymes produced by the plant itself. To do this, *S. purpurea* seedlings were grown under sterile conditions and tissue cultures were established. The plant seedlings and tissue culture grew slowly. Tests on pitcher fluid in plant pitchers, or artificial pitchers containing water, or pitcher fluid, and with or without a cricket as prey showed that the cricket is a very important source of protease and that without induction of proteolytic activity by captured prey, the pitcher is largely inactive. More research is needed to determine the relative importance of the different sources of protease in the *S. purpurea* plant pitcher, particularly pitcher plants grown in sterile conditions will greatly advance this research by permitting investigation of the contribution of the plant pitcher alone.

2.0 Introduction

2.1 Plant Insectivory

All plants have three basic requirements; light, water and nutrients. Most plants acquire these necessities in a general way common to all species. However, some species have evolved specialized mechanisms to deal with deficiencies in any of these requirements. One of these mechanisms is plant insectivory, which is utilized by a variety of plants including the purple pitcher plant, *Sarracenia purpurea* L. When these unusual bog-dwelling plants with pitcher-shaped leaves were first discovered, their insectivorous nature was not recognized (Juniper *et al.*, 1989) because observers did not acknowledge the presence of dead and decaying insects. Among the first to recognize insectivory in plants was Charles Darwin, who studied sundews and published his experiments on them in *Insectivorous plants* in 1875 (Darwin, 1875; Juniper *et al.*, 1989). Plant insectivory, and the digestive enzymes used by the pitcher plant *Sarracenia purpurea* to obtain nutrients from captured insects are the topic of this thesis. The published scientific articles which are relevant to this topic are summarized in the following pages.

Various types of insectivorous plants are believed to have evolved independently several times over the course of evolution (Albert *et al.*, 1992). A common feature of all of these plants is that they are found in environments with abundant water and sunlight, but insufficient nutrients (Juniper *et al.*, 1989). The most common places to find insectivorous plants are bogs, fens and marshes. Species are usually specialized for attracting different types of prey, the most common prey being insects (Juniper *et al.*, 1989). There are some reported

cases of finding small vertebrates such as rats, frogs and even birds in large tropical pitcher plants, but these are considered accidental prey captures (Juniper *et al.*, 1989; Butler, *et al.*, 2005). Thus, in subsequent pages, I will refer to these plants as "insectivorous" rather than by the all-encompassing term "carnivorous."

Insects are rich in nitrogen, phosphorus and potassium, the three macronutrient elements most limiting to plant growth (Adamec, 1997). But prey also contain other macro and micro nutrients needed by the plant such as sulphur, iron, copper, cadmium and zinc, which have been found to be readily absorbed through insectivorous plant traps (Adamec 1997). One of the main preoccupations of plant nutrition research has always been nitrogen, as its available forms are often limited and because this element is needed for synthesis of plant nucleic acids, proteins, and chlorophyll (Hopkins and Hüner, 2004). Protein in prey, rather than inorganic nitrogen in the soil is thought to be the main source of nitrogen for insectivorous plants (Juniper *et al.*, 1989; Ellison and Gotelli 2001).

The nutrients obtained from prey are thought to result in an increase in the photosynthetic rate and ultimately growth rate (Adamec, 1997; Givnish *et al.*, 2000). It is possible that the plants use prey as a heterotrophic source of energy as well, but there is no evidence to support this theory (Adamec, 1997). Although insectivorous plants "eat" their prey, they are believed to do so mainly for mineral nutrients, rather than for carbon and energy. Thus, these plants are not heterotrophs like animals, but autotrophs like the majority of plants and, as such, rely on photosynthesis for energy (Raven *et al.*, 1999).

Although insectivory provides the benefits of increased biomass and photosynthetic rate, it also carries significant physiological and developmental costs (Givnish *et al.*, 2000). To be considered insectivorous, a plant must attract, trap, kill, digest and absorb its prey (Juniper *et al.*, 1989). Prey attractants, such as nectar, cost the plant metabolic energy in the form of sugar (Givnish *et al.*, 1984). The leaves of an insectivorous plant are often shaped for prey capture and digestion, rather than for maximizing surface area for photosynthesis. This decrease in photosynthetic potential is likely possible because carnivorous plants are commonly found in places with optimum sunlight and water (Givnish *et al.*, 1984). It has been suggested that the cost of this decreased photosynthetic rate is too high to allow some insectivorous plants to benefit from their insectivory due to low prey capture rates (Newell and Nastase, 1998; Ellison and Farnsworth, 2005). However, it has been found that trap formation in some species costs less energy than the formation of photosynthetic specialized phyllodes, making insectivory an effective strategy in low nutrient, high sunlight and adequate water conditions (Karagatzides and Ellison, 2009).

2.2 Digestive Process

For the nutrients from prey to be absorbed and used by the plant, the prey must be digested. The mode of digestion by plants is a fascinating topic, because digestion is usually associated with animals. Like animals, insectivorous plants must first break down the food into its components. Proteins, lipids and carbohydrates need to be hydrolysed to their monomers: amino acids, fatty acids and monosaccharides respectively (Hickman *et al.*, 2001). In animals, digestion is completed by extracellular enzymes formed by the animal's digestive tract with

possible contribution of symbiotic bacteria (Hickman *et al.*, 2001). Pitcher-type insectivorous plants are likely to do the same, with digestion happening in the pitcher rather than in a digestive tract. However, the degree of digestion carried out by the plant itself, compared to the digestive contribution by microorganisms, may differ between insectivorous plant species. There are even some plants that are considered to be insectivorous, which may not actively digest their prey, because they do not appear to release enzymes such as protease, chitinase or phosphatase (Juniper *et al.*, 1989). Rather, these insectivorous plants are believed to provide the conditions necessary for microorganisms or insect larvae to digest the prey. The pitcher plant *Sarracenia purpurea* L. was reported to absorb only surplus nutrients, or nutrients originating as waste, produced by the bacteria and larvae in the pitcher (Błedzki and Ellison, 1998).

2.3 The *Sarracenia purpurea* controversy

Some research indicates that *S. purpurea* is unable to secrete proteolytic enzymes and that its digestion of prey is completely carried out by symbiotic bacteria (Ellison, 2001; Ellison and Gotelli, 2002; Ellison and Gotelli, 2001; Ellison *et al.*, 2003; Farnsworth and Ellison, 2008; Gotelli and Ellison, 2002). In contrast, research by Gallie and Chang (1997) suggests that *S. purpurea* does secrete protease into its pitcher, when the growth of bacteria is inhibited by antibiotics (Gallie and Chang, 1997). They also found that protease secretion by *S. purpurea* is highly controlled. According to Gallie and Chang (1997), *S. purpurea* pitchers secrete a large amount of protease as they first open. Then these secretions stop. However, further hydrolase secretion can be induced by adding protein, DNA or RNA to the pitcher as an insect substitute

(Gallie and Chang, 1997). This finding appears logical, since it would be wasteful to make protease continuously in a nitrogen limiting environment. It also appears unlikely that this single species is different from the many tropical pitcher plants known to secrete proteases, which will be discussed below. Conclusions of Gallie and Chang (1997) may explain some observations made in related studies, but their work is largely ignored, or only mentioned in passing by other researchers (Mouquet, *et al.*, 2008; Butler, *et al.*, 2008).

When tracing the origin of the idea that *S. purpurea* does not secrete protease, I found that this notion likely came from the authoritative book *The Carnivorous Plants* by Juniper *et al.* in which it was stated that "... in some *Sarracenia* species it is doubtful whether any enzymes are secreted by the plant at all" (Juniper *et al.*, 1989). Although the species of *Sarracenia* that cannot secrete protease are not specified, Juniper *et al.* (1989) cited a Master's thesis by Parkes (1980), which reported that *S. purpurea* can digest gelatine. This would indicate the secretion of a protease (Parkes, 1980). However, since the Master's thesis is difficult to obtain and not conventionally published, it is often ignored by researchers who interpret *The Carnivorous Plants* as advancing the proposition that some *Sarracenia* species do not secrete any protease (Farnsworth and Ellison, 2008). Parkes' Master's Thesis is available on microfiche from Monash University (Australia) and gives good evidence that *S. purpurea* secretes protease.

A much earlier paper by Hepburn *et al.* (1927), which is frequently cited by Juniper *et al.* (1989), contains some evidence against the protease secretion of *S. purpurea* in its introduction. This introduction begins with a review of German research from the late 19th century. These early researchers placed an unknown meat in the pitchers of *S. purpurea*

(Hepburn *et al.*, 1927) and found that the meat was not digested after two days leading them to conclude that *S. purpurea* was unable to secrete proteases to digest protein (Hepburn *et al.*, 1927). There are two problems with conclusions reached in this early research. The first is that the meat may not have been given enough time in the pitcher to induce protease secretion and begin detectable digestion. Fluid from a mature pitcher that has not been adequately induced would not contain protease (Gallie and Chang, 1997). The second problem is that the large amount of meat given may have had toxic effects on the plant, or may have caused such effects if given more time as reported in Robinson (1908). Another set of experiments by Goebel in Germany on *S. purpurea* showed that fluid removed from the pitcher was unable to digest fibrin in 48 hours (Robinson, 1908). Again, the fluid from an uninduced pitcher would not be able to digest any protein. Furthermore, fibrin has specifically evolved to clot blood and to be refractory to proteolysis in mammalian blood in order to assist in closing a wound (Hickman *et al.*, 2001). It is unlikely that an insectivorous plant such as *S. purpurea,* which has evolved to trap insects, (Ellison and Gotelli, 2009) would be able to efficiently digest meat or fibrin.

Although the article by Hepburn *et al.* (1927) discusses past research which suggests that *S. purpurea* cannot secrete protease, their own experiments demonstrated that *S. purpurea* may secrete protease. The first experiment was almost a reproduction of the early German work, in which liquid removed from a pitcher was used to digest fibrin (Hepburn *et al.*, 1927). The fibrin was digested in these experiments (Hepburn *et al.*, 1927; Robinson, 1908). One notable difference was that the pitcher liquid was given more time, up to 57 days in two experiments, to digest the fibrin (Hepburn *et al.* 1927). It is possible that, in this experiment, the plant had been induced to produce proteases by captured insects prior to fluid removal for

testing, and this extended digestion period resulted in detectable levels of proteolysis. Interestingly, the action of the protease was found to be strongest in alkaline conditions, rather than acidic (Hepburn *et al.*, 1927). Alternatively, since these experiments were not performed aseptically, they are open to the interpertation that bacteria were responsible for the proteolysis.

In order to understand the controversy of protease secretion in *S. purpurea*, the other members of the Sarracenia family should be considered. The *Sarraceniacae* family consists of three genera, the *Sarracenia* found in North America, *Darlingtonia* found in the south western United States, and *Heliamphora* found in South America (Albert *et al.*, 1992; Ellison and Gotelli, 2009). Based on *rbc*L gene sequencing, it has been suggested that the *Darlingtonia* genus is "sister" to the *Sarracenia-Heliamphora* genera (Bayer, *et al.*, 1996). In other words *Sarracenia* and *Heliamphora* are more closely related to each other than to *Darlingtonia* (Albert *et al.*, 1992). Of these three genera, *Darlingtonia* contains only one species, *D. californica* Torrey which, according to Hepburn (1927), relies on bacteria and an inquiline community (organisms that live inside the pitcher, for prey digestion (Juniper *et al.*, 1989; Nielsen, 1990). Similar to *S. purpurea*, there appears to have been little research into prey digestion in *D. californica* since the extensive investigations by Hepburn (1927). In the *Heliamphora* group of six species, only *H. tatei* Gleason is considered capable of producing protease for prey digestion (Juniper *et al.*, 1989). The *Sarracenia* genus consists of eleven species, all of which, except *S. purpurea* and *S. rosea,* are accepted as being able to produce protease for prey digestion (Farnsworth and Ellison, 2008).

Further genetic analysis has shown that *S. purpurea* is most closely related to *S. leucophylla* Rafin., an endangered species found in the southern United States (Juniper *et al.*, 1989; Bayer, *et al.*, 1996). *Sarracenia leucophylla* is capable of secreting digestive enzymes for prey digestion according to Juniper *et al* (1989). Since all members of the *Sarracenia* genus, including its closest relative, are able to secrete protease, it seems likely that *S. purpurea* also has this ability. *S. purpurea* is considered a unique species and can be differentiated from the other members of *Sarracenia*. However, there are natural hybrids found in areas where *Sarracenia* species intersect (Juniper *et al.*, 1989).

S. purpurea is different from the other members of the *Sarracenia* genus in some aspects of its biology, namely its temperature tolerance, morphology, prey and inquiline community. *S. purpurea* has the largest and most northern range, which consists of bogs across Canada and the Eastern United States (USDA, 2011). It is the only member of the *Sarracenia* genus that is able to survive winter in northern Quebec and other parts of Canada. Pitcher formation by *S. purpurea* has been found to change with soil nitrogen levels (Ellison and Gotelli, 2002). In relatively high nitrogen concentrations, more phyllodes (leaves specialized only for photosynthesis) are produced. As the nitrogen concentration decreases, the relative size of the keel, the main area for photosynthesis on a pitcher also decreases. Thus, the morphology of a pitcher depends on the nitrogen availability before leaf development commences (Ellison and Gotelli, 2002).

Because of its large geographic range, there is also a large assortment of prey caught by *S. purpurea* pitchers. The most common form of prey are insects in the order *Diptera* and

Hymenoptera, such as flies and ants (Cresswell, 2000). There is also accidental prey such as frogs, grasshoppers and newt larvae (Butler *et al.*, 2005). Insect species contain different amounts of nitrogen. For example, ants and grasshoppers contain approximately 10.6 and 12.1 percent nitrogen, depending on the species (Siemann *et al.* 1996). Larval newts contain 5 mg of nitrogen, which could contribute for the total seasonal nitrogen requirement for a pitcher plant from prey (Butler *et al.*, 2005; Wakefield *et al.*, 2008).

According to Chapin and Pastor (1995), at least 10% of the nitrogen acquired by *S. purpurea* each season is from insect tissue. This is quite low compared to other insectivorous plants, such as other members of the *Sarraceniace* family which receive 76% of a season's nitrogen from insect prey (Ellison and Gotelli, 2001). Chapin and Pastor (1995) suggest that *S. purpurea* obtains the rest of its nitrogen requirements through soil and rain. However, low nutrient content of soil found in *S. purpurea* habitats makes this unlikely and nitrogen from rain is seen as a threat to *S. purpurea* (Gotelli and Ellison, 2002; Bott *et al.*, 2008). *Darlingtonia californica* and *Heliamphora* spp. are reported to receive 76.4 % and 79.3% of their nitrogen from insects, respectively, over the life span of the plant (Wakefield *et al.*, 2008; Chapin and Pastor, 1995; Schulze *et al.*, 1997). The insectivorous plant that receives the most nitrogen from insects appears to be *Drosera paillida* Lindley at 87.1% (Schulze *et al.*, 1991). These values could be used to suggest that *S. purpurea* is less insectivorous than other insectivorous plants (Ellison and Gotelli, 2001). However, the method used to determine the amount of nitrogen from insects in *Darlingtonia*, *Heliamphora* and *Drosera* is based on the ^{15}N concentration in the plants and is compared to non-insectivorous reference plants and to insects in the same area (Schulze *et al.*, 1991). Sometimes the ^{15}N content of reference plants

was highly variable and could depend on the root depth of the species. Thus, the percentage of nitrogen from insects in these two species is a rough estimate with a high degree of uncertainty (Schulze *et al.*, 1997). The method used to determine the nitrogen content of *S. purpurea* also suffers drawbacks. This calculation was based on the average amount nitrogen in rain and insects captured in one growing season. This amount of nitrogen was then compared to the nitrogen in the plant (Chapin and Pastor, 1995). This study did not include the amount of nitrogen in the plant at the beginning of the growing season. Thus, the 10% insect nitrogen in *S. purpurea* is the annual increase in insect nitrogen over one season, rather than an estimation of total insect nitrogen over the life time of the plant; the measure which is used in the other studies. The age of the plants tested is also unknown. It may be expected that a young plant contains more nitrogen from seed and soil than a mature plant, which has more traps for prey capture and thus has received more insect nitrogen over the course of its life time. Insectivorous plants are good at reallocation of nutrients (Butler and Ellison, 2007). Therefore the recycling and movement of nitrogen from old to new pitchers must also be considered.

A recent investigation by Butler and Ellison (2007) found that the roots of *S. purpurea* are responsible for 2-5% of the nitrogen acquired by the plant in one growing season. Redistribution of nitrogen from previous growing seasons accounted for 65% of nitrogen used that season, leaving 30-33% of the nitrogen to have come from the pitchers (Butler and Ellison, 2007). At the beginning of the growing season, the first new pitchers rely completely on remobilized nitrogen. As the season progresses, new pitchers are grown with newly assimilated nitrogen (Butler and Ellison, 2007). *S. purpurea* insect prey does not appear to directly increase the photosynthetic rate in the pitcher that catches the prey (Farnsworth and Ellison, 2008;

Butler and Ellison, 2007). This is in contrast to the findings that prey addition directly increases photosynthesis in other carnivorous plants (Pavlovič *et al.*, 2009). In summary, ecological evidence for the lack of plant contribution to insect proteolysis is not equivocal. It seems unlikely that *S. purpurea* and possibly a few other pitcher plant species are exceptions to an otherwise general evolutionary pattern in insectivorous plants.

Although the study by Gallie and Chang (1997) supports the idea that *S. purpurea* can produce protease, there is a good argument to suggest that there may be a problem with their conclusions. Gallie and Chang (1997) controlled for the possible presence of bacteria in the test plant pitchers with antibiotics. They also checked for the growth of bacteria in the pitchers by running control bacterial culture plates inoculated with collected pitcher fluid. No bacteria were found in these control plates. However, obtaining bacterial counts by nutrient agar streak plates is not a definitive method of determining the presence of bacteria, although it is a standard method for readily culturable species. Antibiotics prevent population growth, but they do not inhibit metabolism of pre-existing bacteria or antibiotic insensitive microorganisms, such as fungi. Like many other plants, *S. purpurea* has an endophytic population of fungi that grow inside the plant (Juniper *et al.*, 1989). Fungi may have provided protease for prey digestion, but this is not considered in the Gallie and Chang (1997) study. Since it is not stated that the plants used by Gallie and Chang were raised under aseptic conditions from germination, fungi were most likely present. The only solution to this problem is to conduct similar experiments with *S. purpurea* plants grown in sterile conditions, from germination to maturity.

2.4 *Sarracenia* as an ecological microcosm

A lot of research has gone into *S. prupurea* as a model for ecological microcosms (Porembski and Barthlott, 2006). Bacteria are not the only organisms that can live in the pitchers of *S. purpurea* and other insectivorous pitcher plants. *S. purpurea* is commonly used for this research because of its relative abundance and convenient size (Ellison *et al.*, 2003). Macrocosms consist of whole ecosystems, in which it would be impossible to control the input of organisms and nutrients. The small size of the pitcher plant makes it an ideal model for this type of research (Ellison *et al.*, 2003). The predator-prey relationships within the pitcher are also studied because of the quick generation times of the microorganisms, and relatively brief generation times of mosquito larvae (ter Horst *et al.*, 2010). Thus, although there is uncertainty about the role of the plant in the digestion of the prey that it captures, a lot of attention has been given to the organisms, living inside these plants, in regard to the digestion of prey. Other insectivorous pitcher plants such as *Darlingtonia californica* and *Nepenthes bicalcarata* Hooker are host to an inquiline community that could be used as model microcosms (Nielsen, 1990; Cresswell, 2000). However, they are not as readily available as *S. prupurea*.

2.5 *Sarracenia purpurea* inquiline community

There is quite a lot known about the community of organisms that live inside *S. purpurea*. These organisms may thus play a substantial role in the digestion of prey caught by the host plant. When a new leaf is formed by *S. purpurea*, the leaf is closed and soft. As it matures, the leaf opens to form a pitcher and becomes rigid. It has been shown that the inside of the immature, unopened leaf is essentially sterile (Gallie and Chang, 1997). After opening,

microorganisms in the air and rain water will enter the pitcher. The water in the pitcher provides a safe place for aquatic invertebrates such as mosquitoes and midges, which lay their eggs, and possibly bring additional microorganisms to the pitcher carried on their bodies (Harvey and Miller, 1996). When their larvae develop into adults and leave the pitcher to lay eggs in another pitcher, they carry with them microorganisms, which presumably colonize the next pitcher.

The food web starts outside the pitcher, with insects such as ants and crickets that eat plants or act as scavengers (Hickman *et al.*, 2001). These insects are lured into the pitcher, where they drown and die (Schaefer and Ruxton, 2008; Jürgens *et al.*, 2009). The drowned prey is now considered detritus and becomes the base of the food web within the pitcher (Butler *et al.*, 2008). Digestion starts in the pitcher with the shredders (midge and flesh fly larvae) that feed on the solid prey, ripping it into smaller pieces in the process. This makes the nutrients available to the next group in the web, bacteria, protozoa and rotifers (Butler *et al.*, 2008; Heard, 1994; Trzcinski *et al.*, 2005b). These organisms are all consumed by mosquito larvae and bacteria are targeted specifically by mites. The top predators of the pitcher plant are sarcophagid fly larvae that eat mosquito larvae, bacteria, protozoa and rotifers (Butler *et al.*, 2008; Peterson *et al.*, 2008). By hosting this complex food web, the plant receives nitrogen in the form of excrement and the decomposing bodies of prey and inquiline organisms (Butler *et al.*, 2008).

Because this food web development is based on a succession of organisms eating the detritus, it has been suggested that the occupants of the pitcher can be used to determine the

age of the pitcher (Fish and Hall, 1978; Koopman *et al.*, 2010). However, this is not entirely true. The start of the cycle is related to when the first prey was caught, rather than to when the pitcher opened. *S. purpurea* is considered to have a very low prey capture rate (Cresswell, 1991). It is unlikely that prey will be caught on the same day that the pitcher opens, and before the pitcher matures and starts producing attractants and other trapping mechanisms, such as hairs (Juniper *et al.*, 1989). The other problem with this theory is that there may be more than one prey organism in the pitcher at once, again obscuring the initial date on which the food web was established.

The inquiline community clearly plays a large role in the decomposition of prey. It has been suggested that more nitrogen is received by the plant, when the top predators are not present in the plant. Removing the top predators was expected to destabilize the food web and result in a change in nitrogen received by the plant (Butler *et al.*, 2008). However, it was found that the nitrogen received by the plant was not affected by the removal of the top predators from the food web. Butler *et al.* (2008) suggest that this is because the microbial population, which was not manipulated, plays a larger role than expected, making nitrogen available to the plant, rather than the food web as a whole. Bacterial growth should increase with the presence of top predators acting as shredders, thus making the detritus more available to bacteria (Heard, 1994). If bacteria and other microbes make nitrogen available to the plant, then the absence of shredders would decrease their growth and decrease nitrogen availability to the plant. Two significant problems with the above research are that firstly, no evidence exists showing that shredded prey is more available to bacteria and secondly it assumes that the plant has a passive role in nitrogen acquisition. An alternative explanation is that proteases released

by the plant could also digest the detritus and in so doing release nitrogen for the plant. Another study shows that bacteriovores, such as mites, decrease the nitrogen available to the plant, making the relationship between the plant and mite parasitic rather then mutualistic (Mouquet *et al.*, 2008).

2.6 Inquiline species present in the pitcher

There have been several studies into the basic species composition of the pitcher plant inquiline communities. Although the relative contribution of various organisms to pitcher plant nutrition is unclear, some discussion of the species present is in order. There are some generalizations that can be made based on plant characteristics and the mechanism by which new organisms arrive in the plant. For example, water fleas (Cladocerans) arrive by wind dispersed eggs (Harvey and Miller, 1996). Thus the height of the pitcher plays a role in the number of eggs that will land in the plant. Mosquito larva number increases with the visibility of the pitcher (Harvey and Miller, 1996). Midges (Chironomids) will only deposit their eggs in pitchers that contain enough water (Harvey and Miller, 1996; Nastase, *et al.*, 1995). Harvey and Miller (1996) tested the species composition of the inquiline community of *S. purpurea* plants in five geographical locations. They found that the inquiline communities in the five different geographical locations were distinct from each other statistically (Harvey and Miller, 1996). Other studies have shown that some species of the inquiline community are endemic to *S. purpurea,* although the number of individuals living in one plant may vary greatly (Nastase *et al.*, 1995; Miller *et al.*, 1994; Trzcinski *et al.*, 2005a).

The bacterial population of the pitcher has also been studied as part of the inquiline community and in isolation. As part of the inquiline community, the bacterial population is controlled by the keystone predator *Wyeomyia smithii* (Peterson *et al.*, 2008). The mosquito larvae eat the bacteriovores and also provide nutrient-rich feces (Mouquet *et al.*, 2008; Peterson *et al.*, 2008). It has been found that the bacteriovores may eat bacteria selectively, influencing the species abundance within the pitcher. However, this finding could also be explained by differing growth rates between bacterial species (Cochran-Stafira and von Ende, 1998). An emerging area of pitcher plant bacteria research is the formation of bacterial biofilms (Kolter, 2010).

Bacterial species living in the pitchers, have been found to be quite diverse between different *S. purpurea* communities. Although there may be differences in bacterial population between the pitchers on one plant, overall the bacterial population in one community can be distinguished from another (Peterson *et al.*, 2008). Studies of bacteria within *S. purpurea* pitchers are general analyses of species richness (Peterson *et al.*, 2008). *Escherichia coli* Mig. have been found in pitchers but not in the surrounding water, leading to the theory that insect vectors introduce the bacteria (Whitman *et al.*, 2005). Nitrogen-fixing bacteria have also been found, which leads to the suggestion that these bacteria may be a source of nitrogen for the plant (Prankevicius and Cameron, 1991). Mathematically, the number of nitrogen fixing bacteria found in some plants could provide more than enough nitrogen for the plant. However, the rate of nitrogen fixation by the bacteria, and how much of this nitrogen is taken up by the plant remains unknown (Prankevicius and Cameron, 1991).

In a study of *Sarracenia minor* Walter, a protease-secreting relative of *S. purpurea*, thirteen bacterial species were isolated by culturing and identified by DNA sequence (Juniper *et al.*, 1989; Siragusa *et al.*, 2007). This method does not account for viable, but nonculturable, bacteria, or rare species that would not have appeared in the cultures. Thus there are likely more species present than those identified in this study. Siragusa *et al.* (2007) suggest that the origin of some of the bacteria may be from the gut of captured insect, and they do not preclude the possible participation of fungi in prey digestion. The presence of a diverse bacterial population does not rule out the host plant as a contributor to insect digestion.

S. purpurea pitchers may also contain algae which, as alternate primary producers, are not usually considered a part of the inquiline community. Ellison reported to Gebuehr *et al.* (2006) that algae are not commonly found in North American *S. purpurea* pitchers. However, algae can be found in *S. purpurea* pitchers in North Western Ontario (pers. obs.). A study by Gebuehr *et al.* (2006) was conducted in Germany on *S. purpurea* which have an incomplete food web. When these plants were grown from seed in Europe, the invertebrates endemic to *S. purpurea* in North America such as *Wyeomyia smithii* Coquillett did not make the migration. Gebuehr *et al.* (2006) found algae from 78 different taxa growing in these European grown plants, with filamentous algae being particularly common. Gebuehr found that 25% of the total organic and 83 % of the living biomass came from algae (Gebuehr *et al.*, 2006). Photosynthesis by the algae would provide oxygen to the abundant bacterial population in the pitcher. Nitrogen-fixing cyanobacteria may provide additional reduced nitrogen to the host. It has also been speculated that the algae may leach nutrients into the pitcher fluid, adding further to growth of bacteria or the host plant (Gebuehr *et al.*, 2006). Conversely, the algae may absorb

the nutrients in the pitcher fluid, such as nitrogen, leaving less nutrients available to the host plant.

2.7 The role of prey in their own digestion

When considering the digestion of any prey, it must be remembered that there are other sources of hydrolytic enzymes than the predator. All cells contain lysosomal proteases and other hydrolytic enzymes for digesting cellular debris (Alberts *et al.*, 2002). Once the cell is damaged, the cellular mechanisms that kept these enzymes under control are lost, and the cell or entire tissue will start to digest itself. Another source of decomposition, in a multicellular organism, is the large array of digestive enzymes, and bacteria in the organism's digestive tract (Hickman *et al.*, 2001). This decomposition should also be considered as part of the digestion of prey in the pitcher of insectivorous pitcher plants. To my knowledge, the role of decomposition in the digestion of prey by pitcher plants has not been investigated. There are several invertebrates that are commonly caught by *S. purpurea*, this includes crickets which could be considered as a model prey. In studies where the gut of crickets was removed and tested for enzyme activity, the only proteases tested for were trypsin and aminopeptidase, and both were the most concentrated in the cricket's caeca (Biagio *et al.* 2009). The cricket gut was also found to recycle its enzymes and to have many membrane-bound hydrolytic enzymes (Biagio *et al.*, 2009). There are likely enzymes in addition to proteases in the gut of crickets that aid in decomposition of food and lead to a quicker absorption of nutrients through the gut epithelium. These enzymes include: amylase, maltase and lipase (Biagio *et al.*, 2009; Woodring *et al.*, 2009).

Studies on the bacterial population in the cricket gut show that the bacteria present are capable of digesting many different sugars, cellulose and gelatine but not lipids (Ulrich *et al.*, 1981). The gut commensal population also varies with the diet of the cricket (Santo Domingo *et al.*, 1998). It is likely that gut bacteria contribute to decomposing the cricket host once it has died. However, it is not known how long the bacteria survive in the pitcher as the host organism decomposes.

2.8 Plant roots and insectivorous pitchers

Since pitcher plants absorb nutrients through the internal epidermal cell layer of their pitchers, the inside wall of the pitcher is root-like in function. The pitcher is anatomically similar to roots in form as well, since the inside of the pitcher has a layer of suberin that also forms the Casparian strip in roots (Juniper *et al.*, 1989). Functional similarities between roots and insectivorous plant pitchers may exist as well. It was recently discovered that the roots of some plants secrete proteases and can use soil protein as a nitrogen source (Paungfoo-Lonhienne *et al.*, 2007; Godlewski and Adamczyk, 2007). The roots of many vascular plants have a symbiotic relationship with vesicular arbuscular mycorrhizae (VAM) (Hopkins and Hüner, 2004). These fungi extend the area available to the plant for absorption of nutrients. In return for the nutrients, the plant supplies the fungi with photosynthesis products and vitamins (Hopkins and Hüner, 2004). In some environments, there is a large amount of protein in the soil which is considered unavailable to plants without the help of VAM. To determine if angiosperms can secrete proteases from their roots, surface-sterilized seeds were germinated and grown hydroponically in sterile conditions without nutrients (Godlewski and Adamczyk,

2007). The culture medium of each plant was later tested for proteolytic activity. Several plants tested, such as onion, leek and common rue, showed proteolytic activity. In contrast, lettuce, grass lily and some others did not. Inhibition assays were also conducted on the culture media of the three most active plants. Cysteine proteases and aspartate proteases were found in all three samples, although the level of inhibition was different in each plant medium (Godlewski and Adamczyk, 2007). The pH optimum for most of the root proteases in this study was 7 (Godlewski and Adamczyk, 2007).

Paungfoo-Lonhienne *et al.* (2008) investigated to determine whether *Hakea actites* Barker, an Australian shrub which does not have VAM, but forms "root clusters," can use protein as a nitrogen source. *Arabidopsis thaliana* L. was used as a control plant, because it does not form either VAM or root clusters. Surprisingly, the finding was that both *H. actites* and *A. thaliana* could use protein as a nitrogen source when grown axenically on nutrient medium with protein as the only nitrogen source (Paungfoo-Lonhienne *et al.*, 2007). Two pathways may exist for the plants to take up the protein; the first is by proteolysis by extracellular protease, followed by absorption of the produced amino acids. The second may be by endocytic uptake of whole protein or peptide and subsequent proteolysis, within the lysosome of the plant cell (Paungfoo-Lonhienne *et al.*, 2007). The evidence for the first pathway has been found in *Sarracenia flava* L. pitchers in 1964, when these plants were fed with radioactive amino acids (Plummer and Kethley, 1964). More recently, it was confirmed that *S. purpurea* can bypass the inorganic nitrogen cycle and directly absorb radioactively labelled amino acids (Karagatzides *et al.*, 2009). This research suggests that protease secretion from plant roots may be more common than believed. Such pre-existing pathways could be the

evolutionary origin of secretory glands in some insectivorous plants. This suggestion is strengthened by the presence of suberin in both roots and pitcher epidermal cell walls.

2.9 Nepenthesin, a well studied protease of another insectivorous pitcher plant

There has not been much research into characterizing the proteases secreted by any *Sarracenia* spp. In contrast, the protease nepenthesin, from the *Nepenthes* spp. pitcher plants has been studied extensively and characterized well. Several species of *Nepenthes* were included, *N. macferlanei* Hemsley, *N. alata* Blanco and *N. ventricosa* Blanco. All species of *Nepenthes* are thought to secrete protease for prey digestion (Juniper *et al.*, 1989). The purified pitcher fluid from unopened and opened *N. macferlanei* was found to contain two proteases with a molecular weight of 59 and 21 kDa (Tökés *et al.*, 1974). The two proteases were apparently different gene products. The larger protease was named nepenthesin (Tökés *et al.*, 1974). Inhibition assays were conducted on nepenthesin and it was found to be similar to pepsin, indicating that nepenthesin is an aspartic protease. The peptides released by nepenthesin digestion were similar to those released by pepsin. A further similarity was that both pepsin and nepenthesin have a low optimal working pH (Tökés *et al.*, 1974).

More recently, the DNA sequences of secreted aspartic proteases NaAP1-NaAP5 were obtained using a primer based upon consensus sequences for plant aspartic protease (An *et al.*, 2002a). This genome level analysis showed the presence of five different aspartic protease genes. Their sequences were determined by reverse transcription PCR using degenerate probes followed by sequencing. Alignments of the sequences from the *Nepenthes* aspartic proteases with other known plant aspartic proteases showed that NaAP1-NaAP4 contained a

plant-specific insert while NaAP5, which was significantly shorter, did not (An *et al.*, 2002a). The expression of NaAP1-NaAP4 was investigated by detecting the mRNA in different parts of the plant. The highest levels of expression were in the lower part of open pitchers. NaAP1 and NaAP4 mRNA were specifically found to be present in *N. alata* glands, from which proteases are secreted (An *et al.*, 2002a).

Further research by An *et al.* (2002b) indicates the presence of other proteases in *N. alata* fluid. By examining the degradation of oxidized B-chain of bovine insulin over time and identifying the fragments at different times, the step-wise pathway for the substrate degradation was deduced (An *et al.*, 2002b). Based on this analysis, it is suggested that proteases other than aspartic ones are present and that exopeptidases may be present as well (An *et al.*, 2002b).

Nepenthesin was isolated by Athauda *et al.* (2004) from 30 L of fluid collected from *N. distillatoria* L. The purification process consisted of binding and separation on various DEAE-cellulose columns. Fractions exhibiting proteolytic activity (by digesting gelatine), were pooled and concentrated. The progress of purification was determined using SDS-PAGE staining with Coomassie brilliant blue (Athauda *et al.*, 2004). Two distinct proteins with molecular weights of 45-51 kDa and 35-45 kDa by SDS/PAGE that were given the names nepenthesin I and II were the result of the purification process. Based on amino acid sequence, the molecular weights of the proteins were 37.476 kDa and 37.511 kDa (Athauda *et al.*, 2004).

The purified proteins were used to develop probes for DNA sequencing and to prepare antibodies. The DNA sequence was used to build a phylogenetic tree and a theoretical 3D

model of the proteins. The phylogenetic tree showed that nepenthesin is part of a novel sub-family of aspartyl protease that is common in the plant kingdom, with genes for members of this subgroup found in *Arabidopsis*, corn, rice, and green algae (Athauda *et al.*, 2004). However, members of this subgroup appear to have a variety of functions and are active at a variety of pH optima. Nepenthesin is the only known member of this subgroup to be an extracellular protease and the functions of the other, presumably intracellular proteases are currently unknown (Athauda *et al.*, 2004). In the case of *Arabidopsis*, at least, the enzyme could be the putative root extracellular enzyme reported by Paungfoo-Lonhienne *et al.* (2008). Nepenthesin I and II 3-D models suggests many cysteine residues, leading to many disulphide bridges and an extremely stable structure (Athauda *et al.*, 2004).

The antibodies, made to recognize nepenthsin I, were used to track the enzyme within the plant. It was found that the enzyme was synthesized in the parenchyma cells surrounding the secretory glands. However, no protease activity was found in tissue extract, suggesting that active nepenthesin I does not accumulate in the gland tissue and is excreted quickly (Athauda *et al.*, 2004).

The main proteases found in *Nepenthes* pitcher fluid belong to the aspartyl family. However, cysteine protease has also been found by inhibition assays (Stephenson and Hogan, 2006). The aspartyl (NvAP1) and cysteine (NvCP1) protease genes were cloned. NvCP1 is 71% identical to a cysteine protease found in soybeans (Stephenson and Hogan, 2006). Although two proteases were cloned, there were 6 protease proteins with different molecular weights found in the fluid of different pitchers. The spectrum of proteases released did not appear to

change over time. Stephenson and Hogan (2006) suggested that the different proteases are the result of different circumstances of the plant during pitcher development. This is because pitchers develop one at a time and thus none are exactly the same age (Juniper *et al.*, 1989). The authors also speculate that post-transcriptional modifications of a mRNA transcript could result in multiple similar proteases, with somewhat different molecular weights (Stephenson and Hogan, 2006). None of the work on *Nepenthes* considered non-plant sources of enzymes.

N. verticosa pitcher fluid had activity for up to 22 days after opening without induction by prey (Stephenson and Hogan, 2006). This is much longer than the two weeks seen in *S. purpurea* (Gallie and Chang, 1997). Because of this, it has been suggested that *N. verticosa* and thus other members of the Nepenthesin producing genus have a different method of protease regulation than *S. purpurea* and other members of the *Sarracenia* genus.

There appear to be many differences between *Sarracenia* and *Nepenthes*. However, it seems unlikely that *S. purpurea* evolved to use a substantially different strategy for prey digestion. Thus, the research done on characterizing the proteases released by *Nepenthes* can be used as a guide for research on *S. purpurea* and other members of the genus *Sarracenia*.

2.10 Project Objectives

I set out to investigate the types of proteases released by *S. purpurea*. The first approach was to grow plants aseptically from surface sterilized seeds. Then the plants were to be induced with sterilized prey to secrete protease, which would be collected and tested for proteolytic activity. The second approach was to develop a batch tissue culture of *S. purpurea*, which could be induced and also tested for protease release *in vitro*. While the seedlings and

tissue cultures were growing, the protease activity and amino acid assays were developed using plants collected from the wild. These plants were washed out and given water or pitcher fluid. Some were given a model prey, a cricket. The same liquid and insect conditions were emulated in plastic sterile tubes, to eliminate the contribution of the plant pitcher. An attempt was made to investigate the relative contributions of the plant pitcher, inquiline population of bacteria, and the prey model organism to the proteolytic activity in this complex system. This was done by using iodoactamide, a cysteine protease inhibitor; pepstatin A, an aspartyl protease inhibitor; soybean trypsin inhibitor, a serine protease inhibitor; and ethylenediaminetetraacetic acid (EDTA), a metaloprotease inhibitor.

3.0 Seedling and Tissue Culture Development

3.1 Introduction

Since the inquiline community that lives inside the pitchers of *Sarracenia purpurea* has a role in prey digestion, these organisms must be removed in order to observe the digestive actions of the plant itself. Gallie and Chang (1997) used antibiotics to inhibit the growth of bacteria in the plants they tested. However, antibiotics do not necessarily inhibit the actions of pre-existing bacteria or endophytic fungi that may have grown inside the plant since germination (Juniper *et al.* 1989 and Hopkins and Hüner, 2004), or fungi entering from the surroundings. To ensure that there was no interference from fungi, bacteria or other organisms, *S. purpurea* were grown from surface sterilized seeds in aseptic conditions. However, *S. purpurea* is a slow growing species and takes at least two years in natural conditions to reach the size necessary to catch insect prey. Thus, a less ideal but potentially more expedient tissue culture model was also developed.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Plant Material and Collection

Seeds were collected from William's bog, located at 48°23'47.26"N, 89°17'53.99"W, near Lakehead University, in the fall of 2008. Whole *Sarracenia purpurea* plants were collected from the Goodfellow/Gibbard 3 peatland, located at 48°52'31.59"N, 89°58'43.93"W, about 100 km northwest of Thunder Bay, an area which has been used as a test bog for several research

projects concerning mercury levels in peat. During the early summers of 2009 and 2010, plants were collected from the bog and kept in the Lakehead University greenhouse. Plants were watered twice a week and kept at room temperature with a natural photoperiod.

3.2.1.2 Chemicals

Materials for surface sterilizing seeds and explants included commercial Clorox Bleach (Oakland CA) and Plant Preservation Medium (PPM) from Plant Cell Technology (Washington, DC, USA), along with hydrogen peroxide and sodium dodecyl sulphate (SDS) were from Sigma (St. Louis, MO, USA).

For germination and tissue culture medium, I used agar from DIFCO laboratories (Detroit, MI) and sucrose from Bio-Rad (Richmond, CA). Murashige and Skoog (MS), medium components, in the form of individual chemicals for the macronutrients and a powder for the micronutrients, along with the hormones kinetin and indole-3-acetic (IAA) acid were from Sigma (St. Louis, MO).

3.2.2 Aseptic Seedlings

Seeds were surface-sterilized before stratification which prepares the seeds for germination. The sterilization procedure consisted of a rinse in 70% ethanol, followed by a rinse with autoclaved ddH_2O and a one minute wash in 10% commercial bleach. Next, the seeds were rinsed four times with autoclaved ddH_2O. The surface sterilized seeds were placed between moistened autoclaved filter paper and enclosed in a Petri dish which was sealed with parafilm and stored at 4 °C for one month to achieve stratification.

Germination medium consisted of 1.5% MS medium (Murashige and Skoog, 1962), 1.5 mL/L PPM and 6 g/L agar, without added sucrose or hormones. Ten 720 mL glass mason jars were filled with 250 mL of germination medium and autoclaved. Once the agar had cooled and solidified, approximately 4 surface-sterilized and stratified seeds were placed on the agar surface of each jar. The jars were capped with glass Petri dishes and sealed with parafilm. The jars were placed in a growth chamber and given a 16 hour photoperiod, reaching a temperature of 34 ˚C during the day and cooling to room temperature during the night. The growth chamber was lit by a sodium lamp that provided approximately 540 $\mu mol/m^2/sec$ of light at plant level. After 8 months, the seedlings were given a dormancy period by being kept at +5 ˚C for 2 months and receiving low light for 8 hours a day. After the 8 months of growth in the growth chamber and 2 months of dormancy, some seedlings were transferred to 2.5% MS growth medium.

3.2.3 Tissue Culture

3.2.3.1 Tissue culture medium

Tissue culture medium consisting of 2.5 or 5.0% MS medium at pH 3.6, 5.6 or 6.6 with 1.5 mL/L PPM in 6 g/L agar was autoclaved. The pH of the solutions was adjusted with HCl and NaOH before the addition of agar and autoclaving. Once the medium was cool enough to handle, it was pipetted in 50 mL aliquots onto 90 mm diameter glass Petri dishes containing plant hormones. Hormones were aliquoted onto the plates, so that the final hormone concentrations would be 1, 4 or 8 mg/L of IAA and 0.25, 0.5 or 0.75 mg/L of kinetin. This resulted in a total of 54 plates, each with a different combination of hormones, pH and nutrient

concentration. The plates were stored in the laminar flow hood until they were inoculated with *S. purpurea* explants, 1 to 7 days later.

3.2.3.2 Explant preparation

Sarracenia purpurea plant rhizome and leaf petioles were rinsed with water and transferred to a laminar flow hood. In the flow hood, the 5 x 0.5 cm tissue sections were rinsed with 70 % ethanol and then with sterile ddH_2O. The explants were then placed in 10 % hydrogen peroxide for 5 min and again rinsed in sterile ddH_2O. Next, the tissue sections were bathed in 10 % bleach and 10 % SDS for 40 min. The tissue sections were then rinsed with sterile ddH_2O at least 3 times until all traces of the SDS (foaming) were gone. The large explants were cut into 5 mm by 5 mm pieces using sterile technique. Finally, the explants were soaked in 5 % PPM for 4 h and placed on tissue culture medium plates, which were then sealed with parafilm. The tissue culture plates were stored in the dark at room temperature and taken out of the dark once every two weeks for observations.

3.3 Results

3.3.1 Aseptic Seedlings

Growth of aseptic seedlings from surface sterilized seeds was slow and appeared to stop after several months (Fig 3.1 A and B). The seedlings grew into rosettes approximately 5 mm in diameter and 3 mm tall. After a two month dormancy period at 4°C, increased growth was not seen rather, some of the original small pitchers turned brown and new growth was initiated. Half of the seedlings were transferred to higher nutrient medium, 5 % rather than the 2.5 %

M&S medium. More growth was observed for most of the transferred seedlings (Fig 3.1 C). These seedlings grew into rosettes of about 6 cm in diameter and 3 cm tall after 17 months interrupted by a 2 month dormancy period.

3.3.2 Tissue Culture

Successful tissue culture callus growths are shown (Fig 3.2) and an overview of different callus inducing conditions attempted in this experiment is summarised in Table 3.1. Over the course of 4 months, even the largest callus did not reach more than about 5 mm in diameter. The greatest number of explants developed callus on pH 5.6 medium with 2.5% MS medium. Growth was also more likely when the hormone concentration was high on the kinetin scale, although high amounts of IAA were also more likely to induce callus than lower concentrations. The lower nutrient medium (2.5%) also supported more growth overall than the higher nutrient medium (5%). The medium at pH 6.6 induced very little growth and had the highest incidence of contamination.

3.4 Discussion

3.4.1 Sterile seedlings

The majority of the seeds germinated, the stratification and surface sterilization processes were successful, and aseptic seedlings were established. However, their growth was too slow to be useful in experiments. The expected time to maturity for greenhouse-grown *S. purpurea* seedlings is two years, longer than the time the seedlings were given to grow during this project. It is likely that their growth has been retarded by the artificial *in vitro* conditions.

To improve growth, the seedlings were given a dormancy period that was recommended for *S. purpurea* (Ellison, 2001). This did not appear to increase the seedling's growth rate, to the contrary, the older fronds turned brown and a new set had to re-grow (Fig 3.1 C). Perhaps the seedlings were not receiving enough nutrients. I did not move the seedlings to a higher nutrient medium earlier because the seedlings had minimal root development. I thought that as the nutrient medium immediately surrounding the seedlings was depleted of nutrients, the seedlings would grow roots to access nutrients further from the main plant body. *Sarracenia purpurea* and other insectivorous plants have notoriously little root growth. Thus, root growth may have been an unreasonable measure to determine when the seedlings needed more nutrients.

The growth rate of the seedlings could be improved by putting them onto higher nutrient medium or by periodically adding MS medium, without the agar, to the plants in their existing containers. The advantage of using whole new medium would be that the nutrient content would be known. However, removing the seedlings from their current medium might damage or destroy the little root growth they mustered and increase the likelihood of contamination. Medium added to existing jars would flow between the Mason jar walls and the agar, limiting the access of the seedlings to the nutrients. However, the nutrients would slowly diffuse through the agar. This would allow the seedlings to receive nutrients slowly, and avoid nutrient shock.

Another option would be to grow new seedlings without dormancy interruption. This would only take more time and further delay possible experiments on aseptic *S. purpurea*

seedlings grown in sterile conditions. As long as the seedlings do not exhibit signs of fungal or bacterial contamination, I do not recommend starting new ones. However, lack of visible contaminants would not preclude the presence of "subtle" symbiotic organisms growing in close association with the plant. The argument that such contaminants may exist in the aseptic culture will always be difficult to refute.

An alternative explanation for the slow growth of the aseptic seedlings may be that symbiotic relationships with unknown organisms develop in nature. Such heretofore unrecognized symbionts may contribute to accelerating the young seedling growth. Since mature pitchers clearly rely on prey and possibly symbiotic relationships this is not an unreasonable expectation in very young seedlings.

3.4.2 Tissue Culture

It appears that low pH, pH 3.6 and 5.6 and high hormone concentration is preferred by *S. purpurea* explants (Table 3.1). The low pH preference is not surprising as the peat filled bog water, in which *S. purpurea* grows, is usually acidic (Juniper *et al.* 1989). The nutrient parameter is interesting. Although there was more callus development with 2.5 % MS medium, the tissue growth was slow. Explants were given time to grow to 10 mm in diameter before subculturing (Fig 3.2). However, none of the calluses grew to that size. Because there was more growth in the 2.5 % medium (Table 3.1), nutrient content was not initially a concern. However, when the calluses showed signs of dying by turning brown, it became apparent that nutrient levels may be too low. Although the calluses were placed on new medium, they did not resume growth. This suggests that the calluses had already started to senesce and that nutrition was a

problem. The browning of the calluses suggests oxidation and possibly irreversible senescence (George *et al.* 2008).

There were no guidelines in the literature for callus development of insectivorous plants. Most of the tissue culture methods that have been developed for insectivorous plants are for propagation by cuttings, rather than callus growth for experimentation. There was a web site (worldofcarnivores.com, accessed 2009) for home propagation of insectivorous plants that was used as a guide for explant preparation. The extensive sterilization technique worked quite well, but there were a few problems with fungal contamination. I used an antifungal mixture called plant preservation medium (PPM), which is a combination of antifungal agents designed to minimize the impact of the antifungals on the plant, and to maximize fungal inhibition. In the short term, this worked quite well. However, as the tissue cultures grew older, there was an increased likelihood of observable contamination. This may have been because the PPM components degraded while the explants were growing. As the PPM became less effective, the fungi became less inhibited and more fungal growth was observed. Alternatively, the senescing calluses provided ideal medium for fungal growth. *S. purpurea* is considered very difficult to grow in sterile culture, due to its fungal endophyte (Juniper et. al 1989). Because this endophyte grows inside the tissue of the plant, killing or removing the fungi without harming the plant tissue is very difficult. Conversely, this endophyte may be the mycorrhizae-like symbiont required for rapid seedling growth.

3.4.3 Improvements and follow up experiments

Tissue culture development of *S. purpurea* should be repeated with some modifications. The pH range could be narrowed to include only pH 5.6 and 3.6. Only the highest concentrations of hormones, 0.75 mg/L cytokinin, paired with the whole range of auxin concentrations and 8 mg/L auxin paired with the whole range of cytokinin concentrations could be used, as the pairings at low concentrations did not result in callus growth. The range of nutrient concentrations could be increased, rather than decreased, because of the uncertainty of the nutrient component. The callus forming on the explants could be subsultured once every two months to fresh medium instead of testing a range of nutrient contents. In order to reduce contamination, I would suggest using PPM that is not near its expiration date and being vigilant in using aseptic technique. With a smaller range of pH and hormone concentrations, the number of tissue culture plates would be reduced, allowing a greater number of replicates.

Table 3.1. Tissue culture observations. Clear blocks indicate a lack of callus development and dark blocks indicate cultures lost due to contamination introduced with the explant. + indicates observed swelling and ++ indicates callus growth.

PH 3.6

2.5% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1			++ (Fig 2 B)
4			++
8		++	

PH 3.6

5.0% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1			++
4			
8		++ (Fig 2 A)	

PH 5.6

2.5% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1		+	++
4			+
8	++ (Fig 2 D)	+	++ (Fig 2 C)

PH 5.6

5.0% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1			+
4			+
8			

PH 6.6

2.5% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1			
4		++	
8			

PH 6.6

5.0% M&S

Auxin (mg/L)	Cytokinin (mg/L) 0.25	0.5	0.75
1			
4			
8			

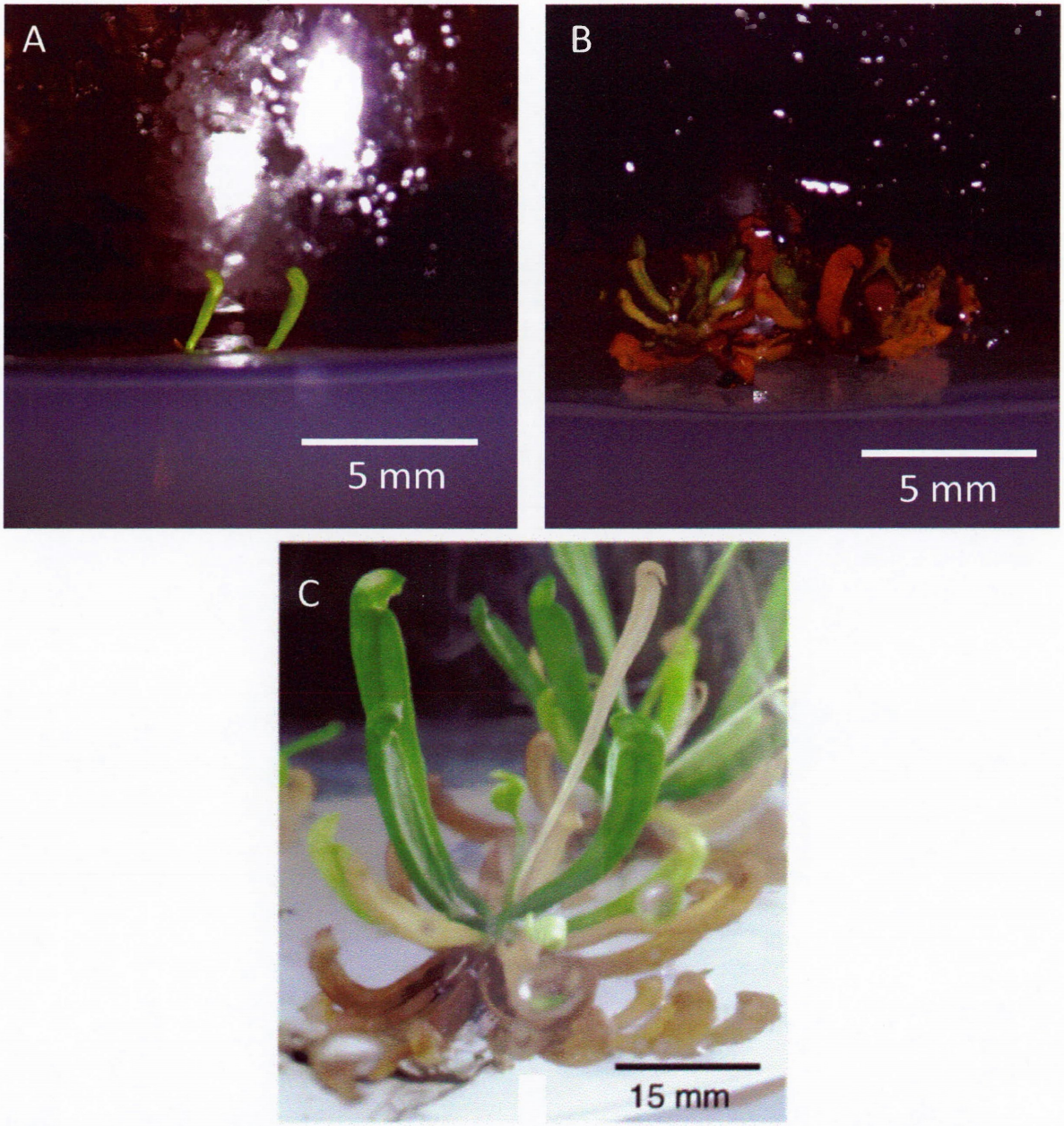

Figure 1: Sterile seedling growth over several months. (**A**) shows newly germinated seedlings at 3 months. (**B**)shows seedlings at 8 months. Finally, (**C**) shows seedlings after 17 months and after transfer onto higher nutrient medium.

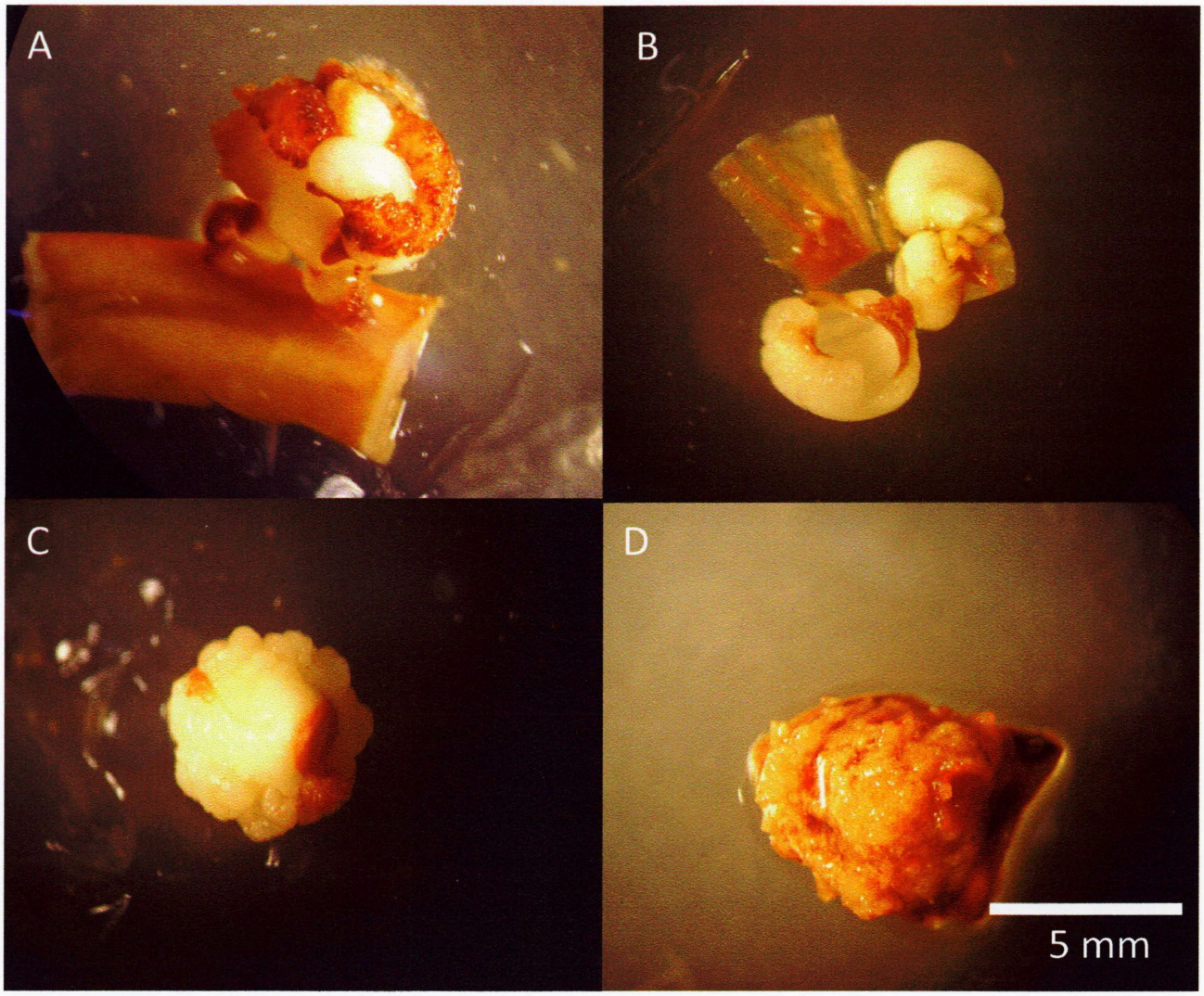

Figure 3.2: *Sarracenia purpurea* tissue culture callus grown over 4 months on different combination s of nutrient concentration, pH and hormones. Callus grown on (**A**) 5.0% Murashige and Skoog (M & S) media, pH 3.6 with 8 mg/L indole-3-acetic acid (IAA) and 0.5 mg/L kinetin. (**B**) 2.5 % M & S, pH 3.6 with 1 mg/L IAA and 0.75 mg/L kinetin. (**C**) 2.5% M & S, pH 5.6 with 8 mg/L IAA and 0.75 mg/L kinetin. (**D**) 2.5% M & S, pH 5.6 with 8 mg/L IAA and 0.25 mg/L kinetin.

4.0 Pitcher fluid experiments

4.1 Introduction

While the model *Sarracenia purpurea* plants and tissue cultures were growing, whole wild *S. purpurea* plants were used to develop methods for characterizing protein digestion occurring within *S. purpurea* pitchers. This consisted of determining caseinolytic protease activity and protein and amino acid content of the pitchers over time and under different conditions. This included controls which removed the influence of the plant by using a plastic centrifuge tube as an artificial pitcher, the presence or absence of a cricket as prey, and the presence of water rather than pitcher fluid.

Casein is a protein that can be hydrolysed by many different proteases and is a common protein used in protease assays, when bonded to fluorescein isothiocyanate (FITC-casein) (Twinning, 1984). As the casein is hydrolysed, fluorescein is released and can be detected by a fluorometer. Two different methods of detecting casein hydrolysis were used during these experiments. The first was a newer method, in which the reaction and fluorescence reading takes place on a micro plate. Low detection of fluorescein release prompted concerns regarding the pH during the reaction. Fluorescence of fluorescein is very sensitive to pH, with maximum around pH 8.5 (Twinning, 1984). Changing the pH of the reaction required a different method, in which the pH could be changed, before the reading. This was possible with the shaker-stir bath method, which more closely resembles the original FITC-casein assay by Twinning (1984), where the reaction occurs in micro tubes and the reaction is stopped before the fluorescence is read. When the reaction is stopped, there is an opportunity to change the pH from the enzyme reaction optimum to the fluorescein reading optimum.

The protein content of the experimental pitchers and tube controls was measured to form a basis for comparison of protein content and specific caseinolytic activity. Most proteolysis assays suffer from competitive inhibition if high background protein is present as a potential substrate. The amount of free amino acids in the pitchers showed the availability of amino acids for absorption by the plant and the progress of protein digestion. I expected that monitoring caseinolytic activity, protein, and amino acid content would give some information about the dynamics of cricket protein digestion.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Plant Material and Collection

During the summer of 2009 and 2010, *Sarracenia purpurea* plants were collected from the Goodfellow/Gibbard 3 peatland, located at 48°52'31.59"N, 89°58'43.93"W, about 100 km northwest of Thunder Bay. The collected plants were kept in the Lakehead University greenhouse. They were watered twice a week and kept at room temperature with a natural photoperiod.

Fluid collected from *S. purpurea* pitchers in William's bog, located at 48°23'47.26"N, 89°17'53.99"W, during the spring of 2009 was filtered and concentrated. The fluid from approximately 20 pitchers was pooled and filtered with suction through a Whatman 1 filter (11µm pore size) followed by a Whatman GF/C filter (1.2 µm pore size) to remove debris. The final filtration step was through an autoclaved Millipore 0.22 µm pore filter into a sterile flask. This fluid sample was concentrated by ultrafiltration (NMWL 5 kDa) to 18.75 times its original

concentration. This concentrated pitcher fluid was stored at 4 °C and used for the caseinolytic activity assay optimization experiments.

4.2.1.2 Other materials

Crickets were obtained from Pet Smart (Thunder Bay) and were killed by freezing. Protein content was determined using Protein Assay Dye Reagent Concentrate from Bio-Rad (Hercules, CA, USA) (Bradford, 1976). Components of the ninhydrin solution, ninhydrin, hydradantin, DMSO and the components of the sodium acetate buffer were purchased from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate-labeled casein (FITC-casein) and inhibitors: iodoactamide, pepstatin A, soybean trypsin inhibitor, and ethylenediaminetetraacetic acid (EDTA) were also Sigma products. Tris(hydroxymethyl)aminomethane, was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.2.1.3 Instruments

Absorbance and fluorescence were detected using a BMG FLUOstar OPTIMA plate reader for the protein and caseinolytic activity assays. The absorbance for the amino acid assays was read using a Bio Tek Synergy HT plate reader. The 96 well micro plates used for the protein and amino acid assays were Corning 96 well UV micro plates (Cat# 3635) and for the caseinolytic assays, Corning 96 well solid black polystyrene micro plates (Cat# 3650). Two microcentrifuges were used during sample preparation; an Eppendorf centrifuge 5410 was used for samples at room temperature and a Fischer Scientific accuSpinmicro R was used for samples at 4 °C.

4.2.2 Pitcher experimental conditions

A summary of treatments is given in Table 4.1 (page 73). Three healthy mature *S. purpurea* plants with 4 intact pitchers each were chosen as replicates. Pitcher fluid was collected and combined from all pitchers in the experiment and pitchers were washed out with ddH_2O. For each plant, the 4 pitchers were given different experimental conditions that were randomly assigned. The first was 10 mL of ddH_2O and a frozen/thawed cricket (PW+); the second was given only 10 mL ddH_2O (PW-); the third was given 10 mL of the collected pitcher fluid and a frozen/thawed cricket (PF+); and the fourth was given only 10 mL of pitcher fluid (PF-). Controls consisting of 10 mL of pitcher fluid or ddH_2O with a frozen/thawed cricket placed in a 50 mL plastic centrifuge tube (TW+, TF+ and TF-) were also replicated 3 times, and one replicate was placed with each plant. A fourth non plant control containing an autoclaved frozen/thawed cricket in 10 mL of autoclaved water in a sterile 50 mL tube (TW+*) was replicated 3 times and stored in a laminar flow hood. The test plant pitchers and tubes were covered with plastic wrap, secured with a rubber band to limit evaporation and contamination. Every 2 to 3 days, 1 mL of pitcher fluid was collected after gentle mixing with a sterile pipette. The unfiltered samples collected were used for determining caseinolytic activity as well as protein and free amino acid content.

4.2.3 Caseinolytic Activity Assay

4.2.3.1 Caseinolytic activity assay method 1, the micro plate method

To set up the reactions using the micro plate reader method, 80 µL of unfiltered pitcher fluid sample was aliquoted in triplicate onto 96 well micro plates. To create sample blanks, 300 µL of each sample was boiled for 15 minutes and plated in triplicate. Immediately before being

placed onto the plate reader, a reaction mixture of 5 µL 1% FITC-casein, 5 µL ddH2O and 10 µL 0.5 M Tris buffer, pH 8.5 was added to each well and was immediately placed into the reader, which recorded fluorescence of each well with an excitation at 360 nm and emission at 460 nm at ten min intervals from time zero to sixty min. The amount of fluorescein released was determined using a standard curve from 0 to 50 nM fluorescein. For inhibition assays using the first method, 70 rather than 80 µL of pitcher fluid sample was aliquoted, in triplicate for each inhibitor and control. Each well received 10 µL of inhibitor: 0.8 mM iodoactamide (a cysteine protease inhibitor), 0.01 mM pepstatin A (an aspartyl protease inhibitor), 8 mM soybean trypsin inhibitor (a serine protease inhibitor), 8 mM EDTA (a metaloprotease inhibitor). Some wells received 10 µL of ddH_2O or DMSO as solvent controls. The well contents were mixed by pipette and allowed to pre-incubate at room temperature (RT) for at least 1 h. The reaction mixture with the FITC-casein substrate was added and the plate read as above.

4.2.3.2 Caseinolytic activity assay method 2, the shaker-stir bath method

The experiment was performed in 1.5 mL microcentrifuge tubes, as above in the micro plate method, with the addition of a 0 time blank. The tubes were vortexed and allowed to pre-incubate at RT for at least an hour. After the pre-incubation, a reaction mixture of 5 µL 1% FITC-casein, 5 µL ddH2O and 10 µL 0.5 M Tris buffer, pH 8.5 was added to each tube, for a total 100 µL. Each tube was quickly vortexed and then placed into a shaking water bath at 37 ˚C except for the time zero control. The reaction in this tube was stopped immediately by the addition of 70 µL 100% TCA (Sambrook and Russell, 2001) and 180 µL 0.5 M Tris-HCl buffer (pH 8.5) and vortexed and placed in the water bath at 37 ˚C. The remaining reactions were stopped after 60 min as above, and all reactions were cooled to 4 ˚C for at least 1 h. Contents were centrifuged

at 4 ˚C for 10 min at 12 800 g. Finally, 100 µL of each supernatant was aliquoted in triplicate onto a 96 well black polystyrene micro plate. The fluorescence of each sample, with an excitation at 360 nm and emission at 460 nm was recorded. The amount of fluorescein released was determined using a standard curve from 0 to 50 nM fluorescein. To verify time-dependent progress of the reaction, additional reactions were stopped at 15, 30 and 45 min.

4.2.3.3 Caseinolytic activity assay pH optimization

Protease activity assays using the second method were carried out on concentrated pitcher fluid using different buffers during the reaction. The concentrated pitcher fluid used in these experiments is described in section 4.2.1.1. The buffers used were 400 mM Tris-HCl buffer (pH 8.5), 400 mM monosodium phosphate-disodium phosphate buffer, pH 7 and 400 mM sodium acetate buffer, pH 5. The reaction was stopped using 180 µL 400 mM Tris-HCl buffer (pH 8.5) and 70 µL 100% TCA. For determining the concentration of fluorescein released, new standards were also prepared using the different buffers. For example, the fluorescein released in the reactions that occurred at pH 5 with the sodium acetate buffer was determined based on standards that been prepared at pH 5 with the same sodium acetate buffer and also received the same 180 µL 400 mM Tris-HCl buffer (pH 8.5) and 70 µL 100% TCA and other processesing before fluorescence reading.

4.2.4 Protein Assay

Protein content was measured by Bradford assay with BSA as a standard (Bradford, 1967). A 10 µL of unfiltered sample was mixed with 200 µL diluted Protein Assay reagent (1:5 dilution) in triplicate on a clear 96 well UV micro plate. After a 15 min reaction, the absorbance was read at 595 nm and compared to a BSA 0 to 5 mg/mL standard curve.

4.2.5 Amino Acid Assay

Total amino acid content of samples was determined by a downsized modification of the method described by Sun et al. (2006). The ninhydrin solution was made by dissolving 0.2 g ninhydrin and 0.03 g hydradantin in 7.5 mL DMSO. Once the solutes had completely dissolved 2.5 mL 4 M sodium acetate buffer, pH 5.2 was added. The ninhydrin solution was then gassed with N_2 for at least 2 minutes. The ninhydrin solution was kept at 4 °C and used the same day it was made. The samples were prepared by removing the protein from the samples by adding 25 µL 100% TCA to 100 µL sample. These were cooled at 4 °C for 1 h, then centrifuged for 10 min at 4 °C and the supernatant, about 100 µL was collected. To this, 100 µL of ninhydrin solution was added. Then the mixture was heated in a boiling water bath for 10 min and cooled on ice. Then 250 µL of 50% ethanol was added to the amino acid solution and vortexed. Aliquots of 100 µL were distributed in triplicate onto a clear 96 well UV micro plate and absorbance was read at 570 nm. Amino acid concentration was determined from a standard curve of absorbance for amino acid mixtures (consisting of 20 "protein" amino acids in equal concentration) from 0 to 500 µM. The amino acid mixture for the standard was created by making stock solutions of each amino acid at a known molarity and combining them in equal amounts to create a master mix with an overall amino acid concentration of 500 µM that was diluted to make the standard curve.

4.3 Results

4.3.1 Caseinolytic assay pH optimization

Due to the standards at different pHs not going to a high enough concentration (zero to 100 ng fluorescein released), determining the absolute amount of fluorescein released past the

first 30 min was unreliable (Fig 4.1). However, the relative amount of fluorescein released at the different pHs showed that the activity decreased with pH and that pH 8.5 was optimal (Fig 4.1).

4.3.2 Caseinolytic activity assay method comparison

When the concentrated pitcher fluid was tested for caseinolytic activity at pH 8.5, using both the micro plate and shaker-stir bath methods, the detected amount of fluorescein released was much greater with shaker-stir bath method (black line) than when the micro plate method was used (grey line) (Fig 4.2 A). The boiled and the non-boiled concentrated pitcher fluid both had caseinolytic activity detected by the micro plate method (Fig 4.2 A, black dotted and grey dotted lines). However, less caseinolytic activity was observed than when the shaker-stir bath method was used. Since the total fluorescein released was measured as the difference between the amount released in the non-boiled sample and the release that occurred in the boiled sample, the total release near zero (Fig 4.2 A, grey line). The time zero blank for the micro plate method contained 116 ng of fluorescein while the shaker-stir bath method time zero blank only contained 6 ng.

Inhibition assays were conducted using an inhibitor of each protease family (aspartyl, cysteine, serine and metalo proteases) to determine the type of proteases present. More inhibition was detected when the shaker-stir bath method was used (Fig 4.2 B). Almost 80% inhibition, seen in the presence of trypsin inhibitor from soybean when the shaker-stir bath method was used, was compared to only about 10 % inhibition by trypsin inhibitor from soybean, when the micro plate method was used (Fig 4.2 B). There was also twice the amount of inhibition seen in the presence of EDTA and a little less than 10 % inhibition by iodoactamide

was detected rather than none. However, less rather than more inhibition was seen in the presence of pepstatin A, where there was around 10% inhibition detected using the micro plate method and less than 5% when using the shaker-stir bath method.

The two methods may be compared by the amount of caseinolytic activity detected from the full range of pitcher plant and artificial plant experiments, with the micro plate method (Fig 4.3) or the shaker-stir bath (Fig 4.4 C and Fig 4.5 C) method. In the micro plate method, fluorescein released was in the pg/μg protein range, while in the shaker-stir bath method, the fluorescein released was in the ng/μg protein range. The shaker-stir bath method was adopted in later experiments because of these comparisons. The difference in standard error shown on the two figures is due to the micro plate method (Fig 4.3) using only three replicates, while the shaker-stir bath method (Fig 4.4 C and Fig 4.5 C) is the accumulation of six replicates.

4.3.3 Pitcher plant experiments

The amount of protein, amino acid and caseinolytic activity increased in the presence of a cricket (Fig 4.4). Pitchers that did not contain a cricket (PW- and PF-, light grey and black bars), consistently had low protein and amino acid content, as well as low caseinolytic activity as compared to pitchers which received a cricket (Fig 4.4 A, B, white and dark grey bars). The protein content in pitchers with a cricket (PW+ and PF+) remained higher throughout the experiment than in pitchers without a cricket (Fig 4.4 A). However, the protein content of pitchers with the original fluid and cricket (PF+, dark grey, Fig 4.4 A) decreased to 0.02 mg/mL from day 6 to 15 compared to about 0.07 mg/mL protein concentration in pitchers with only water and cricket remaining constant, between days 6 and 15 (Fig 4.4 A, white bars).

The amino acid content of pitchers with water and a cricket (PW+, white bars) also remained fairly constant at 60 μM, while the concentration was about double in the pitcher with the original fluid and cricket (PF+, dark grey bars), but highly variable as seen from the large error bars (Fig 4.4 B). The amino acid concentration in pitchers without a cricket remained low, near the detection limit of the ninhydrin assay (PW-, light grey bars and PF-, black bars) (Fig 4.4 B).

The caseinolytic activity of pitchers flushed with water and receiving a cricket (PW+, white bars) was initially high, but decreased over time (Fig 4.4 C). The opposite was seen in cricket-induced pitchers retaining the original pitcher fluid (PF+, dark grey bars), where the activity was relatively low at first and then rose over the course of the experiment (Fig 4.4 C). However, this activity was highly variable among individual pitchers, as reflected in large standard error bars. The pitchers not receiving a cricket (with either water or pitcher fluid) initially had low caseinolytic activity, which disappeared by 9 to 12 days (Fig 4.4 C, light grey and black bars).

4.3.4 Artificial pitcher plant experiments

The "artificial pitcher" experiments were included as a control, in the sense that the long term contribution of the pitcher was eliminated. The sterile water experiments with a cricket (TW+) measured the contribution of the cricket to the system (Fig 4.5, white bars). Similar treatment with the autoclaved cricket (TW+*) provided an indication of the protein and amino acid contribution by the cricket in the presumed absence of any proteolytic activity (Fig 4.5, light grey bars). Dark grey bars in Fig 7 represent a situation where only initial pitcher fluid interacted with the cricket, without the longer-term contribution of the pitcher. The last

treatment evaluates the relative contribution of the cricket to the pitcher fluid (TF-) system (Fig 4.5, black bars).

As in plant pitchers (Fig 4.4), the tube experiments demonstrated the large contribution of the cricket to the system (Fig 4.5). The tubes which contained pitcher fluid but did not contain a cricket (TF-, black bars) had low amounts of protein and amino acids (Fig 4.5 A, B). The autoclaved cricket in a tube (TW+*, light grey bars) released lower amounts of protein relative to the other treatments, but had concentrations of amino acids similar to other tubes containing the cricket (Fig 4.5). Furthermore, tubes which contained water or fluid, along with a cricket (TW+ and TF+, white and dark grey bars), had similar and relatively consistent concentrations of both protein (0.15 mg/mL) and amino acids (300 μM), although the amount of amino acids in the tube with pitcher fluid (TF+) was about double the water control (TW+) on day 6 (Fig 4.5 B).

Regarding caseinolytic activity (Fig 4.5 C), tubes with pitcher fluid (with and without a cricket, TF+ and TF-, dark grey and black bars), had very high activity on day 3 that tapered off over the course of the experiment (Fig 4.5 C). Tubes with water and a cricket (TW+, white bars) consistently had low caseinolytic activity, while similar treatment with autoclaved cricket (TW+*, light grey bars) had the highest level of activity during the middle of the experiment (Fig 4.5 C). However, similar to the activity in the natural pitchers (Fig 4.4 C), there was a high degree of variability among individual tubes (Fig 4.5 C).

The protein and amino acid content were low throughout the experiment in both pitchers and tubes with only pitcher fluid (Fig 4.4 A, B and Fig 4.5 A, B, black bars), although some variability was detected. The caseinolytic activity was similar in that there was

caseinolytic activity of about 30 ng fluorescein released per μg protein on the first day of testing, but this activity decreased over the course of the experiment in both pitchers (Fig 4.4 C) and tubes (Fig 4.5 C), although there was an increase in caseinolytic activity on the final day of testing in the tubes (Fig 4.5 C, black bars) but no such increase was noted in the pitcher (Fig 4.4 C, black bars).

A comparison must be made between a tube which contained pitcher fluid and a cricket (TF+), with a plant with contained pitcher fluid and a cricket in the pitcher (PF+). The protein content of tubes with a cricket (Fig 4.5 A, dark gray bars) was much higher than that of pitchers with a cricket (Fig 4.4 A, dark gray bars), where it remained at about 0.05 mg/mL throughout the experiment. In the tubes, however, the protein content was initially around 0.075 mg/mL, but stabilized at around 0.15 mg/mL, three times higher than in the plant pitchers (Fig 4.4 A and Fig 4.5 A, dark grey bars). The amino acid content was also very different, with the amino acid in the pitchers measuring around 100 μM (Fig 4.4 B, dark grey bars), with little change over time, compared to the tubes, in which the amino acid content was slightly less than 400 μM on the first day and decreased to 225 μM over the course of the experiment (Fig 4.5 B, dark grey bars).

The caseinolytic activity was very different between the pitchers with a cricket and tubes with the cricket. The caseinolytic activity in the tubes started relatively high and decreased over the course of the experiment (Fig 4.5 C, dark grey bars). The opposite was seen in the pitchers, where the caseinolytic activity was moderate during the first 9 days of the experiment but rose to nearly 100 ng fluorescein per μg protein on the final two days of testing (Fig 4.4 C, dark grey bars). Other than on the first day of testing, there was more caseinolytic

activity seen in the pitchers with a cricket than in the tubes with a cricket throughout the experiment (Fig 4.4 C and 4.5 C, dark grey bars), about 3X more an days 6 and 9, and up to 10X more on day 15.

A comparison of the pitcher plants with water and a cricket, and tubes with water and a cricket, revealed that the protein and amino acid content of the tubes was about three times higher than that of the plants (Fig 4.4 A, B and Fig 4.5 A, B, white bars). The increase in amino acid content was up to seven times higher on day 9 in the tubes (Fig 4.4 B and Fig 4.5 B, white bars). The caseinolytic activity of tubes containing water and a cricket was low throughout the experiment, just above the detection limit, until the final day of testing when it rose to about 20 ng fluorescein per µg protein (Fig 4.5 C, white bars). In contrast, the pitchers with water and a cricket had the highest caseinolytic activity detected in the entire experiment on the first day of testing (day 3), which decreased but remained at or above the caseinolytic activity measured in the tubes with water and a cricket (Fig 4.4 C and Fig 4.5 C, white bars).

4.3.5 Inhibition experiments

The inhibition of serine proteases (trypsin inhibitor sensitive) was very high throughout the experiment (Fig 4.6 A to E) except for a tube with sterile water and cricket after 20 days (Fig 4.6 C, black bar).

Inhibition of metallo proteases (EDTA sensitive) was between 25% and 60% in the cricket containing pitchers (PW+ and PF+, Fig 4.6 A and B) and the tube containing water and a cricket on day 3 (TW+, Fig 4.6 C) and at least doubled by day 20 in all tubes (Fig 4.6 A to E) except for the tube containing water and an autoclaved cricket, where the inhibition was almost complete on day 3 (Fig 4.6 D). The tubes containing pitcher fluid and a cricket (TF+)

showed the most dramatic change in metalloprotease inhibition between days 3 and 15 (Fig 4.6 E).

Aspartyl protease inhibition (pepstatin A sensitive) was low throughout the experiment, with the exception of pitcher fluid on day 3 (Fig 4.6, B and E). The enzymes in pitcher fluid on day 3 were inhibited by pepstatin A more than 60% on day 3, but this inhibition diminished to less than 25% on day 20 (Fig 4.6, B and E).

Inert tubes (Fig 4.6, C, D and E) which would not be a source of any enzyme, revealed no inhibition of cysteinyl protease by IAA at 3 days, but high inhibition by day 20. This was irrespective of whether they contained water or pitcher fluid. No cysteinyl protease was inhibited even in tubes with pitcher fluid and a cricket, which had high specific caseinolytic activity on day 3 (Fig 4.5 C, dark gray bar). The common denominator in C, D, and E was the presence of a cricket, even an autoclaved one (Fig 4.6 D).

4.3.6 Statistical analysis

The data was compared in terms of standard error on a case by case basis for the results and discussion. For example, I considered the caseinolytic activity on the first test day in the plants to be the same for PW-, PF+ and PF- (Fig 4.4 C, light grey, dark grey and black bars) since the standard error of each overlap the standard error of the others. The activity of PW+ was higher than the rest on that first test day (Fig 4.4 C, white bars). The standard error of PW+ did not have any overlap with the standard error of the other treatments. Another situation is shown on the first day in the tubes. The caseinolytic activities in TW+ and TW+* (Fig 4.5 C, white and light grey bars) are much lower than in TF+ and TF- (Fig 4.5 C, dark grey and black

bars). However, TF+ and TF- could also be considered to be the same since the lower standard error of TF+ overlaps the upper standard error range of TF- (Fig 4.5 C, dark grey and black bars).

4.4 Discussion

4.4.1 Caseinolytic activity assay optimization

Method development was a major component of my research. Since plant proteases were ultimately the main focus, optimizing the protease activity assay was very important. However, only small amounts of fluorescein released were detected in the initial experiments, which suggested that there was little specific caseinolytic activity, or that the assay was not working. For this reason, I performed tests to determine the optimum pH for specific caseinolytic activity in pitcher plant fluid. The pH of pitcher fluid is in the acid range, around 3.8 (Juniper *et al.* 1989) and I expected the pH optimum for the enzymes in the pitcher fluid to be acidic as well. However, the optimum pH for hydrolysis of casein found by Twinning *et al.* (1984), and the pH used in my initial experiments (Fig 4.3) was 8.5. The specific caseinolytic activity tests at pH 5, 7 and 8.5 indicated that pH 8.5 was the optimum (Fig 4.1). Pitcher plant fluid is a complex mixture, that is likely to contain many different enzymes which may cover a range of pH optima.

During the pH optimization tests, I made new fluorescein standards at pH 5 and 7. The range of fluorescein standards was based on the range seen in the initial experiments, using the micro plate method (Fig 4.3), so the range of new standards, made for pH 5 and 7, only went from zero to 100 ng of fluorescein. However, after 30 min of the 60 min incubation, more than 100 ng of fluorescein was released, which meant that the standard at pH 5 and 7 was unreliable

after 30 min. This indicated that the shaker-stir bath method used for the pH optimization tests might yield better results than the micro plate method.

The reaction conditions in the shaker-stir bath and the micro plate method were identical, with the same volume of enzyme mixture, buffer and FITC-casein substrate and with the same temperature maintained throughout the reaction. The two methods differ in blank preparation and the processing of the sample after the reaction. The final volumes of the reactions in the shaker-stir bath method were greater than the micro plate method due to addition of TCA and Tris-HCl for protein removal. The standards used to determine fluorescein released in the shaker-stir bath method also received this treatment, making the results from the shaker-stir bath method comparable to the micro plate method.

The blanks used in the micro plate method did not work. To prepare the blank, I boiled an aliquot of each sample for 15 min to denature the proteins in the sample. This was so that background fluorescence could be detected and separated from the actual fluorescein released by non-boiled samples. However, I was not successful in denaturing the proteins. The boiled, concentrated pitcher fluid had more specific caseinolytic activity than the non-boiled sample, by the end of the 60 min (Fig 4.2, black dotted line and grey dotted line). This meant that there appeared to be no fluorescein release and resulted in the extremely low total fluorescein release calculated in the initial experiments (Fig 4.3). Since the amount of fluorescein released, in boiled and non-boiled samples, was so similar, the total release reported was extremely small. The blank created in the shaker-stir bath had proteins chemically denatured and did not appear to interfere with the total amount of fluorescein release calculation.

In the shaker-stir bath method, after the reaction, the reaction was stopped and the protein in the sample was removed by TCA, before measuring fluorescein. Neither of these steps occurred in the micro plate method. The time zero blank for the micro plate method contained 116 ng of fluorescein, while the shaker-stir bath method time zero blank only contained 6 ng. The extremely high blank of the micro plate method suggests that either the excitation or emission wavelengths could have been obscured by protein in the samples. The removal of the protein in the shaker-stir bath method may have allowed more of the released fluorescein to be detected.

The inadequate blank and the potential interference by protein may be the cause of the differences between the results obtained by the two methods when conducting inhibition assays, and may have made the samples appear to be less inhibited than they actually were (Fig 4.2 B), than when I used the micro plate method. I used the shaker-stir bath method, at pH 8.5, for the rest of my experiments because this method provided a reliable blank, avoided optical interference by protein, and was more similar to the original method by Twinning (1984) than the micro plate method.

I found greater standard error in the results of the shaker-stir bath method than when I used the micro plate method (Fig 4.3, 4.4 C and 4.5 C). The higher values measured give a broader range of values to be included in the error calculation. Also, the micro plate data (Fig 4.3) calculation is based on 3 replicates, while the data for the shaker-stir bath method (Fig 4.4 C and 4.5 C) was the result of 6 replicates. Unfortunately, there was not enough of the concentrated pitcher fluid, which I used in the optimization test, to repeat these experiments

for error comparison. However, I do not think that the increased standard error from the shaker-stir bath method was due to the method, but rather was caused by the natural variability of the complex pitcher plant system.

4.4.2 Pitcher fluid experiments

I used the data in Figures 4.4 and 4.5 to determine and evaluate the relative contributions of the various biological components to the protein hydrolytic process. In nature, a complete system for prey digestion by *S. purpurea* consists of a plant pitcher filled with pitcher fluid and trapped insect prey, with introduced microbial populations and possibly other inquiline organisms. Each of these components is a potential source of protease. The plant pitcher may secrete protease, which was the main focus of this study. Finally, the insect prey itself had proteases in its own cells and its digestive system. Protease producing bacteria also enter the pitcher either directly from air, or on the bodies of prey or inquiline organisms.

In this study, I attempted to control these sources of protease to determine the relative contribution of each. The transfer of existing pitcher fluid into the sterile test tubes eliminated further contribution by the plant. The use of sterile water, rather than pitcher fluid, reduced the amount of bacteria and other inquiline organisms in the plants or tubes, although complete removal of pitcher fluid from the plant pitchers could not be certain. The inclusion of the cricket provided a potentially enzyme inducing condition for the plant, was a food source for inquiline bacteria, and introduced additional bacteria on and in the body of the cricket. I used an autoclaved cricket, in which the enzymes would be expected to be denatured, and the bacteria killed by heat, as a control. The following discussion is divided into sections dealing with

specific variables, starting with the simplest "control" scenarios (4.4.2.1) to the most complex, involving the role of the plant pitcher (4.4.2.4).

4.4.2.1 Water in tube or autoclaved cricket only

The water by itself in a tube and the autoclave cricket in a tube with water (TW+*) represent controls, in which I did not expect proteolytic activity to exist. I did not perform a sterile water in a tube control since, double deionised water is not expected to contain protein or amino acids, or to exhibit any specific caseinolytic activity. I used the autoclaved cricket in the tube with sterile water as a comparable control, assuming no enzymatic activity, no source of bacteria, and only protein or amino acids from the cricket leaking into the tubes (Fig 4.5, light grey bars). Thus, the rate of protein and amino acid release could be detected without the influence of self digestion by protease and bacteria. I found that low levels of protein and amino acids were released from the autoclaved cricket that could be considered background levels (Fig 4.5 A and B, light grey bars). There has been very little research into the decomposition of crickets in nature. Seastedt *et al.* (1981) found that cricket carcasses on the forest floor are quickly eaten by other arthropods. Using the crickets that were not eaten by other arthropods, Seastedt and Tate (1981) found that the nutrients leaving the cricket carcasses started with potassium and phosphorus, nitrogen loss was not calculated. Unfortunately, the cause of nutrient loss, likely self digestion, bacteria and fungi was not explored. The organisms consuming the crickets on the terrestrial forest floor would be quite different than the ones consuming the crickets in the aquatic environment of the pitcher plant. Research regarding insect decomposition has largely been directed toward the effect of insects

on decomposition in forensic science (Catts and Goff, 1992). Although chitin decomposition has been thoroughly researched (Bhattacharya *et al.* 2007), Gallie and Chang (1997) found that *S. purpurea* is unable to digest chitin, making chitin relatively uninteresting in terms of my research.

Surprisingly, it appears that not all of the enzymes inside the autoclaved cricket were denatured, as there was still specific caseinolytic activity measured on and after day 6 (Fig 4.5 C, light grey bars). There are heat resistant enzymes in milk from bacteria that can survive pasteurization, although the bacteria are killed (Cogan, 1977). Similar heat resistant enzymes may exist inside the cricket. The relatively high specific proteolytic activity is in part due to the low levels of protein leaking out of the cricket (Fig 4.5 A and C, light grey bars). The detection of high inhibition of protease in the fluid (Fig 4.6 D), suggests that serine and metalo proteases survived the autoclave sterilization and were quickly released into the water and cysteine protease may have leaked more slowly from the cricket or been introduced by contamination. The heat stability of cricket digestive enzymes, the possible survival of gut bacteria, in spite of the autoclaving process, and most of all the previously unrecognized contribution of the insect prey protease, deserve further detailed consideration in future experiments.

4.4.2.2 The presence of the cricket

The research of Gallie and Chang (1997) showed that protein, DNA or RNA is necessary to induce *S. purpurea* pitchers to produce protease, after the pitcher has been open for more than two weeks. I looked for the presence of a trigger to induce protease production by using a model prey organism in the form of a cricket. I considered using a protein such as bovine

serum albumin (BSA). However, I chose to use the whole prey organism for two reasons. By using a cricket, I could observe the release of protein and amino acids from the prey, rather than the disappearance of BSA, while the plant and bacteria were hydrolyzing the BSA. Also, a cricket is a more natural target prey for *S. purpurea* protease and such natural prey was not tested by Gallie and Chang (1997).

Pitcher fluid on its own in a tube (TF-) contained protein or amino acids at the detection limit of the assay (Fig 4.5 A and B, black bars) suggesting it did not contain previously captured insects, when removed from the pitchers on experiment day 1. Similarly, in both pitchers with fluid or water, but with no cricket (PW- and PF-), the amount of protein or amino acid was very low (Fig 4.4 A and B, light grey and black bars). The addition of a cricket to the pitcher or tube greatly increased the protein and amino acid content of both pitcher and tube (Fig 4.4 A and B, white and dark grey bars, Fig 4.5 A and B, white and dark grey bars). This is consistent with the conclusion that both protein and amino acids can leak out of the dead cricket, likely by simple physical diffusion. Shredding by inquiline organisms did not appear to be required.

The small amount of specific caseinolytic activity in pitchers without a cricket (PW- and PF-), which I found in the first days of the experiment, likely was due to pre-existing activity in the pitcher (see 4.4.2.3) (Fig 4.4 A and C, light grey and black bars). In addition, low protein concentration likely exaggerated the apparent level of specific caseinolytic activity detected at this time. When a plant pitcher contained a cricket, there was much higher specific caseinolytic activity in the pitcher after day 9 of the experiment (Fig 4.4 C, white and dark grey bars),

demonstrating the inducibility of the pitcher protease(s) as reported by Gallie and Chang (1997).

In the tubes, there was little proteolytic activity with either water or pitcher fluid and a cricket (TW+ and TF+) (Fig 4.5 C, white and grey bars). This is due to the inability of the inert tube to contribute any enzymes and in part also a result of the high amount of protein, that decreased the measured specific activity, in the same way that low protein content would inflate the specific activity. The first day of testing, during which the tube with a cricket and pitcher fluid (TF+) had the most (presumably residual) activity, was also the test day that its counterpart (TF-), without a cricket, had its highest specific caseinolytic activity (Fig 4.5 C, dark grey and black bars). This suggests that the cricket is not the source of the high specific caseinolytic activity seen in the tubes on the first day of testing. The likely reason for the high protein will be discussed in section 4.4.2.4.

In the inhibition assays, only the experimental treatments that contained high specific caseinolytic activity were tested. This meant that only the treatments that contained a cricket were tested in the inhibition assays and there are no direct comparisons without a cricket. In the tubes, the inhibition of cysteine proteases by iodoacetamide was low on day 3 of testing and increased greatly by day 15 (Fig 4.6 C, D and E). This increase was seen in all the tubes, including tubes with water and tubes with pitcher fluid. Serine proteases were strongly inhibited in each experimental condition, at the beginning and end of the experiment (Fig 4.6). The one exception is the tube with water and a cricket (TW+), where there was a drop in serine protease inhibition on the final test day, for which I cannot account (Fig 4.6 C). This strong

inhibition suggests that the cysteine and serine protease originated in the cricket. This was likely, since the serine protease trypsin is commonly tested for in the digestive tract of crickets, while the other types of protease are not (Biagio *et al.* 2009). The cricket may not have been the major source of aspartyl protease. This is because aspartyl protease inhibition appeared to be related to the presence of water or pitcher fluid (Fig 4.6 A and C versus Fig 8B and E) and will be discussed in 4.4.2.3. However, it is possible that all of these types of protease are present in small amounts within the cricket.

4.4.2.3 The role of pitcher fluid and replacement with water in tubes

Pitcher fluid potentially contains pre-existing enzymes in addition to bacteria and insect residues. In order to remove the influence of past secretions of individual pitchers, inquiline organisms and other detritus, I collected and pooled the pitcher fluid from all the test pitchers during experiment, so that the pitcher fluid used in this experiment was homogeneous. During this time, I washed out some plant pitchers with sterile water to remove as much of the inquiline organisms and residues as possible, and to remove any residual agent in the pitcher that might induce the plant to secrete protease. I used these washed out plant pitchers in experimental conditions involving a plant pitcher with water and a plant pitcher with water and a cricket. I found that pitcher fluid on its own (TF-) only contained a small amount of amino acids and protein, but had relatively high specific caseinolytic activity at the beginning of the experiment that decreased over time (Fig 4.5 A, B and C black bars). Since the tube is inert, these proteases and amino acids originated in the pitcher fluid and the decrease in specific

caseinolytic activity is due to the actions of the organisms in fluid rather than unlikely contributions of the inert tube.

While the protein content in the tubes with either water or pitcher fluid and a cricket (TW+ and TF+) was similar throughout the experiment, there were differences in amino acid content. On the first day, I measured an increased amount of amino acids in the tube with pitcher fluid and a cricket (TF+) compared to the tube with water and a cricket (TW+), but these amounts were similar for the rest of the experiment, with a decrease in amino acids on the last day (Fig 4.5 B, white and dark grey bars). In the tube with pitcher fluid and a cricket (TF+), this decrease in amino acids likely was due to inquiline organisms and microorganisms, on and in the cricket, consuming the amino acids. In the tube with water and a cricket (TW+), the decrease likely was caused by microorganisms, on and in the cricket, since I found less of a decrease when the cricket was autoclaved (Fig 4.5 B, light grey bars).

I observed little specific caseinolytic activity in the tubes. I found an increase in specific caseinolytic activity in the tubes with pitcher fluid and a cricket (TF+) on the first day of testing, but did not find a similar increase in the tube with water and a cricket (TW+), in which water rather than pitcher fluid was used (Fig 4.5 C, white and dark grey bars). Since the tube is considered inert and cricket is unlikely to be the cause of this increase, it is possible that the pitcher fluid was the source of this initial high specific caseinolytic activity. The types of protease present in the pitcher fluid, initial high inhibition of aspartyl proteases by pepstatin A was only observed in tubes that contained pitcher fluid (Fig 4.6 B and E). This initial aspartyl protease likely originated in the pitcher fluid.

4.4.2.4 The role of the plant pitchers

The most important comparisons in this experiment are the differences between the plant pitchers and the inert tubes. The sterile plastic tubes acted as a control, since the inert plastic was not expected to contribute to the pitcher fluid or cricket digestion. In contrast, the living pitchers are able to absorb amino acids and release proteases (Karagatzides *et al.*, 2009; Gallie and Chang 1997). This resulted in the large, fourfold difference in protein content between the plant pitchers with a cricket (Fig 4.4 A, white and dark grey bars) and the tubes with a cricket (Fig 4.5 A, white and dark grey bars). This is because the cricket proteins and amino acids were absorbed by the plant, while the inert tube was unable to do this. A complex dynamic state exists in the pitcher characterized by protein input, breakdown, into amino acids and their simultaneous uptake. Periodic (every 3 days) analysis only provides a "snap shot" of the current contents of the pitcher, not a detailed understanding of the dynamic kinetics of amino acid use by the pitcher and inquiline organisms.

The protein content of the tubes with a cricket appeared to be fairly stable by the 6^{th} day of testing (Fig 4.5 A, white and dark grey bars). This may indicate that the decomposition of the cricket had stopped, or that the fluid had reached the protein saturation point, but these explanations are unlikely, because there was still visible cricket to be hydrolyzed. Although different proteins have different solubilities, 0.28 mg/mL (Fig 4.5 A, white bar, day 6) is low compared to the solubility of BSA which is 40 mg/mL (Sigma). Compared to BSA, I think that the maximum concentration of protein found in the pitcher fluid is too low for the solution to be considered saturated. It is much more likely that the inquiline organisms were consuming

the protein at a rate which made it appear that there was no new protein release. Consumption by inquiline organisms is the likely cause of the decrease in amino acid content in the tubes on day 15 of testing, as consumption is the most likely mode of removal (Fig 4.5 B, white and dark grey bars).

In the plant pitchers with water and a cricket (PW+, Fig 4.4 C, white bars), there was more specific caseinolytic activity at the beginning of the experiment that decreased over time, but in the pitcher with pitcher fluid and a cricket (PF+, Fig 4.4 C, dark grey bars), activity increased over time. The inquiline organisms in the pitcher fluid may increase specific caseinolytic activity over the long term by growing and using the cricket as a food source. The protein content of the plant pitcher with pitcher fluid and a cricket (PF+) had decreased by the end of the experiment (Fig 4.4 A, dark grey bars). This suggests that most of the protein in the cricket had been hydrolysed. In contrast, the protein content of the plant pitcher with water and a cricket (PW+) had not dropped, suggesting that the pitcher fluid in the plant pitcher with pitcher fluid and a cricket (PF+) had resulted in quicker digestion than the plant pitcher with water and a cricket (PW+). This decrease in protein content may have also contributed to exaggerated specific caseinolytic activity, since the protein level was low.

In the plants, pitcher fluid appeared to increase specific caseinolytic activity over time, while in the tubes, the pitcher fluid had high activity only on the first day. This does not change the finding that there is much more specific caseinolytic activity in the plant pitchers, than in the tubes (Fig 4.4 C and 4.5 C) and much more activity in the plant pitchers containing a cricket (Fig 4.4 C, white and dark grey bars compared to light grey and black bars). In terms of protein,

amino acids and specific caseinolytic activity, the uninduced plant pitchers (PW- and PF-, Fig 4.4, light grey and black bars) are more similar to the tube with pitcher fluid (TF-, Fig 4.5, black bars) than to the plant pitchers that received a cricket (PW+ and PF+, Fig 4.4, white and dark grey bars). However, given the difference in protein, amino acids and specific caseinolytic activity between the plant pitchers that contained a cricket (PW+ and PF+, Fig 4.4, white and dark grey bars) and the tubes that contained a cricket (TW+ and TF+, Fig 4.5, white and dark grey bars), it cannot be said that the plant pitchers and inert tubes are the same. This strongly suggests that the plant is being induced to action by the cricket. The action of protease to decrease the protein content and the absorption of amino acid in the plant pitchers is clearly demonstrated. Although the identity of proteases released by the plant cannot be ascertained at this time, my evidence suggests that the plant is being induced and the increase in specific caseinolytic activity suggests that the plant may be providing some of that protease.

The types of protease released by the plant are difficult to determine, due to the presence of the cricket in all the inhibition assays. Thus, from these tests, it cannot be determined if anyone specific type of protease originated in the plant. In studies of insectivorous plant *Nepenthes macferlanei* L., Tökés *et al.* (1974) only found aspartyl protease production. Godlewski and Adamczyk (2007) found that the roots of corn and sunflowers were able to secrete cysteine and aspartyl protease. From my findings, it is unlikely that *S. purpurea* is producing aspartyl protease. There was little inhibition by pepstatin A in the plant pitcher with water and a cricket (PW+) on any test day (Fig 4.6 A) compared to the greater inhibition by pepstatin A in the plant pitcher with pitcher fluid and a cricket (PF+, Fig 4.6 B). In the tubes, there was also a greater amount of inhibition by pepstatin A in the presence of pitcher fluid and

a cricket (TF+, Fig 4.6 E) compared to a tube with water and a cricket (TW+, Fig 4.6 C). This suggests that the origin of the aspartyl protease was the inquiline organisms in the pitcher fluid. If the origin had been the plant, an increase in aspartly protease inhibition would have been seen on day 15 of testing in the plant pitcher with water and a cricket (PW+). Regarding the comparison to plant roots, only corn and sunflower were tested for protease type (Gidkewski and Adamczyk, 2007). With more testing, other types of protease may be discovered to be released by plant roots. Insectivory in *Nepenthes* and *Sarracenia* is known to have evolved independently of each other (Albert *et al.*, 1992). It is not surprising that, they may secrete different types of protease to digest prey. A comparison of protease secreted by *S. purpurea* to protease secreted by other members of the *Sarracenia* family would be ideal. However, no studies of the types of protease secreted by *Sarracenia spp.*, that are known to secrete protease, have been reported.

The amino acid content of the plant pitchers showed a balance of amino acid synthesis by protein hydrolysis seen and the disappearance of amino acids as they were absorbed by the plant. The synthesis of amino acids from the cricket by pitcher fluid protease and self digestion was seen in the tubes (Fig 4.5 A, white and dark grey bars). The absorption of amino acids by the plant was seen in the form of the almost threefold decrease in amino acids in the plant pitchers (Fig 4.4 B, white and dark grey bars). The amino acid levels remained high in the tubes, because they had less absorptive surface to remove them. In the plant pitchers, protein and amino acids were likely being removed by the plant, as they were released by protein hydrolysis (Karagatzides *et al.*, 2009). The 200 μM difference in amino acid content in the plant pitchers and tubes may represent the amount of amino acids and thus nitrogen absorbed by the plant

over the course of the experiment (Fig 4.4 B and Fig 4.5 B). This is clear support of the conclusion that *S. purpurea* can absorb amino acids (Karagatzides *et al.*, 2009). The actual amount of amino acids absorbed by the plant pitchers was likely to be greater than the 200 μM difference due to the increased amino acid synthesis by the increased rate of proteoyltic activity seen in the plant pitchers compared to the tubes (Fig 4.4 C and Fig 4.5 C).

4.4.3 Improvements and follow up experiments

There are many ways in which these experiments could be improved in the future. As the results of the tests were analysed, I considered new experimental conditions but I was unable to initiate further experiments due to time limitations. One of the future goals of this research is to conduct similar pitcher fluid experiments on *S. purpurea* plants grown from seed in sterile conditions and on *S. purpurea* tissue culture. The results of the experiments presented in this paper provide useful information regarding the experimental conditions required in such future *S. purpurea* experiments.

Throughout the series of experiments contained in this paper, I found the self digestion of the cricket difficult to separate from digestion by inquiline organisms or by the plant, even with the comparison of the cricket in a tube (TW+) to an autoclaved cricket, also in a tube (TW+*). Because I recorded caseinolytic activity in the tube with water and an autoclaved cricket (TW+*) (Fig 7C, light grey bars), I suggest that future researchers consider other ways to look at self digestion. In general, more research on the decomposition of crickets or other arthropods would be helpful, particularly research on bacterial and fungal decomposers of the whole organism, rather than components such as chitin. In the experiments by Biagio *et al.*

(2009) into the cricket digestive system, the researchers removed the cricket's gut to determine the enzymes present in different sections of the gut. A de-gutted cricket and the cricket gut may provide useful controls for determining self digestion of the cricket. The role of this self digestion could be clarified by autoclaving the cricket for a longer period and quantifying the microorganisms and the degree of inactivation of the proteases, resulting from the autoclaving. In order to reduce possible contamination of the autoclaved cricket, other precautions could be taken, such as storing sterile experimental treatments in a biosafety cabinet rather than in a laminar flow hood.

Unlike Gallie and Chang (1997), I decided not to use antibiotics to inhibit the growth of bacteria in the plant pitchers, because of the eventual goal of performing similar experiments on sterile plants, with these tests as method development and the goal of gathering preliminary data in regard to prey digestion by the pitcher plant. Since antibiotics would not be required in the sterile seedlings, I decided that it would not be useful to add antibiotics as a variable in these experiments. When I realized that the seedlings would not be grown in time for use in these experiments, I decided not to change the protocol, so that the older data could be compared to the new. If I were to conduct a new set of experiments on non-sterile plants, I would consider the use of antibiotics.

If I were repeating these experiments, I would consider using azure hide powder as a substrate rather than FITC- casein, in my protease activity assays. Azure hide powder has a hydrolysis optimum at pH 6.0 (Gallie and Chang, 1997), which is much closer to the pH 3.8

(Juniper *et al.*, 1989) of pitcher fluid than the 8.5 pH for casein hydrolysis. I expect that the pitcher plant enzymes have evolved to work best at the pH of the pitcher fluid.

Although it would be ideal to test the samples for caseinolytic activity, protein and amino acid content on the same day that the samples were collected, I was unable to do such timely testing. For consistency and convenience, the samples for the amino acid assay were stored at -20 °C until they could be processed together. The samples were frozen to stop protease from hydrolysing the proteins and increasing their amino acid content before they were analysed. I suggest removing the protein from the amino acid samples before freezing the samples, if the analysis cannot be completed on the same day because, when the samples are frozen and thawed, the proteins may degrade and release amino acids which may result in an increase in amino acid concentration.

The procedure for the inhibition assays could also be improved. For the data represented in Figures 4.4 C and 4.5 C, I incubated the time zero and time 60 control samples separately. Samples that are to be directly compared, such as time 60 controls compared to corresponding inhibition assays, should be incubated together. This eliminates the possibility of small differences in incubation time and FITC-casein reaction mixture. Future researchers may consider at least partially purifying the samples before the inhibition assays, to facilitate the identification of the types of protease in the mixture. This would be a first step towards isolating the various proteases. Future researchers may also consider using different inhibitors. Godlewski and Adamczyk (2007) used two different inhibitors for each type of protease and Tökés *et al.* (1974) used the same inhibitors at different concentrations. Using multiple

inhibitors or inhibitors at different concentrations may be useful in determining the types of protease present in the samples. I used a single inhibitor in these experiments because I did not have access to the volume of sample that the extra tests would have required.

The caseinolytic activity data could be analysed for statistical differences. The data would be analysed using split plot design repeated measures ANOVA, if found to be parametric. The experimental design is considered to be a split plot since the individual treatments are not independent of each other. Rather, they are dependent, each plant in the test had 4 pitchers, with each pitcher on the plant having a different, but randomly assigned treatment condition. I was unable to transform the caseinolytic activity data to be parametric. The appropriate method to use would be a split plot Friedman's test however, the variance was too great for this testing.

My primary recommendation is to conduct these pitcher fluid experiments using *S. purpurea* plants grown in sterile conditions or using *S. purpurea* tissue culture. However, the experimental conditions would require adaptations to accommodate either of these model systems.

Wild *S. purpurea* plants, that have not been exposed to rain, contain very little fluid (Hepburn *et al.*, 1927). *S. purpurea* plants grown in sterile conditions, would require sterilized water to be introduced by the researcher, in order to create pitcher fluid. To prepare this fluid for testing, I recommend washing out the pitchers with sterilized water, using aseptic technique and allowing sterilized water to sit in the pitchers for several days. Before beginning the experiment, the researcher would collect water from the pitchers and wash the pitchers out

with sterilized water again. This collected water would be analogous to the pitcher fluid, used in the tests in my research with wild plants.

It might be necessary to transfer the plants to nutrient medium that has little or no nitrogen. The presence of easily absorbed forms of nitrogen, such as nitrate or ammonium, should not be included in this new nutrient medium (Hopkins and Hüner, 2004). If more easily accessible nitrogen is available, the plant may not be induced to secrete protease, in oder to use protein as a nitrogen source (Godlewski and Adamczyk, 2007).

The experimental conditions for this research, using plants, inert tubes, sterile water, pitcher fluid and BSA rather than a cricket, could be set up similarly to experimental conditions used in my research. An easily sterilized protein such as BSA would eliminate possible contamination from crickets and is easier to regulate. As a free protein, BSA would undergo less digestion than would be required in the digestion of the whole cricket by the plant, so the plant may be induced more quickly with BSA than with the cricket. For this reason, the researcher might choose to test the fluids more frequently, or on a daily basis.

To adapt this research to *S. purpurea* batch tissue culture, the cricket would also need to be replaced with BSA and the fluid collection and preparation would need to be adapted to accommodate the changes in the experimental conditions. In batch tissue culture, the cells are suspended in liquid nutrient medium. The cells would need to be removed from the liquid and resuspended, in new nitrogen reduced medium. As with the sterile plants, nitrogen deficiency would ensure that the cells would secrete protease to obtain nitrogen. The cells should be left in the low nitrogen medium for several days to emulate the equivalent of a pitcher fluid.

The researchers could the expose the cells into different experimental conditions, with some being induced with BSA. The results of such a study with tissue culture would not be as conclusive as with the sterile plants, because cells in tissue cultures have been known to secrete protease differently than the whole plant (Simmons *et al.*, 2004).

More research is needed to determine the origins of the different proteases found in this experiment and to determine the types of protease released by *S. purpurea*. My research has shown that if *S. purpurea* can produce protease for prey digestion, it can be induced to do so by prey. Perhaps more importantly, this research also shows that the prey itself has a role in its own digestion, either by microorganisms or the enzymes in the prey's digestive system and cells. This is a direction that has not been investigated in terms of insectivorous plant prey digestion. Rather, researchers tend to focus on bacteria in the pitchers that is difficult to differentiate from the action of the plant. The methods developed in these experiments and the development of sterile seedlings or tissue culture, will assist future researchers in determining the types of protease released by *S. purpurea* for prey digestion and solving the question of the roles of different sources of digestive enzymes.

Table 4.1. Pitcher fluid experimental conditions

Code		PW+	PW-	PF+	PF-	TW+	TF+	TF-	TW+*
Vessel	Pitcher/Tube	P	P	P	P	T	T	T	T
Medium	Water/Fluid	W	W	F	F	W	F	F	W
Cricket	presences/abscence	+	-	+	-	+	+	-	+*

*the cricket has been autoclaved

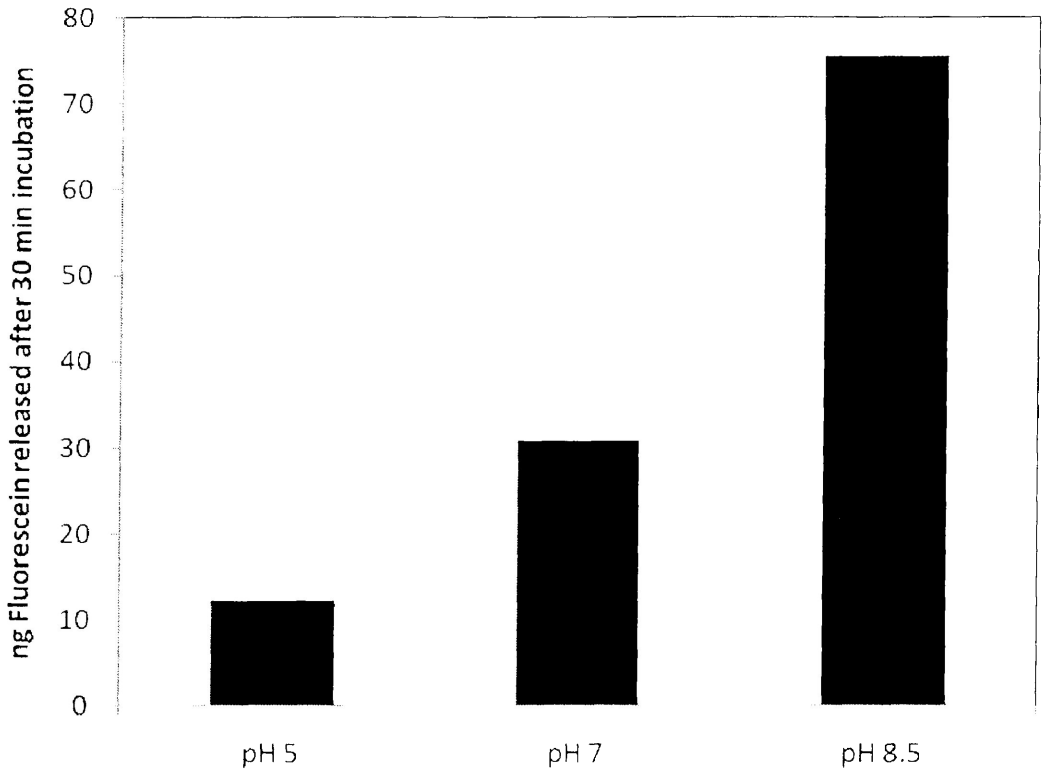

Figure 4.1. Caseinolytic assay, pH optimization. Fluorescein released after 30 min incubation with FITC-casein substrate with concentrated pitcher fluid and three different buffers, 400 mM Tris-HCl buffer, pH 8.5, 400 mM sodium phosphate buffer, pH 7 and 400 mM sodium acetate buffer, pH 5. Protein concentration was not considered since the samples are the same.

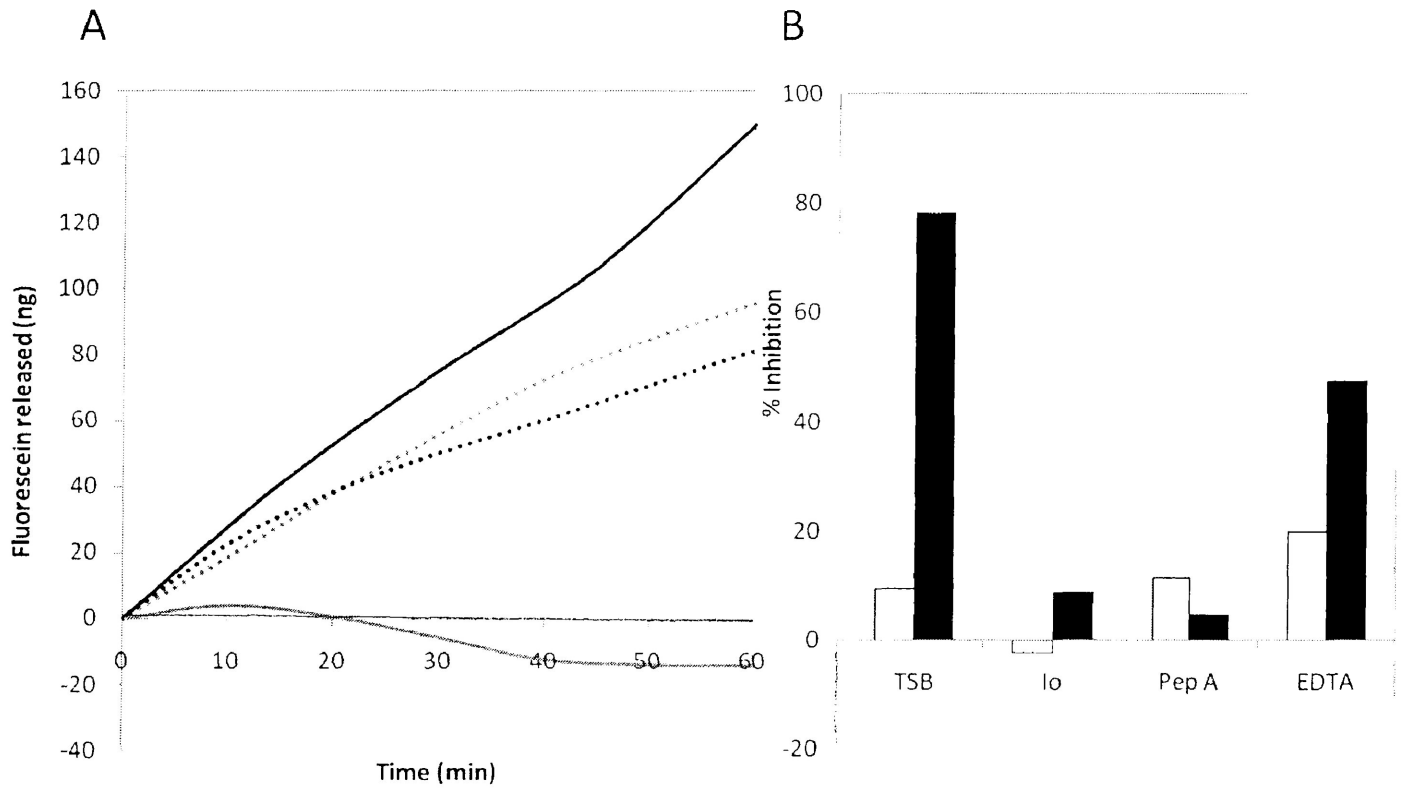

Figure 4.2. Caseinolytic assay, micro plate vs. shaker-stir bath methods. (**A**) The difference in fluorescein released over the course of an hour incubation of concentrated pitcher fluid with FITC-casein substrate using the shaker-stir bath method (solid black line) and the micro plate method, the total fluorescein released (grey line), with the time zero blanks removed. The black dotted line shows the fluorescein released in the non-boiled sample and the gray dotted line showes boiled sample, the difference between the two create the total fluorescein released (grey line). (**B**) Inhibition assays of FITC-casein hydrolysis by micro plate method (white bars) and the shaker-stir bath method (black bars) with the protease inhibitor indicated, Io (Iodoactamide), Pep A (Pepstatin A), TSB (soybean trypsin inhibitor) and EDTA (Ethylenediaminetetraacetic acid).

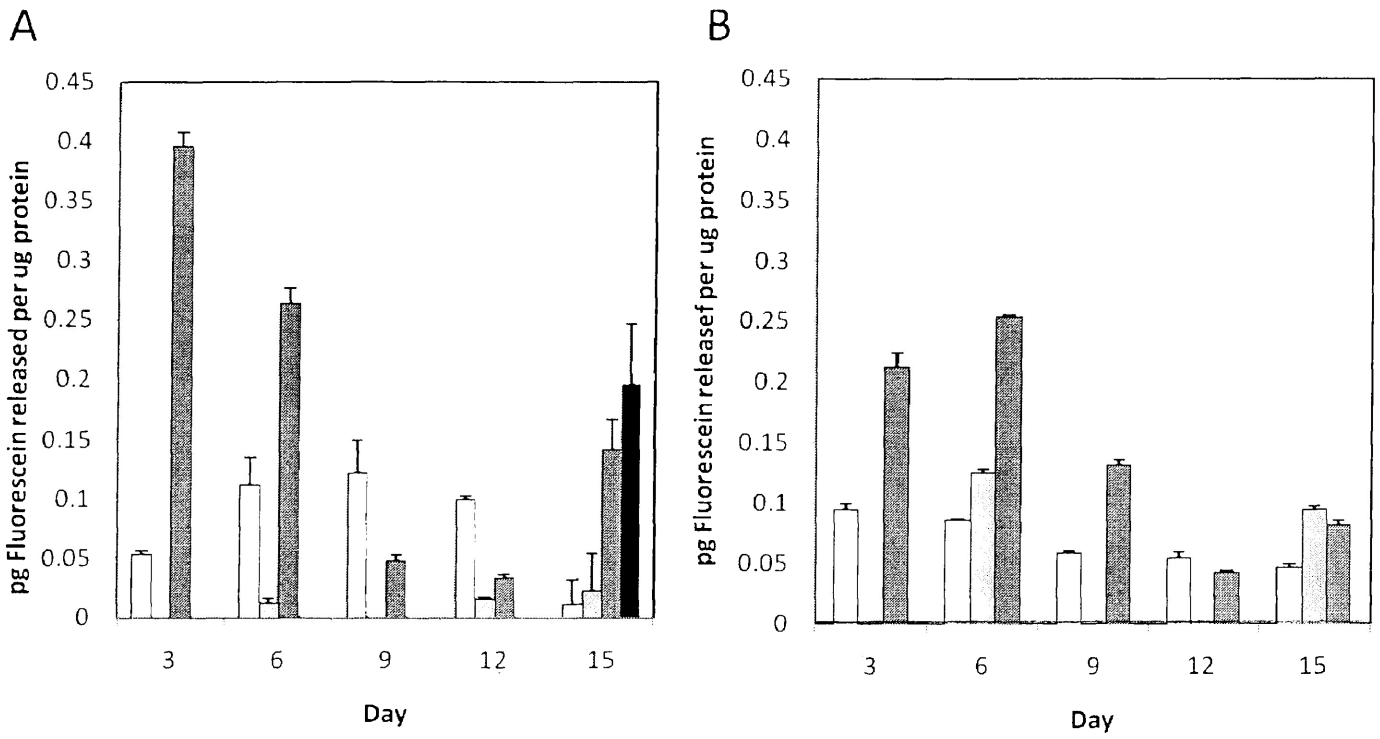

Figure 4.3. Plant and tube fluid caseinolytic activity, micro plate method. The amount of fluorescein released after one hour incubation of FITC-casein substrate with fluid from pitchers, determined using the micro plate method. In (**A**) open bars indicate a pitcher filled with water and induced with a cricket (PW+). Light gray bars indicate a pitcher with fluid replaced with distilled water (PW-). Dark grey bars represent pitchers that received both pitcher fluid and a cricket (PF+) and those represented by black bars only received pitcher fluid (PF-). In (**B**) open bars indicate a tube filled with water and induced with a cricket (TW+). Light gray bars indicate a tube filled with autoclaved water and an autoclaved cricket (TW+*). Dark grey bars represent tubes that received both pitcher fluid and a cricket (TF+). Error bars indicate standard error for three replicates.

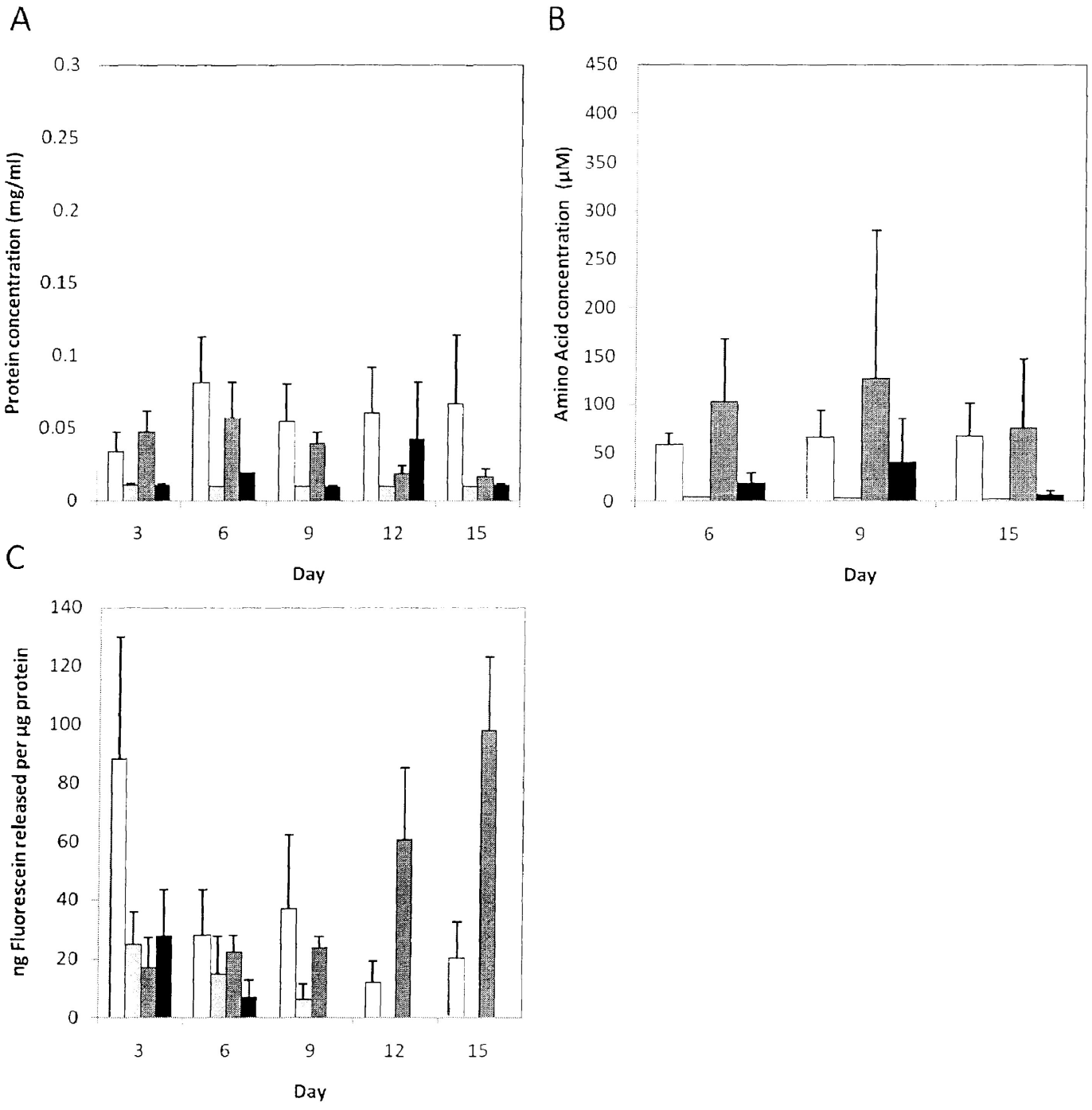

Figure 4.4. Plant fluid experiments shaker-stir bath method. (**A**) the amount of protein and (**B**) free amino acids in pitcher plant fluid under different conditions. (**C**) the amount of fluorescein released after a one hour incubation of FITC-casein substrate with fluid from those pitchers. Standard errors are the result of three replications. Open bars indicate a pitcher filled with water and induced with a cricket (PW+). Light gray bars indicate a pitcher with fluid replaced with distilled water (PW-). Dark grey bars represent pitchers that received both pitcher fluid and a cricket (PF+) and those represented by black bars only received pitcher fluid (PF-).

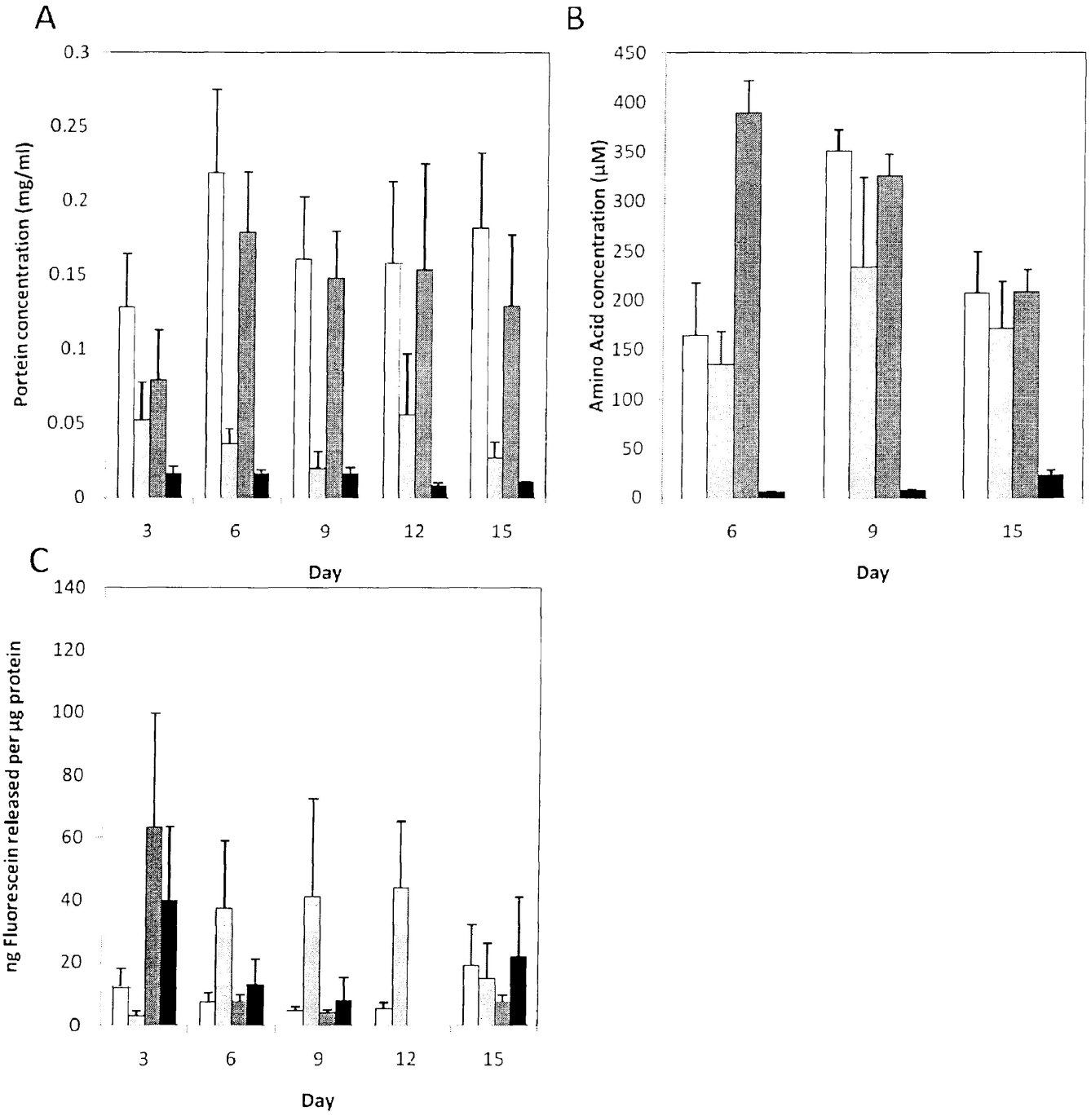

Figure 4.5. Artificial pitcher fluid experiments shaker-stir bath method. (**A**) the amount of protein and (**B**) free amino acids found in artificial pitcher plants (plastic tubes) under different conditions. (**C**) the amount of fluorescein released after a one hour incubation of FITC-casein substrate with fluid from those tubes. Error bars indicate standard error for 6 replicates. Open bars indicate a tube filled with water and induced with a cricket (TW+). Light gray bars indicate a tube filled with autoclaved water and an autoclaved cricket (TW+*). Dark grey bars represent tubes that received both pitcher fluid and a cricket (TF+) and those represented by black bars only received pitcher fluid (TF-).

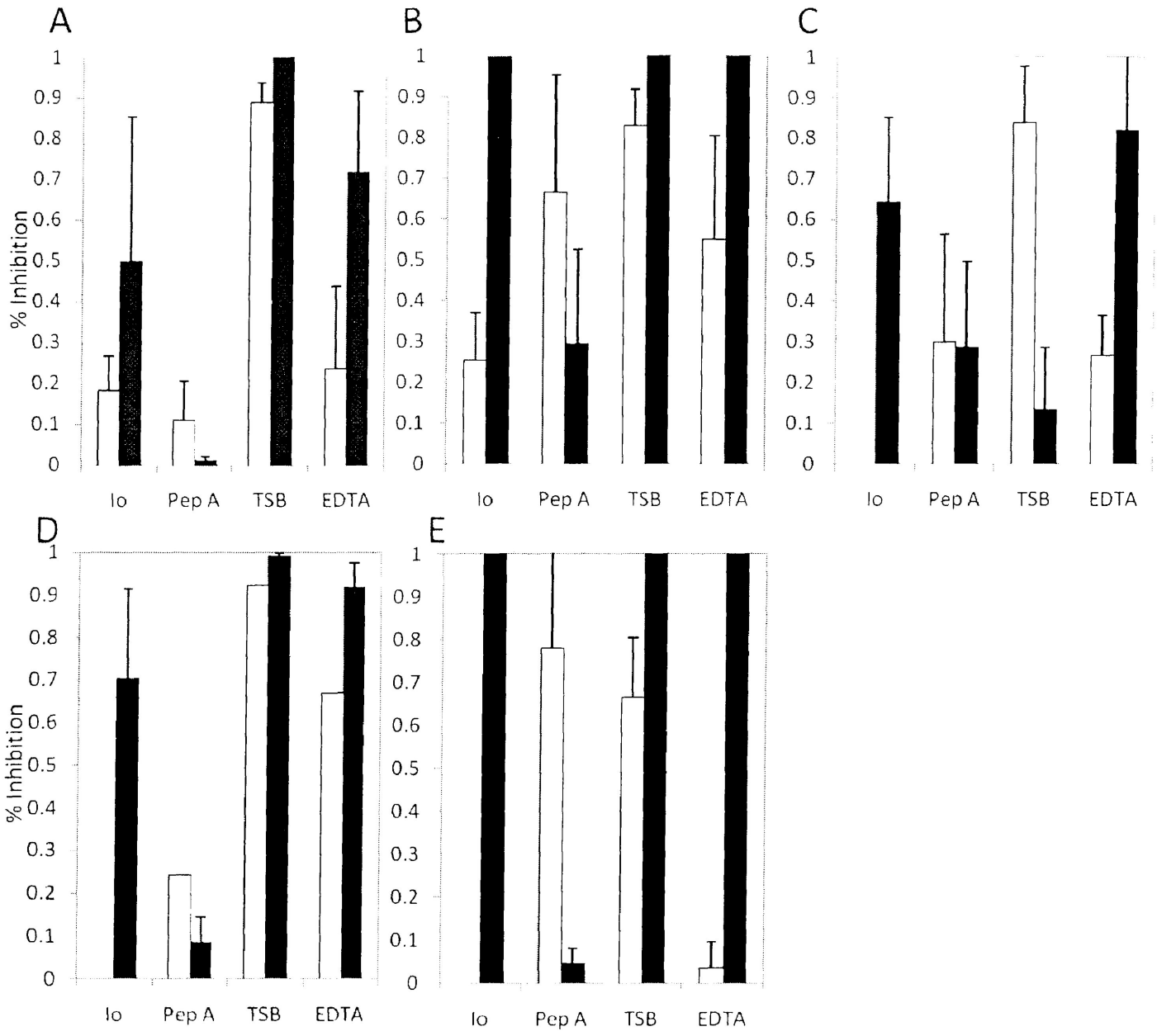

Figure 4.6. Inhibition experiments. Inhibition assays of FITC-casein hydrolysis with the protease inhibitor indicated, Io (Iodoactamide), Pep A (Pepstatin A), TSB (soybean trypsin inhibitor) and EDTA (Ethylenediaminetetraacetic acid). Samples were collected and tested on day 3 (open bars) and day 20 (solid bars). Bars are the mean of up to 6 assays with standard error bars shown. (**A**) shows the inhibition profile of fluid collected from a pitcher that contained water and a cricket (PW+). (**B**) pitcher that contained pitcher fluid and a cricket (PF+), (**C**) tube with water and a cricket (TW+), (**D**) a tube with water and an autoclaved cricket and (TW+*), (**E**) tube that contained pitcher fluid and a cricket (TF+).

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