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A Comparison Of The Enzyme Profiles Of Two Pitcher Plant Species (*Sarracenia jonesii* and *Sarracenia purpurea* var. *montana*) And Their Hybrids

A. Allyson Brandon
Biology
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Caroline Kennedy

Abstract

Although the effects of hybridization have been documented in various plant and animal systems, the consequences of this phenomenon in pitcher plants is not known. Two species of carnivorous pitcher plants (*Sarracenia purpurea* var. *montana* and the endangered *Sarracenia jonesii*) endemic to western North Carolina and northwestern South Carolina are known to hybridize in sympatry resulting in several small populations of hybrid progeny. These two species have very different ways of acquiring growth-limiting nutrients (nitrogen and phosphorous), with *S. purpurea* var. *montana* relying on products from phytotelma communities within their pitchers, and *S. jonesii*. trapping and digesting prey. Hybrids of these two species are morphologically intermediate, but it is unclear which trophic strategy they employ. I sampled pitcher fluid from *S. purpurea* var. *montana*, *S. jonesii*, and their hybrids from two sites in western NC and used colorimetric protease and phosphatase assays to compare their enzyme profiles. I hypothesize that hybrids will have protease and phosphatase activity levels that are intermediate between the parental species. These analyses will shed light on the potentially significant ecological and evolutionary impacts of natural hybridization in pitcher plants.

1. Introduction

The North American pitcher plant family, Sarraceniaceae, consists of 30 species comprising 3 genera of carnivorous plants[1]. The genus *Sarracenia* contains 10 species found mainly along the southeastern Atlantic coast of the United States. These pitcher plants inhabit sunny wetland fens, marshes, and bogs[2] that feature nutrient-deficient, acidic soils[3]. *Sarracenia* spp. have evolved carnivory, primarily as a mechanism to supplement the plant with the growth-limiting nutrients[4], like nitrogen and phosphorous, that their habitat lacks[5]. These plants produce specialized pitcher leaves that passively trap infalling prey items[6], such as insects or other small invertebrates[7], and obtain nutrients via prey digestion[2,8,9].

The widely distributed purple pitcher plant, *Sarracenia purpurea*[10] and the mountain variety, *S. purpurea* var. *montana*, have short, squat pitchers that fill with rainfall and drown trapped prey[6]. The rain-filled pitchers also host several species of protozoans[11], bacteria[12,13], copepods[14], algae[15], midges[16], mosquito larvae[16,17], and other invertebrates[7,18]. This inquiline community provides mature *S. purpurea* individuals with both mechanical[19,20] and chemical breakdown of prey in the form of secreted digestive enzymes[21]. The ratio of plant-produced versus inquiline-produced enzymes in *S. purpurea* is unclear. Researchers have shown that several hydrolytic enzymes such as RNases, proteases, nucleases, and phosphatases were produced by newly opened juvenile *S. purpurea* pitchers. They found that these enzymes were directly regulated by the plant based on the presence or absence of prey in the pitcher. As plants aged, these enzymes were only detected when prey items were added to pitchers[22]. Other studies have suggested that the inquiline community is the ultimate source of enzymes, and that digestive peptides are only produced by unopened *S. purpurea* pitchers. Two separate studies found culturable bacteria in *S. purpurea* pitchers that secreted

proteolytic enzymes[13,23]. Bacteria in pitchers of the similar South American pitcher plant genus, *Nepenthes*, have also been found to produce significant amounts of digestive enzymes[24]. Phosphatase activity was also found in over 20 species of insectivorous *Utricularia*[25]. It is evident that the inquiline communities in *S. purpurea* are crucial for its ability to obtain and absorb nutrients from prey items[12,20,26,27], despite the murky origin of digestive enzymes.

The mountain sweet pitcher plant, *Sarracenia jonesii*[28], is limited to only a few isolated populations in the southeast and is federally endangered[29]. Unlike *S. purpurea*, *S. jonesii* produce tall, slender pitchers that remain mostly dry[30]. Much is unknown about the ecological role of this species, and no studies have investigated digestive enzymes in *S. jonesii*.

In sites where they co-occur, *S. purpurea* and *S. jonesii* produce morphologically intermediate hybrid offspring[31]. Natural hybridization in plants can have many ecological and evolutionary impacts[32], possibly acting as a bridge through which speciation occurs[33]. Although hybridization is common in plants[34], hybrid crosses are not always phenotypically intermediate to the parent species. For example, in reciprocal transplant experiment, F_1 hybrids of parent beardtongue (*Pentsemon* spp.) were equivalent or more fit in all fitness parameters tested (seed set, time to flower, growth, etc.), and exceeded the parent species in reproductive output[35] . Similar results were found in the analysis of cottonwood plants and their hybrids, with F_1 hybrids producing the same amount of seeds and exceeding the parent species in vegetative growth[36]. In contrast, in slender wild oak (*Avena barbata*) late-generation hybrids were significantly less fit overall, but a few hybrid genotypes enabled some individuals to survive and match the reproductive output of the parents[37]. Experimental evidence shows that intermediacy is not the only outcome of hybridization, nor can it be assumed from hybrid morphology[34].

Despite a wealth of observations on naturally occurring hybrids, no study has focused on hybridization between *S. purpurea* and *S. jonesii*. Previous work has shown that hybrids have inquiline communities, but their pitchers have fewer macroinvertebrates than *S. purpurea* var. *montana*[38]. It is also unclear how the presence of hybrids will impact the few remaining populations of the vulnerable *S. jonesii*. To determine the trophic strategy of hybrid offspring, I analyzed the enzyme profiles of *S. purpurea* var. *montana*, *S. jonesii*, and their hybrids in two western NC sites where they co-occur. I expect *S. jonesii* to show the highest enzymatic activity because I assume *S. jonesii* synthesizes and secretes appreciable amounts of their own digestive enzymes due to the lack of commensal inquilines. I hypothesize that the hybrids will have intermediate phosphatase and protease activity. Results of this study will provide insight into the consequences of hybridization in sympatric populations of pitcher plants and could potentially provide vital information in managing the few remaining populations of the federally endangered *S. jonesii* in those areas.

2. Materials and Methods

2.1 Sampling Sites

Samples were collected at two sites in Henderson and Transylvania counties, North Carolina between June and August of 2018. The Transylvania county site (SF) borders a small pond and contains sympatric populations of *S. purpurea* var. *montana*, *S. jonesii*, and *S. purpurea* x *S. jonesii* morphological hybrids. Overall, this site is relatively dry with dense sandy soil. The Henderson county site (MC) is adjacent to a cow pasture with rosettes of pitcher plants scattered throughout a shady wetland. The predominant pitcher plant species in MC is *S. jonesii*, with only a few rosettes of *S. purpurea* var. *montana* and *S. jonesii* x *S. purpurea* var. *montana* hybrids found. The habitat of MC varies considerably from that of SF, containing water-saturated soil.

2.2 Sampling

At SF, 8 rosettes of both parental species and hybrids were identified along a ~25 m transect and marked with a beaded monofilament necklace to ensure the same rosette could be located for subsequent sampling. The MC site had ongoing sampling with established plot boundaries, so I used their pre-existing PVC pipe markers to identify the sampling locations. Beaded necklaces were placed on marked rosettes for future sampling. In all, 6 *S. jonesii*, 3 *S. purpurea* var. *montana*, and 8 hybrids were sampled at MC. At both sites, native pitcher fluid was removed with either a disposable plastic bulb pipet (for large traps, like those of *S. purpurea* var. *montana* and hybrids) or a 3cc syringe attached to a rubber catheter (for long narrow traps, like those of *S. jonesii*) and replaced with 2 mL distilled water. After1 h of incubation, the distilled water was removed and evenly distributed into 3 microcentrifuge tubes per pitcher sampled,

temporarily stored on ice, and permanently stored at -80°C. Pitcher fluid samples were collected a total of 3 times at each site between June 15, 2018 and August 3, 2018 following the protocol listed above.

2.3 Enzyme Activity Assays

Phosphatase and protease enzyme analyses were performed in triplicate on the pitcher fluid samples collected using colorimetric spectrophotometry via a BioTek© ELx808 microplate reader and Gen5© 3.03 software. The phosphatase assays were performed using a GBiosciences© Phosphatase Assay Kit. Frozen samples were thawed, centrifuged briefly (~30 seconds) to pellet any prey or debris present, and 50 µL of the pitcher fluid was loaded into 3 experimental and 3 control (blank) microplate wells. The blank wells received an additional 50 µL of PA Buffer (pH 7.5) while the experimental wells received 50 µL of PA Substrate. Activity readings occurred at 405 nm λ every 10 minutes over a 7-hour period.

A Pierce® Protease Assay Kit was used to perform the protease assays. Frozen samples were thawed, centrifuged, and loaded into 3 experimental and 3 blank microplate wells as before. Additionally, 50 µL of 5% 2,4,6-trinitrobenzene sulfonic acid (TNBSA) was added to each well. Finally, blank wells received 100 µL of 50 µM borate buffer (pH 8.5) while experimental wells had 100 µL of succinylated casein substrate added. Samples were read at 450 nm λ in two separate rounds with the first reading occurring over 1 hour in 1-minute intervals, and the second reading occurring over 6 hours in 10-minute intervals immediately upon completion of the first reading.

2.4 Data Analysis

Before statistical analysis, all enzymatic data were averaged based on the triplicate absorbance values compared against the averaged triplicate blank wells using Microsoft Excel™. I analyzed the protease assays after 2 hours, and phosphatase assays after 5 hours because enzyme activity plateaued at those times. One-way analysis of variance (ANOVA) tests were performed on both the protease and phosphatase data. Tukey's HSD post hoc tests ($p \leq 0.05$) were executed if the ANOVA showed significant differences among the variables tested. All analyses were conducted using R statistical software[39] and the tidyverse[40] and ggplot2[41] packages.

3. Results

3.1 Protease Activity

There were significant differences in protease activity among the 3 plant types ($F_{2,97}=25.32$, $p < 0.0001$). Protease activity in *S. jonesii* was significantly higher than in *S. purpurea* var. *montana* and the hybrids (Fig. 1). Overall, the hybrids were more like *S. purpurea* var. *montana* than *S. jonesii* in protease activity (Table 1).

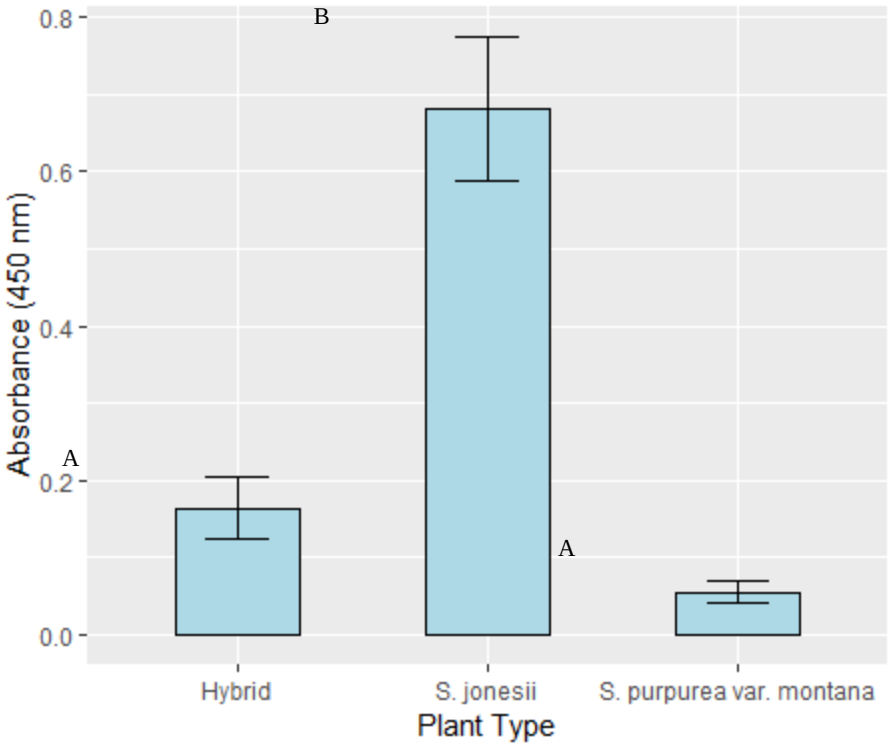

Figure 1. Protease activity (mean ± 1 SE) in the pitcher fluid of *S. jonesii*, *S. purpurea* var. *montana*, and hybrids after two hours elapsed time. Activity was significantly different between *S. jonesii* and hybrids, and *S. jonesii* and *S. purpurea* var. *montana*.

Table 1. Descriptive statistics for the protease activity of three pitcher plant types.

Plant Type	Mean Absorbance	Standard Error	Standard Deviation
S. jonesii	0.681	0.0941	0.588
S. purpurea var. *montana*	0.055	0.0149	0.078
Hybrid	0.164	0.0396	0.228

3.2 Phosphatase Activity

Although the phosphatase activities among the 3 plant types were not statistically different ($F_{2,118}$=2.8159, p=0.0639), they were nearly so (Fig. 2). Hybrids and *S. purpurea* var. *montana* had the highest and lowest phosphatase activity respectively, while *S. jonesii* had intermediate activity (Table 2).

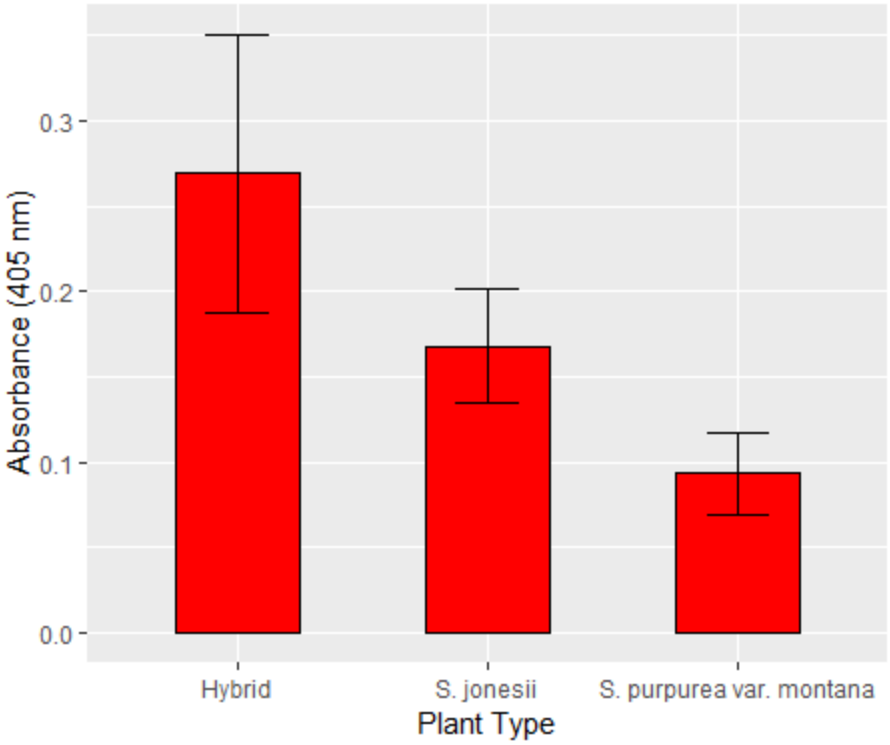

Figure 2. Phosphatase activity (mean ± 1 SE) in the pitcher fluid of *S. jonesii*, *S. purpurea* var. *montana*, and hybrids after five hours elapsed time. Phosphatase activity was not significantly different between plant types.

Table 2. Descriptive statistics for the phosphatase activity of three pitcher plant types.

Plant Type	Mean Absorbance	Standard Error	Standard Deviation
S. jonesii	0.168	0.033	0.215
S. purpurea var. *montana*	0.093	0.023	0.151
Hybrid	0.269	0.081	0.514

4. Discussion

The results of this study were mixed. On one hand, the protease assays produced the expected trend, in which *S. jonesii* showed the highest protease activity, *S. purpurea* var. *montana* showed the lowest activity, with the hybrids showing intermediate activity (Fig. 1). This is likely due to *S. jonesii* either producing a larger quantity of proteases leading to higher absorbance values during the assay, or because *S. jonesii* produces proteases that are more active than those of the hybrids and *S. purpurea* var. *montana* while not necessarily producing a higher quantity of enzymes. Either way, the proteases present in *S. jonesii* lead to higher activity readings than that of its co-habitants. The low protease activity of *S. purpurea* var. *montana* supports the consensus that the main source of digestive enzymes in this species are its aquatic pitcher inhabitants[12,20,26,27]. Removal of these pitcher residents and subsequent addition of distilled deionized water would likely not remove all bacteria and invertebrates present[42], and thus the low absorbance reading is most probably the result of residual inquilines and perhaps a small amount of plant-produced proteases.

On the other hand, the phosphatase assay results were unexpected. The hybrids, not *S. jonesii*, showed the highest phosphatase activity out of the three plant types (Fig. 2). Most interesting of all is that the difference among the three types was just outside the threshold of statistical significance. Is this a fluke? Does this mean the hybrids are exhibiting a novel adaptation allowing them to secrete higher levels of phosphatases that give them a competitive advantage over their parent species? This is not an unusual phenomenon in hybrid plants. F_1 *Iris* hybrids were significantly more fit than their parent species as a result of increased photosynthetic efficiency and nutrient allocation[43]. In fact, hybrids

showing simultaneous vigor for one trait, and intermediacy for another, have been observed numerous times [34,44,45] and fall in line with the results of this study.

It would be interesting to repeat this experiment over multiple growing seasons, or on both newly opened and older pitchers to determine if phosphatase (and other enzyme) expression is developmentally regulated in the hybrids as it appears to be in *S. purpurea*[22]. Further research may elucidate if hybrids possess novel recombinant genes that allow them to produce higher quantities of phosphatases, and if this allows them to absorb and utilize nutrients more effectively and efficiently than the parent species. Nutrient allocation experiments like those performed on *S. purpurea* may provide answers to these questions[46].

Despite the unexpected results of this study, one thing is clear: although they appear a perfect 50/50 mix of each parent species morphologically, *S. jonesii* x *S. purpurea* var. *montana* hybrids are not intermediate in all traits. To the contrary, they may possess superior traits that enable them to pose a real ecological[47] and evolutionary[32,33] threat to their vulnerable parent species. This makes the need for managing the remaining populations of *S. jonesii* and *S. purpurea* var. *montana* even more pressing[48]. Results from this study coupled with genetic analyses performed on interspecific *Sarracenia* hybrids[49] reveal an extinction risk currently facing both species[50], and that immediate action is necessary if both are to be conserved in the future.

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