

PICKING PICKY FLOWERS: TESTING COMPATIBILITY SYSTEMS IN *Physalis acutifolia*
AND THE PREDICTABILITY OF OUTCROSSING SUCCESS

By

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Abstract

Although the overarching process of plant breeding biology has received considerable attention in the plant sciences, few studies thoroughly examine the complexities of breeding system variability. *Physalis acutifolia* is a self-incompatible (SI) flowering plant in the *Solanaceae* family native to the southwestern United States. Self-incompatibility (SI) is a reproductive adaptation that serves to promote outcrossing and increase genetic fitness, and it is the opposite of self-compatibility (SC), which permits self-fertilization. Occasionally, certain species will be capable of employing more than one reproductive strategy. In *P. acutifolia*, specimens were collected from a variety of locations in the southwest U.S., and SI and SC populations were identified through preliminary crossing experiments in a greenhouse. Using these data, I focused on pollination crosses of SI and SC populations of *P. acutifolia* with three other species in the *Physalis* genus. These designed crosses contained a variety of SI and SC populations, and were intended to test the SI x SC hypothesis, which states that species that are self-compatible are also more likely to be compatible with an SI species, but the converse would not be true. Based on this previous work, I hypothesized that a SC female (the flower being pollinated) would be successfully fertilized by an SI male (the pollen donor), but that the reciprocal would not be true. Flowers received one of four treatments: emasculated and not crossed (negative control); crossed with individuals from the same location (positive control); pollinated with self pollen (experimental treatment 1); and crossed with individuals of a different species (experimental treatment 2). There was a statistically significant difference between the crossing outcomes of SI and SC species (p-value: 0.00247). However, there is not sufficient evidence to suggest that *P. acutifolia* consistently behaves as predicted by the SI x SC

hypothesis. This experiment addresses the complexities of breeding systems, and is a necessary first step for understanding how and why such complexities occur.

Keywords: Plant breeding systems, plant reproductive biology, Physalis acutifolia, SI x SC rule, self-incompatibility, self-compatibility, outcrossing, self-fertilization

Introduction

Plants and pollinators have long shared an intimate relationship, and this deeply-seated coevolution has affected plant breeding system traits. The vast majority (~75%) of angiosperms (flowering plants) have a self-incompatible (SI) breeding system (Knapp, 2010), and is found in most major plant families (Pandey, 1960, Goldberg & Iqic, 2012). SI is a prezygotic breeding system that is reliant on pollinators to promote outcrossing because SI plants are not receptive to pollen from their own flowering (Nasrallah, 2017).

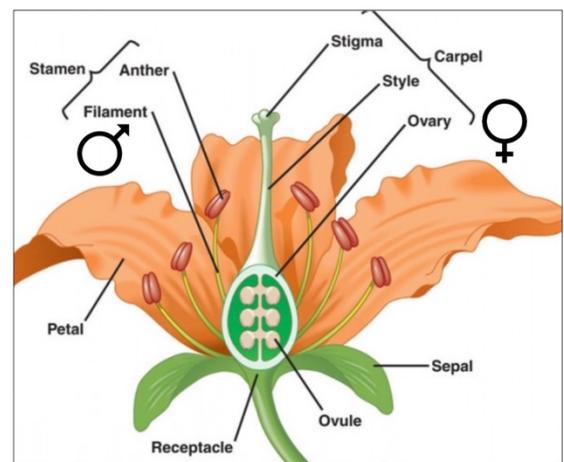


Image 1: Hermaphroditic flowering plant reproductive anatomy (gametophytic).

The evolution of ensures high quality offspring, as many studies have demonstrated that outcrossed species yield greater fruit size, growth rate, and germination rates than self-compatible (SC) species (Chauta-Mellizo et. al, 2012, Goldberg & Iqic, 2012). In contrast to SI, SC can utilize self-fertilization and therefore does not require pollinators, wind, or water to move pollen from the male anthers to the female stigma (Baker, 1955).

However, sometimes a species is capable of using both SI and SC for its mode of reproduction, and this trend is referred to as “intermediate SI” (Pandey, 1957). One factor that might be responsible for this reproductive variation among species is different microclimates across population ranges (Sakai & Weller, 1999). For species that thrive across a large range,

there can be notable ecological differences such as the amount of rainfall per year and frequency of pollinator visits (Berry & Calvo, 1989). Moreover, SI is thought to be the ancestral state of flowering plant reproduction (Ilgic et al., 2008). Overall, plants have been shown to make the shift from an SI to an SC system, but the reverse has not occurred (Goldberg, 2010). With these strong adaptive factors, populations may adapt their breeding strategies to meet the needs of their environment over evolutionary time (Goldberg, 2010).

In this study, we seek to investigate plant breeding systems by testing a common hypothesis, the SI x SC rule. This rule is a suggested relationship which states that SC species will unilaterally accept pollen from SI species, but SI species will not accept pollen from SC species (Baek et al., 2015). Testing this hypothesis is critical for not only gaining insights into evolution, but also into the genetic and molecular forces that play a large role in selecting for these breeding systems. In summation, understanding patterns in plant reproductive behavior is an imperative first step for designing future plant reproduction experiments.

There has been no prior research on the reproductive behavior of our selected species, *Physalis acutifolia*, thus making this an ideal system to work with. *Physalis* is an important genus that includes edible crops with a role in farming and agriculture (Valdivia-Mares et al., 2016). Even though *P. acutifolia* is a wild species, understanding its breeding system can assist with crop improvement of the species in this genus that have been domesticated.

This project consists of two experiments. First, I assessed the crossability within *P. acutifolia*, and then I tested crossability of other species to *P. acutifolia*. The results of experiment one revealed *P. acutifolia* to have an intermediate SI system (Figures 2, 3). These data prompt the question at the center of this research project: does breeding system variability in

P. acutifolia predict the ability to cross with other species in the genus *Physalis* as in the SI x SC rule?

Background

The vast majority of flowering plants utilize self-incompatibility (SI) as their breeding system (Igic & Busch, 2013). SI is favored because it increases gene flow, thus increasing genetic fitness and diversity (Nasrallah, 2017). Because SI systems are not receptive to pollen of their own flowers, they rely on pollinators to promote outcrossing (Sullivan, 1984). This reproductive pressure is likely to be a reason for the evolution of self-compatible (SC) systems, as a way to provide reproductive assurance for the plant when resources are limited (Grossenbacher et al., 2017).

Furthermore, the transition from outcrossing to SC, a ‘selfing’ mechanism, is one of the most prevalent evolutionary transitions in flowering plants (Nasrallah, 2017). SC created reproductive assurance for the plant (Cruden, 1976). However, while SC may seem like a convenient strategy, SC lineages eventually fail, either due to a narrow capacity to adapt to changing environments, or increased susceptibility to an accumulation of deleterious mutations (Igic & Busch, 2013).

Recently, evidence surrounding mixed-mating systems within a single species has been growing (Roda & Hopkins, 2018). A single species can employ both SI and SC strategies, with this variation being attributed to several environmental and molecular factors (Igic & Busch, 2013). Preliminary data of this research detected that in *P. acutifolia*, one sample population collected from New Mexico exhibited SI, while the second sample population collected from Arizona displayed SC behavior. Such a mixed-mating system is also referred to as an

intermediate breeding system, which refers to the “leaky” nature of the genes that regulate reproduction.



Image 2: Native range of *P. acutifolia*. (Taken from <https://plants.usda.gov/core/profile?symbol=phac6>)

There are several factors worthy of investigation to fully understand intermediate breeding systems. In certain families of flowering plants, it is the S-locus (located in the pistil) that prevents self-fertilization (Thompson & Kirch, 1992). In Solanaceae, SI depends on the expression of S-RNases located in the S-locus (Takayama & Isogai, 2005). This expression depends on messages received from the F-box proteins expressed in pollen (Baek et al., 2015). When reproduction begins, a haploid pollen grain must arrive at the tip of the sticky stigma, which then forms a pollen tube that may grow through the diploid style and into the ovary (Mulato-Brito, 2007). In an SI population, if the pollen was self-pollen, then the S-locus would reject the pollen. This is believed to happen because the self-pollen carries an allele that is also expressed in the style, which can be indicated by an increase in S-RNase concentration (Richman & Kohn, 1999). Additionally, the expression of S-alleles in the pistil can also selectively reject any cross pollen carrying alleles that are identical to the pistil (Lu, 2001). The interaction of alleles at the S-locus are inconsistent in species that show an intermediate breeding system, that is likely from one or more of the genes that regulate these processes becoming non-functional at some point in the lineage (Lu, 2001). Lastly, one species under study in this experiment is *Physalis philadelphica*. *P. philadelphica* is a cultivated species of tomatillo that has been found

to have a “loss-of-function” mutation in the S-gene at the S-locus, the gene that regulates compatibility. For this reason, *P. philadelphica* is a strictly SC species because it does not have a functioning expression of genes that reject non-self pollen (Li, 2011).

My research utilizes the knowledge of the known molecular basics while examining the behavior of a cross-breeding population. Thus, the results of this research provide a necessary first layer to make sense of what happens during reproduction at the molecular level. Furthermore, in order to investigate breeding behavior, this research employs the SI x SC hypothesis to investigate crossing behavior in *P. acutifolia*. *P. acutifolia* is a species deemed to have an intermediate SI system through preliminary crosses that will be discussed in this study. While the SI x SC rule is an established hypothesis among plant biologists, no research has applied this rule to *P. acutifolia*.

Specifically, the SI x SC rule states that an SI species will not be receptive to pollen from an SC species, but the reciprocal crosses are compatible (Baek, 2015). By taking into consideration the native range, microclimates, and locations on the phylogeny of the selected species of *Physalis*, we can predict the potential for hybridization within *Physalis* by performing crosses from which we can make further analysis.

To highlight this, we can also make predictions about hybridization by applying a biogeographical lens. Populations of *P. acutifolia* used in this study were obtained from three different microclimate locations: Southeast Arizona, New Mexico, and Southern California. With this information, we can interpret our data in light of several biogeographical factors. For example, ecological conditions dictate many generations of trait selection (Knapp, 2010), which impact whether *P. acutifolia* will exhibit either SI or SC behavior. Also, specific environmental

conditions affect plant adaptations, which can help us understand differences in populations who use SI or SC strategies.

Materials and Methods

(I) *Sample Collection*: Given that *P. acutifolia* is an annual plant, specimens need to be recollected every year. In the summer of 2017, *P. acutifolia* specimens were collected from natural settings in Deming and Las Cruces NM, and Sonoran Desert, AZ. These specimens were used for hand pollinations from September 2017-March 2018 in a controlled greenhouse setting (Ramaley Greenhouse, EBIO, Boulder, CO).

In the summer of 2018, specimens of *P. acutifolia* were both ordered from a seed bank (USDA Germplasm Resources Information Network (GRIN)) and collected from a natural setting. Seeds sent from GRIN were native to California. Specimens from the natural setting were collected in Tuscon and Cochise County, AZ, and used for hand pollinations from July 2018-September 2018 in a controlled greenhouse setting.

(II) *Controlled Hand Pollinations in the Greenhouse*: Specimens collected from natural settings were brought into the greenhouse to be used for controlled hand pollinations (Image I). Every flower that was given a treatment (Table I) was marked with a jewelry tag and treatment was recorded into a database. The female (the plant being pollinated) and the male (the pollen donor) were recorded into a database along with treatment.

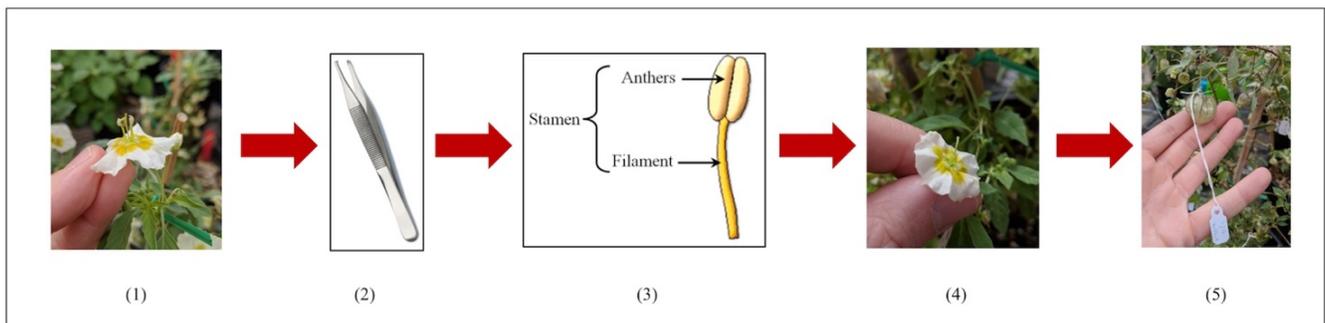


Image 3: Procedure for hand pollinations. (1) Select flower to pollinate prior to dehiscence (2) Remove stamen using forceps (3) Obtain a stamen from a flower that has already dehisced (4) Apply pollen from stamen to emasculated flower (5) Tag flower with treatment type and wait for fruit to set (if applicable).

Four species within of *Physalis* were used for pollinations (*P. acutifolia*, *P. philidelphica*, *P. cinerascens*, *P. peruviana*). This experiment began by selfing *P. acutifolia* (see overview of treatments – Table I) by applying self-produced pollen to the stigma of the same flower (Experimental Treatment 1; “Selfing”), by crossing between two different species (Experimental Treatment 2; “Hybrid”), crossing between populations from the same geographic location (Positive Control 1; “Between”), and by removing the anthers and not crossing the flower (Negative Control 1’ “Removed”). Data was collected and analyzed after these steps and further crosses were made incorporating new species to test for hybridization (Figure).

Treatment Type	Selfing	Hybrid	Between	Removed
Description of Treatment	Experimental 1: Self-pollinate individuals	Experimental 2: Cross one species with a different species	Positive Control 1: Pollinate between populations of the same species	Negative Control 1: Remove anthers only
Species Involved	<i>P. acutifolia</i> , <i>P. cinerascens</i> , <i>P. philidelphica</i> , <i>P. peruviana</i>	<i>P. acutifolia</i> , <i>P. cinerascens</i> , <i>P. philidelphica</i> , <i>P. peruviana</i>	<i>P. acutifolia</i> , <i>P. cinerascens</i> , <i>P. philidelphica</i> , <i>P. peruviana</i>	<i>P. acutifolia</i> , <i>P. cinerascens</i> , <i>P. philidelphica</i> , <i>P. peruviana</i>

Table 1: Description of the treatments and species involved.

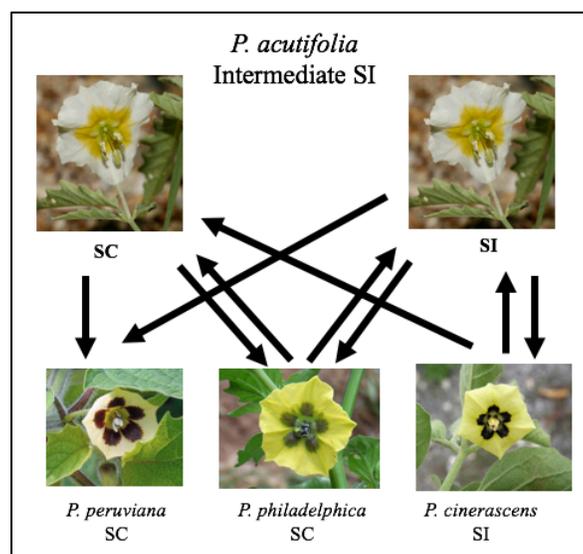


Figure 1: Experimental Treatment 4; “Hybrid”. These are all of the hybrid crosses that were attempted in this experiment. The crosses can be read as Male x Female. The male is the pollen donor and the female is the pollen recipient. The base of the arrow represents the male, which gives pollen in the direction of the arrow, leading to the female.

Once sufficient data to infer breeding system was obtained on *P. acutifolia*, hybrid tests were performed. This experimental treatment aimed at crossing populations of *P. acutifolia* that had self-compatibility (SC) with species of *Physalis* that had either SC (*P. peruviana* and *P. philidelphica*). An SI species of *Physalis* (*P. cinerascens*), was crossed with the SC population of *P. acutifolia*. Populations of *P. acutifolia* that were designated as SI were crossed with all the experimental species of *Physalis* as well (*P. cinerascens*, *P. peruviana*, and *P. philidelphica*).

(III) Fruit Weight, Seed Output, and Survived vs. Died Measurements: In experiment one, Fruits that formed as a result of hand pollinations were collected and labeled in a small envelope and brought back to the lab for examination. Before opening the fruit, fruits were weighed on electronic balancing scales and the weight was recorded in grams. Seed output was measured by cutting a fruit in half, taking out the seeds, and counting the seeds manually on a paper towel. Seed output was recorded, and seeds were dried and placed back into envelopes to be saved for later use.

For the hybrid crosses and second round of selfing crosses, the success of the cross was measured by if the flower survived after one week since the cross was performed. A survived flower received a 1, and a dead flower received a 0.

(IV) Plant Rearing and Maintenance: Greenhouse plants were carefully monitored and watered regularly. Thrips and aphids occasionally interfered with plant health and were moderated accordingly.

(V) Analysis: The results of this study were analyzed using R to obtain averages, conduct a one-way analysis of variance, and two-way analysis of variance for measurements of offspring

viability (survived vs. dead, seed output, fruit weight, and germinations). The independent variable of this study is female population that receives pollination, and the dependent variable is the species that the pollen came from. When running an analysis of variance test, we used our positive control, “Between Treatment” as the intercept for obtaining a p-value specific to the population being tested. In this context, using our positive control as an intercept gave us the most accurate representation of modeling variance, because our positive control is what we would expect to be the most common form of crossing in nature. Therefore, strong deviations from this positive control give us the best hint at detecting a significant difference.

(VI) Supplemental Data

(i) Pollen Tube Growth (Images 4,5,6) – Crosses were performed 48h prior to being collected for pollen tube growth examination. After the according time increment, the flower was collected, and the pistil was extracted to be placed in a softening solution. After the pistil was softened, pistils were placed in a staining solution. Pistils were left in staining solutions until the color was completely taken up by the pistil. Pistils were then mounted onto a slide and covered with a microscope slip. Microscope slides were taken to be viewed by a Leica Microsystems Fluorescent Microscope (HQ: Wetzlar, Germany).

(ii) Pollen Viability (Image 7) – Pollen was collected from *P. acutifolia* flowers, using a random number generator to collect the flowers. The pollen was then placed into a staining solution, centrifuged, and pipetted onto a microscope slide for viewing. These tests were performed in order to feel confident that pollen infertility was not a possible reason for a pollination to fail.

(iii) Fruit Weight Against Total Seeds (Figure 9)

This figure refers to crosses done in *P. acutifolia* experiment one only. This figure is meant to show that fruit weight tends to increase as seed output increases. However, there are outliers within the self-treatment. This graph was made in R in order to highlight heavy fruit weight does not always imply high seed output.

(iv) All crosses attempted for experiment two (Tables 2,3)

This is a supplemental figure meant to be used as a reference for taking sample size into account when viewing the results of experiment two. This table reveals the exact number of crosses performed in experiment two.

Results

Breeding System in P. acutifolia

This experiment set out to evaluate the breeding system of *P. acutifolia*. The prediction for this experiment was that a statistically significant difference in seed output outcomes for “selfing” treatment among different populations of *P. acutifolia* would indicate that *P. acutifolia* has an intermediate SI breeding system. This is because “selfing” treatment was deemed the most accurate way to determine if a population was capable of self-fertilization. If the population displayed self-fertilization, then it was deemed a SC population.

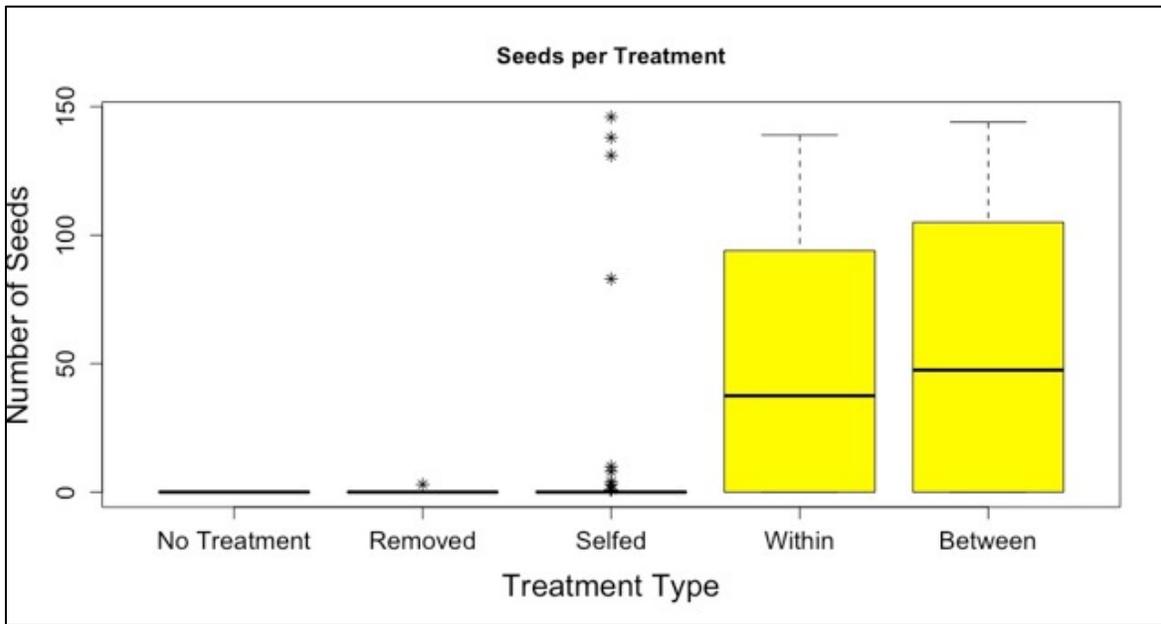


Figure 2: Seed output resulting from treatment given (p-value: $<3.7e-11$ with R-squared 0.247). “No Treatment” means no treatment was given to the flower, but the flower was tagged and taken in for collection after several weeks. “Within” refers to crosses within a single population, all of the same species. See table 1 for a description of the remaining treatments. Data is measured by the number of seeds produced for every given treatment.

Figure 2 provides the results of all crossing data performed on *P. acutifolia* only, using the sample populations from New Mexico and Arizona (Methods I, II, III). The black line above each assigned treatment stands for the mean. In the “Selfed” treatment column, the mean is zero, therefore the starts just above the mean line represent a cluster of values less than five, with the stars greater than 50 number of seeds representing outliers. There is not a significant difference in the means of “within” and “between” treatments.

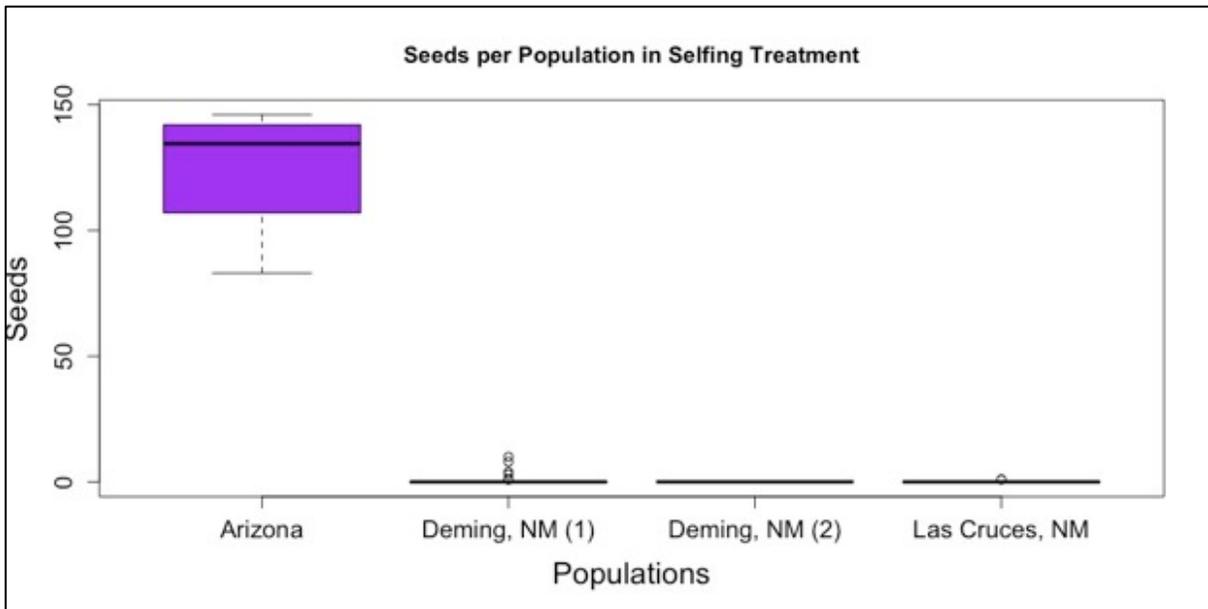


Figure 3: Seed output resulting from “selfing” treatment (p-value: $<2.2e-16$ with R-squared 0.96). Specimens in this graph were collected from two different areas in Deming, NM.

Figure 3 represents results from “selfing” treatment on *P. acutifolia* only. The results of this graph frames the research question for experiment two of this research, because we have a statistically significant value that suggests a significant difference between the populations from New Mexico and the population from Arizona. The results of experiment one allowed us to feel confident that *P. acutifolia* has an intermediate breeding system of only Arizona having the ability to self, while the other population are self-incompatible.

The Crossability of SC/SI Species to Similar Breeding Systems:

Experiment two uses interspecies crosses, deemed “hybrid” treatment, in order to test the SI x SC rule in *P. acutifolia*. Rather than use fruit weight and seed output as a viability measurement, experiment two measures viability by if the flower survived or died five days to two weeks after the cross was performed.

Two-Way Analysis of Variance:

P. peruviana was omitted from the two-way analysis of variance (ANOVA) because no crosses that went to *P. peruviana* survived. When the means from all of the *P. acutifolia* SI population crosses are ran with all of the *P. acutifolia* SC population crosses in a two-way ANOVA, we obtain p-value: 0.0247.

One-Way Analysis of Variance:

SC and SI populations of *P. acutifolia* were pollinated with pollen from different species. A one-way ANOVA was run to obtain a p-value for each hybrid treatment cross.

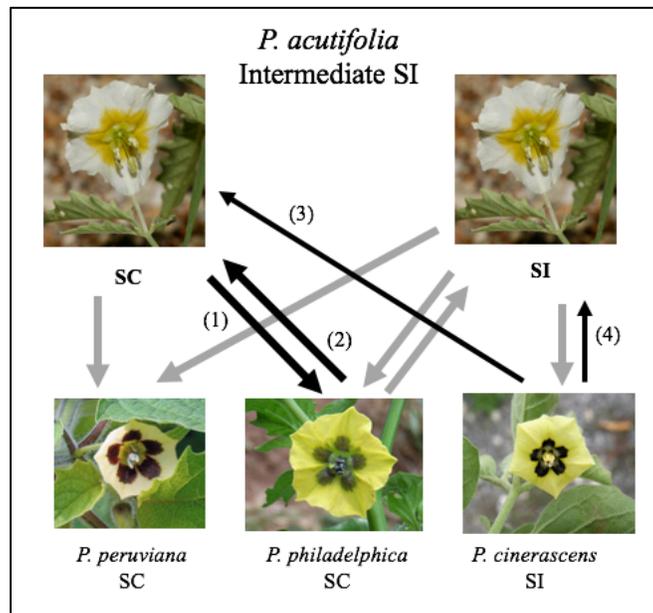


Figure 4: The crosses in which the flower survived. Crosses are read as Male x Female, with the male represented by the base of the arrow. Gray lines represent that no flowers survived out of all of the crosses performed. Average success is the average amount of times a flower survived when that specific cross was performed. Of the survived crosses: (1) *P. acutifolia*, SC x *P. philadelphica*; average success = 1. (2) *P. philadelphica* x *P. acutifolia*, SC; average success = 1. (3) *P. cinerascens* x *P. acutifolia*, SC; average success = 0.667. (4) *P. cinerascens* x *P. acutifolia*, SI; average success = 0.5.

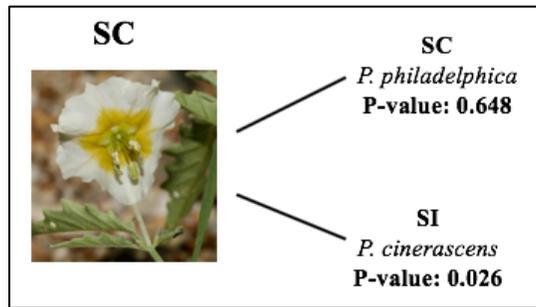


Figure 5: The self-compatible population of *P. acutifolia* was pollinated with *P. philadelphica* and *P. cinerascens*. The significance level differs by species cross.

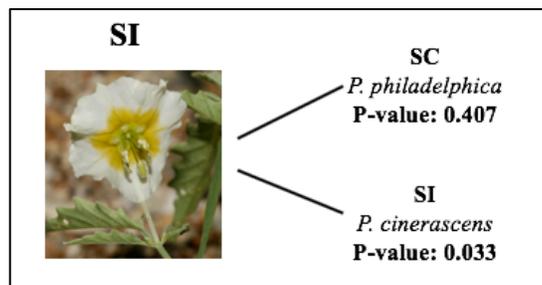


Figure 6: The self-incompatible population of *P. acutifolia* was pollinated with *P. philadelphica* and *P. cinerascens*. The significance level differs by species cross.

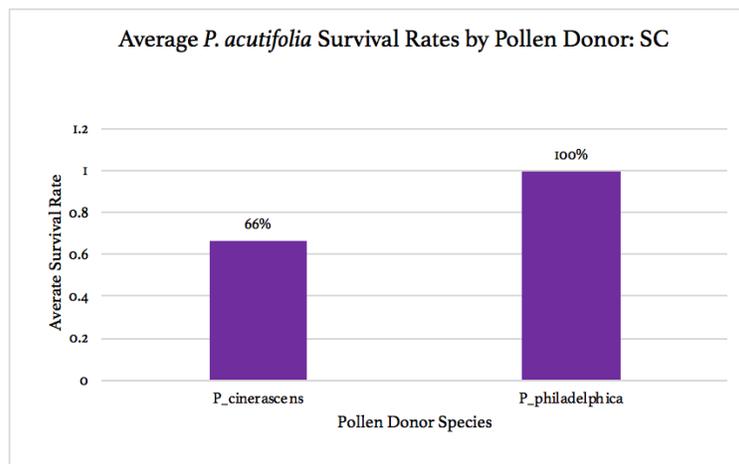


Figure 7: The average SC *P. acutifolia* survival rates differs between species.

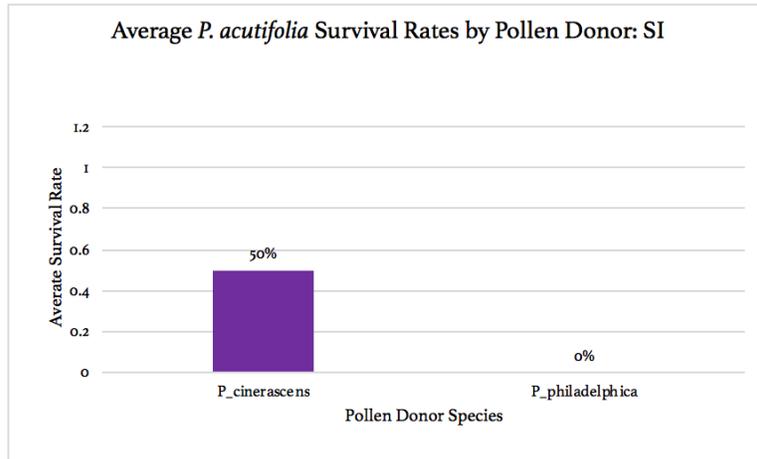


Figure 8: The average SI *P. acutifolia* survival rates differs between species.

Discussion

Breeding System in P. acutifolia

In this study, our data suggests that *P. acutifolia* has an intermediate SI breeding system, and is sometimes capable of crossing with other species in the *Physalis* genus. In Figure 2, the highest means are in the “Within” and “Between” treatment box and whisker plots. These treatments simulated what a natural pollinator would do, which is to move pollen around. This graph is a representation of crossing treatments of *P. acutifolia* before we had detected a difference in our SI and SC population. We would expect high means for “Within” and “Between” treatment because the majority of self-incompatible plants will produce a high seed output when outcrossed (Goldberg, 2010). While we observe a high seed count for the selfing treatment as well, this treatment group did not receive nearly the same amount of crossing attempts as “Within” and “Between” treatment did.

Furthermore, we applied our understanding of *P. acutifolia* as an intermediate SI system by testing the SI x SC rule, which states that self-compatible species will be more receptive of

pollen from a self-incompatible species than the reverse (Baek et al., 2015). There are several explanations behind *P. acutifolia*'s ability to be a selfing plant, an outcrossing plant, and a potential hybridizing plant.

One factor that may explain *P. acutifolia*'s intermediate SI system is collecting site location. In Figure 3, the population capable of producing seeds from self pollen was from Arizona, while three populations from New Mexico produced mean = 0 seeds from self pollen (Figure 3). Plants have the ability to adapt reproductive needs in order to survive in the surrounding environment (Grossenbacher et al., 2017). The collecting site in Arizona was a drier and more desolate location than sites in New Mexico. Because self-compatibility provides reproductive assurance, especially in the absence of resources and pollinators, (Nasrallah, 2017), it is possible that *P. acutifolia* made a transition from SI to SC somewhere in its lineage in order to survive in its desolate location.

To build from that, it is important to acknowledge that *P. acutifolia* has a large native range (Image 2). In experiment two, our SC population of *P. acutifolia* came from a seed bank, where it was collected in California (Methods 1). California is the end of what is known to be *P. acutifolia*'s native range. With this, we can infer the possibility of an edge effect. Numerous ecological studies document edge effects on species behavior (Angert et al, 2011). In plants, edge effects can change the composition of pollinators, as well as other animals (Cunningham, 2000). If the composition of natural pollinators was less abundant at the species' edge than middle range, then it would not be surprising that we found a self-compatible system. This inference could also explain why the population from the desolate location in Arizona exhibited SC breeding behavior. Both scenarios highlight a behavioral adaptation in response to an ecological shift, which is very common strategy in nature (Murcia, 1995).

Additionally, another explanation that has little to do with pollination ecology is possible. In an intermediate SI system, molecular factors may be playing a large role in explaining breeding system variability. In the pistil – the female reproductive organ – the S locus is the site that can prevent self-fertilization (Thompson & Kirch, 1992). This happens due to a very specific interaction between the S-gene contained in the S-locus, and the S-gene products that are expressed by the pollen (Thompson & Kirch, 1992). In an intermediate SI system, the genetic signals at the S-locus are inconsistent, which is visible when intermediate SI behavior is examined. In summation, the intermediate SI system we observe in *P. acutifolia* might not be the product of its environment, but rather an inconsistency of gene functioning at the S-locus. From here, we can speculate about how these genes have been carried throughout evolutionary history, and future research could search for a time in the lineage that one or more of these genes became broken (Lu, 2001). Further investigation is necessary in order to conclude if the intermediate SI system in *P. acutifolia* is due to ecological factors, genetic factors, or a random combination of the two.

The Crossability of SC/SI Species to Similar Breeding Systems:

In experiment two, we sought to use our designated SI and SC populations of *P. acutifolia* to determine if *P. acutifolia* followed the SI x SC rule. Because several studies in the *Solanaceae* family have experimented with this rule, we hypothesized that *P. acutifolia* would align with this rule. Our obtained p-value = 0.0247, which suggests that there was a statistically significant difference in the mean rate of survival sample group; the populations in *P. acutifolia* SI were accepting pollen at different rates than populations in *P. acutifolia* SC. However, our p-value does not provide us with enough information to suggest that *P. acutifolia* followed the SI x

SC rule. For this reason, I speculate on the data on a per species cross basis. Curiously, some of our results were surprising because they challenged the SI x SC rule.

In a *P. acutifolia*, SI x *P. cinerascens* hybrid cross we obtained a percent mean = 50% cross success rate (p-value: 0.033). We would not expect any SI x SI cross to be successful in any scenario under the SI x SC rule (Baek, 2015). Additionally, SI plants have strong pollen-discriminating factors, which contributes to their ability to be highly selective (Takayama & Isogai, 2005). Thus, these results would be surprising even if we were not experimenting with conditions under the SI x SC rule. To build off of that, the reciprocal crosses, *P. cinerascens* x *P. acutifolia* SI did not result in any survivors (percent mean = 0%, supplemental data, table 3). Because of these differences, we might turn to using theories on directionality. The relationship between *P. acutifolia*, SI and *P. cinerascens* could be explained through unilateral incompatibility (UI). UI states that crosses can be compatible going in one direction, though reciprocal crosses may not work (Broz et. al, 2017). UI relationships comprise a part of the overall SI x SC mechanism that the rule is based on, although UI is not synonymous with the SI x SC rule (Broz et. al, 2017).

Moving forward, results from *P. acutifolia* SC x *P. philadelphica* (p-value: 0.648) and their reciprocal crosses (percent mean = 100%, supplemental data, table 3) all maintained livelihood. It is likely that we receive a statistically insignificant p-value from these crosses because a there were a great deal of survivors, which suggests this data are not significantly different from our intercept value, “Between” treatment. Moreover, these results do not align with the hypothesized SI x SC rule. The SI x SC rule does not predict that SC x SC crosses will yield fruitful offspring (Baek et al, 2017). However, this data hint that a bilaterally compatible relationship exists between the two species.

These results are not surprising because SC species do not have as strong of a pollen discriminating mechanism as SI species, therefore making them more likely to be receptive of a wider range of pollen (Pandey, 1960). The species of interest involved in explaining this bilaterally symmetric cross is *P. philadelphica*. As a highly cultivated tomatillo plant, *P. philadelphica* contains a loss-of-function mutation that limits its ability to discriminate between self and non-self pollen at the stigma (Li, 2010). Because *P. philadelphica* was being crossed with the self-compatible population of *P. acutifolia*, it is not surprising that discriminating mechanisms were low, and the crosses maintained survivorship.

Moving along, we observed a statistically significant difference in *P. acutifolia* SC x *P. philadelphica* (p-value: 0.026) This is the one result that followed the SI x SC rule. The SC population of *P. acutifolia* was receptive of pollen from an SI species (percent mean = 50%, supplemental data, table 3). Although statistically significant, this crossing design would benefit from increasing sampling size, because then we could feel more confident in our inference that *P. acutifolia* exhibits the ability to follow the SI x SC rule.

Lastly, we did not get a statistically significant value for *P. acutifolia* SI x *P. philadelphica* (p-value: 0.407, percent mean = 0%). However, this data is negligible because we had a sample size of one for this particular cross. An increase in sample size is absolutely crucial for this population specific cross. As a result, there is some evidence to suggest the *P. acutifolia* is, at the very least, capable of following the SI x SC rule, however we do not have enough samples to make any conclusive statements, or extend this research to imply how populations of *P. acutifolia* from other geographic areas might behave.

In this experiment, there were a few limitations that prevented gaining more progress in the repetition of crosses, and that might have been a confounding factor in a flowers' survival

after being crossed. First, basic physiology might have been at play. It requires a great deal of energy to produce fruit, but only a small amount of energy to produce pollen (Sullivan, 1984). If resources are not available for making fruit, then fruit cannot set. For many weeks during experimentation, nearly all plants were affected by aphid infestation and mildew, which resulted in damage as well as receiving a heavy spraying treatment. Unhealthy plants are less likely to produce quality offspring than healthy plants, and many populations had long periods of recovery after spraying. Second, there is a possibility that species selected for hybrid crosses were too distantly related on the phylogenetic tree to be able to produce fruit. According to the biological species concept, species that are too distantly related face a pre-zygotic barrier to mating (Queiroz, 2005). We may speculate that genetic barriers played a role in stopping pollen from reaching the ovule. Third, the lack of fruit production may have happened because of random chance. Generally speaking, there may be nothing technically erroneous with the plant, but for a combination of energetic factors, the flower chooses not to make fruit.

This experiment would benefit by performing crosses such that there is an even spread of crossing attempts for each population. This is the best measure to permit certainty that the results from each population are different. In addition, this experiment would benefit through greater repetition, such as increasing crossing attempts to a minimum of fifty crosses for each population. It would also improve this study to test out the same procedure in either a natural setting, or a garden. Doing so would help gain better insight on how plants reproduce in more realistic conditions.

Additionally, this experiment was limited by testing the SI x SC rule by measuring if a flower survived or died after a cross. The most accurate way to measure crossing success is to follow a cross all the way through from pollination to fruit output, seed set, and seed

germination. By using these measurements, the data would be a more accurate representation of the success of crossing.

Finally, the results of this study could be expanded by examining what happens at the molecular level in incompatible systems. Previous breeding system research on Solanaceae plants have examined the concentration of S-RNase as a way to understand the mechanism of incompatibility in plants (Thompson & Kirch, 1992). Many compatibility mechanisms are found in this S-locus of flowering plants, so more research is needed to connect the interaction between the S-locus and fruit formation.

In conclusion, the insight gained from this experiment may prove to be incisive to any plant biologist. Conducting crossing experiments is a necessary first step to understanding an organism's behavior, which paves the way for lucrative future research. An overarching theme gathered from this research is that studying systems on a population level yields a great deal of complexity. The main idea is that plants will utilize a strategy that ensures the best production of offspring, and survival of that offspring. In most cases, strong offspring comes from parents with genetic diversity, but plants may forego this and resort to self-fertilization for reproductive assurance. More research is necessary to understand the molecular biology, biogeography, and phylogenetics of the *Physalis* genus in order to pull together conclusive evidence about testing if the SI x SC rule holds. Just as necessary, the SI x SC rule needs to be tested in other genera in Solanaceae if scientists are to best understand breeding system variability, because the ability to make comparisons between systems can provide much needed insight.

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Supplemental Data

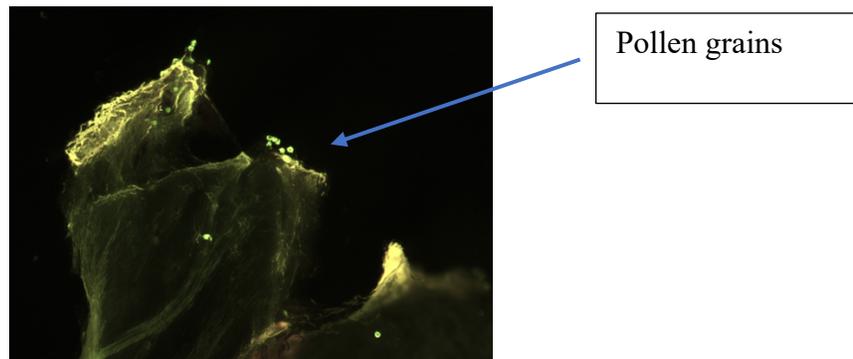


Image 4: Image using fluorescent microscopy. This is the tip of the stigma in *P. cinerascens*. The glowing green circles represent pollen grains.

Stigma

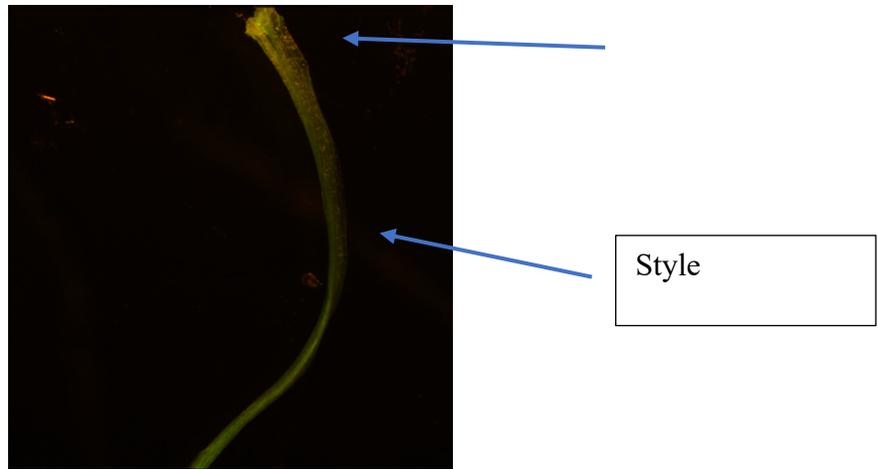


Image 5: The style of a self-incompatible flower in *P. acutifolia*.

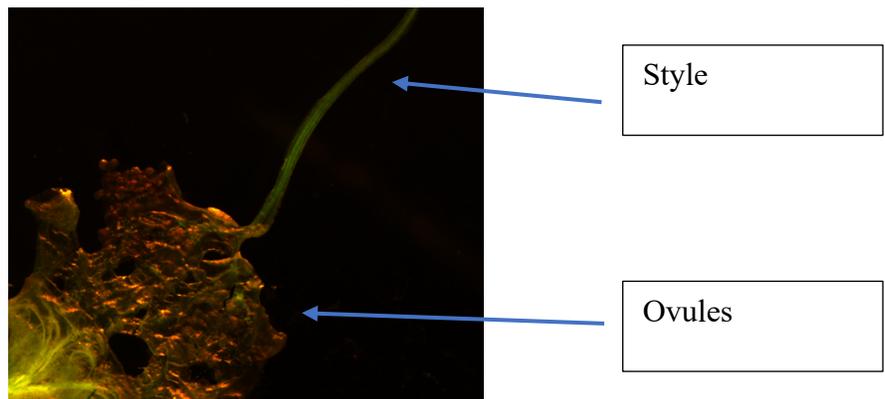


Image 6: The ovules, contained in ovary, connecting to the style in an SI *P. acutifolia*

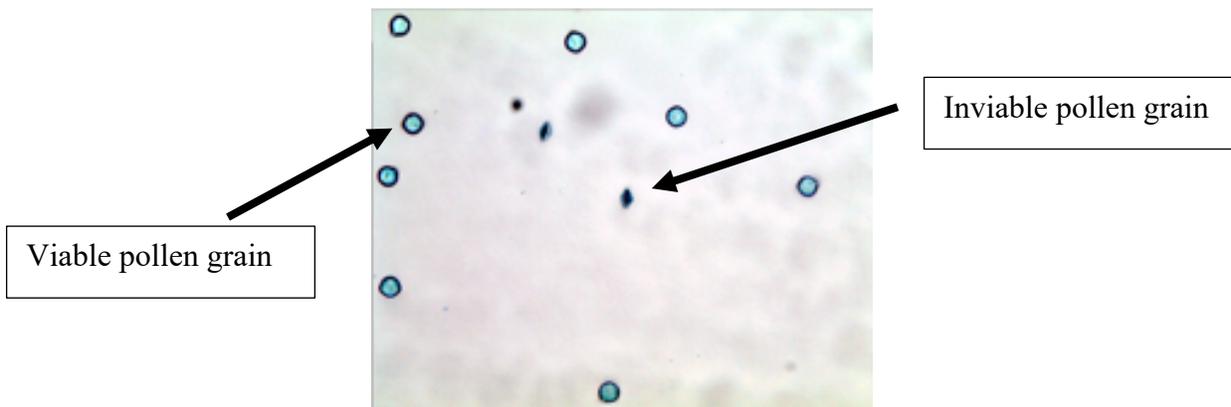


Image 7: Stained pollen grains from *P. acutifolia*. The less discernable, less circular figures represent pollen grains that are not viable and did not absorb the stain properly.

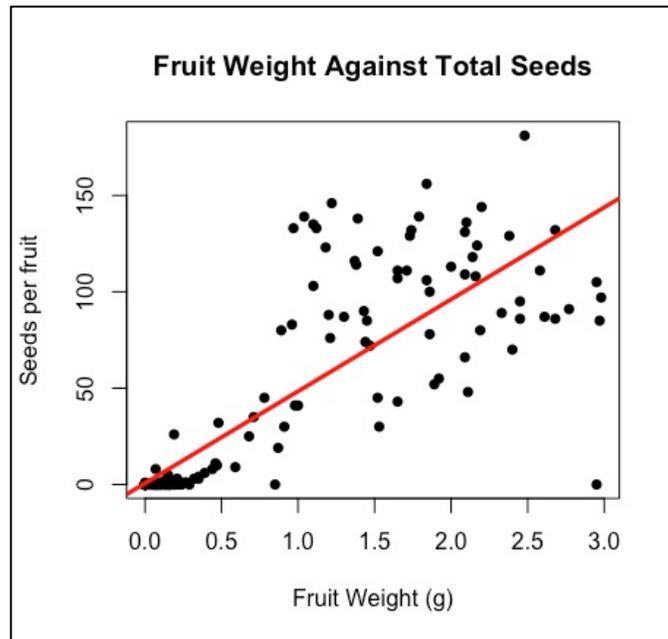


Figure 9: Fruit weight against total seeds (p-value: $<2.2e-16$ with R-squared 0.7082). Seeds per fruit tends to increase as fruit weight increases, though it is possible to have outliers. Crosses done in *P. acutifolia* experiment one only.

Female	Male	Attempts	Successes	Mean
<i>P. acutifolia SC</i>	<i>P. cinerascens</i>	15	10	0.667
<i>P. acutifolia SC</i>	<i>P. philidelphica</i>	1	1	1
<i>P. acutifolia SI</i>	<i>P. cinerascens</i>	8	4	0.5
<i>P. acutifolia SI</i>	<i>P. philidelphica</i>	1	0	0
<i>P. acutifolia SI</i>	<i>P. peruviana</i>	2	0	0

Table 2: The number of times a cross was attempted, succeeded, and calculated mean. Female refers to the species receiving pollen, male refers to the species donating pollen.

Female	Male	Attempts	Successes	Mean
<i>P. cinerascens</i>	<i>P. acutifolia SI</i>	4	0	0
<i>P. philidelphica</i>	<i>P. acutifolia SI</i>	1	0	0
<i>P. philidelphica</i>	<i>P. acutifolia SC</i>	4	4	1
<i>P. peruviana</i>	<i>P. acutifolia SI</i>	5	0	0
<i>P. peruviana</i>	<i>P. acutifolia SC</i>	3	0	0

Table 3: The number of times a cross was attempted, succeeded, and calculated mean. Female refers to the species receiving pollen, male refers to the species donating pollen.

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