April 2014

Potential Heritable Aphid Tolerance and Resistance in Phaseolus vulgaris

Jeannette Marie Gerry
Worcester Polytechnic Institute

Poonam S. Barot
Worcester Polytechnic Institute

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WPI

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

Jeannette Gerry
Poonam Barot

Submitted to
Professor Lauren Mathews
Professor Michael Buckholt

Date:
April 29, 2014

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Abstract

The common bean, or *Phaseolus vulgaris*, is used often in both agriculture and scientific research, but the plant is frequently found infested with the insect pest *Aphis fabae*, the black bean aphid. The current project is the beginning of an endeavor to research the evolutionary relationship between the parasite and host. We hypothesize that tolerance to infestation by the black bean aphid in the common bean is a genetically heritable trait, allowing the plant to reproduce successfully.

Acknowledgements

We would like to thank our professors Lauren Mathews and Mike Buckholt for their patience and guidance throughout this project; we would especially like to thank the professors for their assistance with data collection when we were unable to be on campus and advice on the analysis of our data. We would also like to thank Abbie White for her guidance and constant assistance to us inside and outside of the greenhouse.

Introduction

Though genetic diversity among beans plants has been detailed extensively and the specific effects of aphid infestation on bean plants has been investigated as well, the exploration of the coevolution of the two is limited. The common bean, or *Phaseolus vulgaris*, has had its genome sequenced, and the relatedness between common bean populations has been investigated as well (Blair et al, 2003; Blair et al, 2006; Kami et al, 1995; Kwak and Gepts, 2009; Chacon et al, 2007; Sonnante et al, 1994). Aphid infestation of bean plants is affected by conditions ranging from host plant root bacteria to host plant genome variation (Pineda et al, 2011; Utsumi et al, 2011). The current project is the beginning of an endeavor to research the coevolutionary relationship between host and parasite. We hypothesize that tolerance to infestation by the black bean aphid in the common bean is a genetically heritable trait, allowing the plant to reproduce successfully.

1.1 Coevolution

Coevolution as a concept was first introduced by Ehrlich and Raven (1964) to describe butterfly larvae and host plant interactions; they suggested that the plant hosts had developed defenses as a result of the larvae feeding habits and that the larvae have developed their feeding habits to successfully feed on the host plants even with the plant defenses. This process is the stepwise model of coevolution, where one species evolves in direct response to pressures from another species, and vice versa (Fox, 1988). An alternative model, called diffuse coevolution, has been proposed as well. Diffuse coevolution has been proposed to occur along the same lines as stepwise coevolution, except many species at different levels in the food chain are involved and exert selective pressures directly or indirectly on each other, causing evolutionary changes in all species (Fox, 1988). Stepwise coevolution is most applicable in situations involving direct interactions, such as the contact resulting from aphids feeding on plants (Futuyma and Slatkin, 1983). Coevolution that can be visibly observed over time results from novel gene interactions that cause a change in the phenotype of the species; the novel phenotype then exerts selective pressure on the second species to adapt down to the genetic level as well (Stearns and Hoekstra, 2005). Finally, another hypothesis, called the Red Queen Hypothesis, explains coevolution as a never-ending struggle between species, which results in no reduction in the probability of either species becoming extinct (van Valen, 1973).

1.2 Coevolutionary Relationships between Phytophagous Insects and their Host Plants

There are several types of relationships that can be distinguished between phytophagous insects and their host plants. Phytophagous insects, such as aphids, are insects that either in their larval or adult stage feed on plant tissues or plant saps, excluding nectar and pollen (Jermy, 1984). Aphids are mono- or oligophagous on distantly related plant species. Monophagous describes an animal that eats only one kind of plant for food. On the other hand, oligophagous describes an animal that eats a few specific kinds of food (Bernays & Chapman, 1994; Jermy, 1984). Oligophagous insects are also capable at identifying the best individuals in a plant species as opposed to identifying the host plant species as a whole (Jaenike, 1990). In other words, oligophagous insects are less likely to recognize a specific plant species as their constant host; instead, they feed on plants that are easily available or healthier, regardless of species (Bernays & Chapman, 1994).

Many experiments have been conducted to explore the role evolution plays in the insect/host plant relationship. One such experiment yielded the classic coevolutionary hypothesis that is highly accepted in relation to specific insect/host plant relationship, the hypothesis first developed by Ehrlich and Raven (1964). The hypothesis states that the selection pressure exerted by phytophagous insects enhances the development of the defense mechanisms in the host plants. These newly defensive plants will then enter a new adaptive zone, a term that refers to a set of ecological niches or the response of an organism or population to the distribution of resources and competitors, which may be occupied by a group of species that use the same resources in a similar fashion (Mitter, 1988). This shift may be followed by an evolutionary radiation, in which adaptive change drives an increase in diversity of groups of biological organisms that share characteristics and an increase in disparity of morphology, the form and structure of organisms and their specific structural features, due to adaptive change (Wesley-Hunt, 2005). As the plants evolve and adapt, sometimes the insects do as well. In this way, the insects could also enter a new adaptive zone where they can diversify through speciation into new biological lineages due to the lack of other competing phytophagous insects. Both the insects and their host plants can diversify due to these reciprocal selective responses.

Richard and Guedes (1982) state that coevolution, in terms of host plants and insects, has occurred when the phytophagous insect species adapts to a new plant taxa as the insects' host with no reciprocal effect; this is quite different from how Ehrlich and Raven define coevolution (Jermy, 1984). There are several other authors such as Smiley (1978) and Fox (1981) who define coevolution differently from Ehrlich and Raven and furthermore, there are even authors who claim that there is no evolutionary feedback between the insects and their host plants (Eastop, 1973; Strong, 1979; Hendrix, 1980; Powell, 1980).

Some evolutionary experiments have attempted to test the hypothesis that an evolutionary increase in the performance of an insect lineage on one plant species will result in a reduction in adaptation of that insect lineage to other potential hosts (Jaenike, 1990). As an insect species feeds on a plant species, that insect species will not be exposed to defenses of other plant species. The use of a single host will then lead to an absence in the insect species' evolutionary response to the other plant species. If this cycle between insect and single host continues, the insect species will stop requiring other food sources besides the single host plant and therefore the insect will not need to adapt to other potential hosts. Surprisingly enough, in almost every case, the correlation of single host and insect species was not only disproved but the studies also suggested a positive correlation; performance on one host species has no effect or a positive effect on the performance on others (Via, 1991). This correlation was not expected as feeding on a specific plant species host could eventually lead the insects to focus only on that plant and no other, leading to decreased ability to adapt to other plant species.

1.3 Aphids and their Hosts

Many species of aphids are common and well known pests in the eyes of farmers and gardeners. They feed on various plant species, including many agricultural crops, primarily on the plant sap which deprives the plant of its vital nutrients. Additionally, aphids can transmit plant viruses such as bean common mosaic virus, turnip mosaic virus, and carrot virus Y, among many others (Persley, 2009). The aphids also secrete a sticky honeydew liquid that attracts mold. Aphids feed on the plants by inserting their microscopically thin stylet into the plant phloem, the living tissue that carries organic nutrients, particularly sucrose (Giaquinta, 1983). The aphid spits out gelling saliva that sets around the stylet, and this gel protects the stylet as it punctures through the plant tissue. The aphid then sucks out the sucrose rich sap. As the aphids feed on the

plants, the aphids also inject saliva which causes some plants to develop deformities such as hard stems. The honeydew liquid secreted by the aphids can often completely cover leaves and stems, depending on the severity of aphid infestation. This coverage not only facilitates the growth of fungi called sooty molds, but the honeydew also attracts ants, flies, wasps, and other insects, which further destroy the plant.

This project focuses on a particular aphid species known as the black bean aphid (*Aphis fabae*). *A. fabae* are polyphagous, and their hosts include the many varieties of the common bean, *Phaseolus vulgaris*. The wingless form of the black bean aphid has a large round abdomen with short legs and short antennae (Moran, 1992). Members of the Aphidinae subfamily feed on primary and secondary hosts during the aphid life cycle, which vary depending on the time of year and the life stage of the aphids (Figure 1).

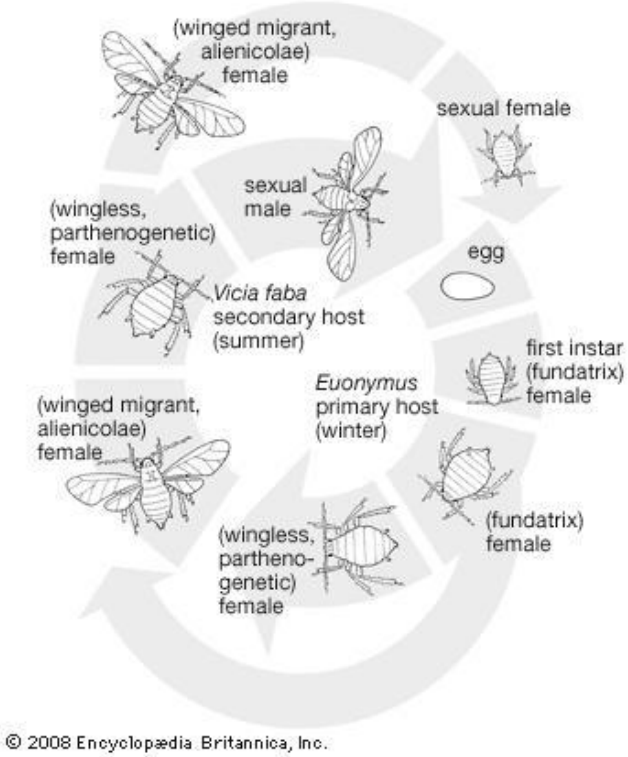

Figure 1: General Aphid Life Cycle

http://www.britannica.com/EBchecked/media/110813/Life-cycle-of-the-black-bean-aphid

Aphidinae family members typically have three different morphs: the fundatrix, also known as the wingless morph; the winged morph; and the sexual morphs (Braendle et al, 2006; Moran,

1992). During the spring and summer months, the female aphids (fundatrix) reproduce parthenogenetically; in other words, they reproduce asexually to produce embryos without fertilization by males. The offspring produced by female aphids are also viviparous; the offspring, known as wingless parthenogenetic aphids, are fully developed at birth (Braendle et al, 2006; Moran, 1992). Lastly, the oldest embryos also contain embryos. Adult parthenogenetic aphids carry not only their immediate offspring but also some of their offspring's offspring within them. During the fall, individuals of the winged morph travel from the primary host to the secondary host and also between secondary host individuals due to declining daily photoperiod and temperature (Braendle et al, 2006; Moran, 1992; Simon et al, 2002). This environmental change induces the development of the sexual morphs, females and males. These aphids reproduce sexually to produce yolk-rich eggs that undergo diapause, or a delay in development, to endure the harsh winter environment (Braendle et al, 2006; Caillaud et al, 2002; Moran, 1992).

The development of wings in aphids is due to either environmental changes (polyphenism) or genetics (polymorphism). Many studies have been conducted to distinguish between the two causes for wing dimorphism (Braendle et al, 2006; Caillaud et al, 2002; Simon et al, 2002). Primarily, wing polyphenism occurs in parthenogenetic females and wing polymorphism occurs in sexual males (Braendle, et al 2006; Simon et al, 2002).

Observable behavioral changes associated with mating, oogenesis, and oviposition can be seen in the sexual females (Moran, 1992). These final stages of the cycle are the only stages where haploid eggs are laid for reproduction instead of reproduction through parthenogenesis of diploid females (Moran, 1992). The determining factor for which morph is present depends on the environment. For example, crowding can induce winged parthenogenic females to develop, or the day length can cause formation of the sexual morphs on a primary host (Moran, 1992). The final morph that is present will be determined postnatal after molting of the instar occurs.

While they might differ from their mothers in morphology, daughters are genetically identical to their mothers. The daughters are produced via parthenogenetic vivipary (Moran, 1992). The females are conventionally labeled as having XX sex chromosomes, whereas the males produced are labeled as XO, or only having one X sex chromosome (Moran, 1992). The

development of the XO males is through specialized meiosis to provide only one X chromosome but a complete set of autosomal chromosomes (Moran, 1992). With the exception of the missing X chromosome, the mothers and the sons are genetically identical as well. The eggs that are laid by the sexual females are guaranteed to be female since the eggs will receive a second X from the male.

1.4 Phaseolus vulgaris

Phaseolus vulgaris, or the common bean, is widely known as the most important legume worldwide for direct human consumption (Jones, 1999). Not only is the common bean important for its nutritive value, but it has been part of many discoveries of important genetic principles (Broglie et al, 1986; Koenig, 1989; Aragão, 1996). For example, Johannsen (1911) utilized *P. vulgaris* in order to demonstrate the quantitative nature of inheritance of traits such as seed weight. A long history of research and recent advances using molecular genetics make *P. vulgaris* a rich resource of basic information for genetic experiments. For example, a number of studies have already carried out analyses involving quantitative trait loci (QTL) for various traits including phenological traits, seed size traits, and seed quality traits (Pérez-Vega et al, 2010). QTLs are stretches of DNA that contain or are linked to genes that underlie a quantitative trait, or a phenotype that varies in degree of expression and can be attributed to multiple genes (Gepts, 1988). Gepts (2001) observed that the location for the domestication syndrome QTL on the linkage map shows clusters of resistance genes and resistance gene analogs that relate to viral, fungal, and bacterial diseases. Domestication syndrome is when a plant displays symptoms such as reduced seed dispersal, reduced seed size, increased seed dormancy, abnormal coloration and color pattern, compact growth habit, and photo-period insensitivity (Koinange, 1996). Although resistance genes to viral, fungal, and bacterial diseases have been mapped and identified, other genes in *P. vulgaris*, such as genes that could influence resistance to pests, have not been explored.

1.5 Resistance

Resistance can be defined generally as never experiencing infection when exposed to a pathogen or parasite, which can be insects or other animal pests, fungi, bacteria, or viruses. Specifically for plants, resistance can be defined as the action of a group of heritable

characteristics to confer to the organism a lower likelihood of infection by any undesirable and detrimental pest or parasite that would use the organism as a host (Beck, 1965). If a plant or any host species is resistant to infection, then infection rates are low and most individuals of the species are never infected. A low natural selection pressure therefore exists for the host to evolve in response to infection; with low selection pressure, the plant species is unlikely to evolve tolerance to infection (Ehrlich and Raven, 1964).

Painter (1958) identified three forms of what he referred to as resistance. First, a plant may simply be undesirable to the insect for food, shelter, or oviposition, for reasons ranging from chemical and nutritional to structural. The second form of resistance is that plants may confer harmful side effects to the insect by adversely affecting biological processes, also known as antibiosis. Holt and Wratten (1986) found that plants can use antixenosis, or the preference for a non-resistant host plant by an insect when the option for a resistant host is also present, in defense as well.

The final form of resistance, according to Painter (1958), is tolerance; however, most biologists consider tolerance to be a separate concept from resistance. Tolerance is the ability of the organism to survive and produce viable offspring while some degree of damage is being inflicted on the organism; the organism continues to grow successfully after the infestation has passed (Beck, 1965). Contrary to the evolution of resistance, tolerance evolves under conditions of high infection rate. The individual organisms that have a natural ability to be infected, but receive little negative impact in terms of reproductive ability, will be under positive selective pressure to create the most offspring (Ehrlich and Raven, 1964). The end result is for the species to evolve to contain the traits conveying tolerance to the very prevalent infection and allowing the species to propagate successfully.

Two main factors affect resistance and tolerance: the plant's abiotic or biotic environment and the plant's genome. When any of the many possible abiotic and biotic environmental factors change, the expression of plant defensive traits is also affected (Cronin and Abrahamson, 1999; Fritz and Simms, 1992; Herms and Mattson, 1992; Hutha et al, 2000; Maddox and Root, 1987; Rosenthal and Kotanen, 1994; Strauss and Agrawal, 1999; Stowe et al, 2000; Strauss & Irwin 2004, Strauss et al, 2005; Valverde et al, 2001). Abiotic factors such as temperature and rainfall

change the ability of a plant to be resistant or tolerant to an infestation or herbivory (Mwangi et al, 2008; Pruter and Zebitz, 1991). While it is known that the abiotic environment affects the resistance and tolerance of a plant, the extent of the environment's influence is not yet well understood (Núñez-Farfán et al, 2007). Biotic factors such as the herbivores themselves affect the plant's tolerance and resistance. Pruter and Zebitz (1991) found that faba bean (*Vicia faba*) rust, in combination with aphid infestation, causes a plant to have a lessened shoot area, leaf area, and dry weight of roots; this indicates that the combination of pest and disease greatly damaged the *V. faba* plant's ability to grow, so the plant therefore did not have a successful resistance or tolerance strategy. Conversely, mycelial networks of fungi among the roots of a population of bean plants can induce resistance or tolerance strategies in neighboring plants (Babikova et al, 2013; Heil and Bueno, 2007). The induction of resistance or tolerance strategies is caused by the release of plant volatile organic compounds that deter pests and attract pest predators (Babikova et al, 2013; Heil and Bueno, 2007). The mycelial network allows the plant population, in which many individuals may be clones or closely related to one another, to survive and reproduce more successfully as a whole, even if the individual plant does not reproduce most efficiently. The presence of predators to the herbivores also influences the fitness effects of resistance and/or tolerance in a plant as well; when the herbivores are removed by the presence of their predators, the plant has a lower selective pressure from the herbivores to become resistant and/or tolerant to the herbivores (Mutikainen et al, 2000; Garrido-Espinosa and Fornoni, 2006). Interactions between plants and their pollinators in mating systems may also play a role in the adaptive value of resistance and tolerance, depending on how well-adapted the herbivores are to using the plant (Núñez-Farfán et al, 2007). Additionally, these various environmental pressures add costs to the expression of any resistance or tolerance traits; these costs are observed in a reduction of plant growth and reproduction, resulting in a negative correlation between the expression of resistance or tolerance and the plant's growth (Koricheva 2002; Marak et al, 2003; Osier and Lindroth, 2006; Fornoni et al, 2004; Hochwender et al, 2000; Pilson, 2000; Siemens et al, 2003; Simms et al, 1987). However, one study by Tiffin (2002) found little evidence that changes in environmental and seasonal conditions constrained the plants' evolution.

Resistance and tolerance traits in plants may have a substantial genetic component. For example, Meng et al (2011) discovered two QTLs that conferred resistance to the soybean aphid,

Aphis glycins, via antibiosis that was mediated by the compound isoflavone. These QTLs were on two separate chromosomes and were useful in explaining phenotypic variations in resistance or tolerance in a portion of the soybean plant population. The expression of these and other specific genetic loci in any bean plant directly affects the ability of the herbivore to successfully reproduce on the resistant or tolerant plant (ten Broeke et al, 2013; Dahleen et al, 2012; Fanli et al, 2011; Meng et al, 2011; Zang et al, 2010). It is also important to note that the hypothesis that tolerance and resistance have a negative genetic correlation does not have much empirical evidence, suggesting that plant resources are allocated to developing both tolerance and resistance in a natural population (Fineblum and Rausher, 1995; Fornoni et al, 2003; Pilson, 2000; Stowe, 1998; Núñez-Farfán et al, 2007).

1.6 Trait Variation and Heritability

Generally, heritability refers to the degree to which observable, or phenotypic, differences in a trait between individuals within a population is caused by genetic differences. However, environmental factors can also lead to variation between individuals in their observable phenotypes. Therefore, heritability studies analyze the relative contributions of genetic and non-genetic factors on the total variability in phenotypes in a population (Hunt et al., 1989; Hallmayer et al, 2011; Raj and Van Oudenaarden, 2008).

In terms of plants, breeders aim to identify and select plants that have genotypes which result in certain desirable phenotypes, as opposed to plants with favorable phenotypes that arise due to environmental effects (Burton and Devane, 1953; Chuang and Meyerowitz, 2000; Collard and Mackill, 2008). In order to predict the outcome of selection in a collection of genotypes, a breeder must know the level of correspondence between phenotypic and genotypic values, or the heritability of that phenotypic trait. There are many ways to estimate heritability, but there can be no single measurement of the contribution of genetic factors to a phenotype. There are two main methods of estimating heritability: analysis of correlation and regression, and analysis of variance (Dudley, 1969; Holland et al, 2003; Johnson et al, 1955).

Studies of heritability are also important for a basic understanding of how evolution occurs naturally. Specifically, many studies have explored the idea that the degree to which traits are heritable affects a species' ability to adapt to their environments and in turn their ability to

survive (Hoffmann et al, 2003; Meyer and Di Giulio, 2003; Nussey et al, 2005). If a member of a species develops a morphological, behavioral, or physiological trait that helps it survive better than its peers then it is more likely to pass on that trait to its offspring. This offspring will then have a better chance at survival than its peers, as it has adapted to the new environmental hardship, while its conspecifics have not.

This type of adaptation may allow plants to resist insect pests. Plant resistance to insect pests is in part the consequence of heritable plant traits that result in insect damage reduction relative to a plant without those qualities. There have been several studies that suggest that certain traits have larger heritable components than others (Houle, 1992; Pilia et al, 2006). These qualities then reflect on which type of resistance the host plant will exercise.

1.7 Hypothesis and Rational

This report presents the results of our experiment to test the hypothesis that tolerance to infestation by the black bean aphid in the common bean is a genetically heritable trait, allowing the plant to reproduce successfully even under some degree of infestation. While the degree to which resistance or tolerance traits may be heritable is unknown in *P. vulgaris*, research in other plant-insect systems has empirically shown that heritable tolerance and resistance has evolved between phytophagous insects and their hosts (Dahleen et al, 2012; Fanli et al, 2011; ten Broeke et al, 2013; Meng et al, 2011; Zang et al, 2010). Our project yielded preliminary data on potentially heritable tolerance between generations of *P. vulgaris* Velour. This data will be useful for directing further study of the heritability of tolerance to aphid infestation as well as for future research into the *P. vulgaris* genome to find genes that confer the tolerance to aphid infestation.

Methodology

2.1 Aphid Colonies

The black bean aphids that were used in our experiments originated from Whitesfield Farm in Hardwick, MA on three different plants of *Chenopodium album*, commonly known as lamb's quarters. The black bean aphids were transferred to a laboratory at Worcester Polytechnic Institute (WPI) and were cultured on *V. faba* plants. Pea aphids (*Acyrthosiphon pisum*) were obtained from a supplier and were also reared in the same laboratory and on the same *V. faba* plants. Approximately five weeks before the experiment began, we established pure black bean aphid colonies in the preparation room of the greenhouse located at WPI. These aphids were always fed on *Vicia faba* bean plants; the supply of plants was constantly replenished as older plants died.

2.2 Phaseolus vulgaris Preparation

P. vulgaris Velour seeds were obtained from Johnny's Selected Seeds, an employee-owned seed merchant and producer based in Winslow, ME; the seeds were ordered from http://www.johnnyseeds.com/. The Velour cultivar was chosen because it has the shortest time to maturity; Johnny's Select Seeds estimates that the *P. vulgaris* Velour's time to maturity is 51 days. In order to prepare plants for the start of the parent generation experiment, we used a specific process for planting. First, we soaked the seeds in water for 18-24 hours to allow for rehydration. After soaking and before planting, we coated the wet seeds in rhizome bacteria (N-Dure soybean/legume inoculant from INTX Microbials Inc.) because legumes require these commensals to provide nitrogen fixation capability. We placed the coated seeds in holes approximately three centimeters deep in plastic pots (7.5 cm^2) pre-filled with non-sterilized Sun-Gro Horticulture's Metro-Mix 360 growing medium. The seeds were covered with the soil already in the pot. The arrangement of seeds depended on how many seeds we planted in a pot. If we planted four, then one seed was in each corner. When we planted five seeds, we used the same four corner placement and additionally planted the fifth seed in the middle of the pot. Once we had planted all the seeds in a pot, we watered the seeds until the soil was visibly wet. We added water a little bit at a time so that the water could be absorbed by the soil. After initial watering, the pots received water via it seeping into the bottom of the pot after the water was

released from stakes interspersed on the tray. Each pot was labeled with the date the seeds were planted, the species of seed, and then the cultivar. When potting seeds, we always planted twenty pots of the *P. vulgaris* Velour cultivar.

We planted our parent generation experimental plants in the greenhouse on November 24, 2013, using the *P. vulgaris* Velour cultivar. By December 13, 2013, all plants definitely had open cotyledon leaves and appeared healthy; forty Velour plants were then repotted in individual pots. Each plant was placed in a new pot with new soil, which was watered until it was visibly wet. The pots were labeled with the plant species, cultivar, original seed planting date, and repotting date, and each received a unique number. The plants were allowed to readjust to being in a new pot for two days and the infestation and experiment start was on December 16, 2013.

After the experiment was started, the plant care was slightly different than described previously. The pots were arranged in the greenhouse on level trays that had watering spikes resting on the tray among the plant covers. The plants received water via it seeping under the cover bottom and into the bottom of the pot. We did not hand water plants during the experiment. On January 22, 2014, the greenhouse water supply was turned off by accident, so some plants were dry for a short period of time and spikes were re-arraigned. Some plants of both experimental and control groups were given additional water via the tray as well; however, we did not note any visible permanent or temporary damage to plants as a result of this dry period. The final change in care during the experiment was use of wood support stakes. As the plants grew, many became top heavy both before and after bean pod development began. Support of a fifteen centimeter long stake was added by inserting the stake into the soil next to the plant and tying the plant loosely to the stake with string.

For the second generation of plants, the same planting protocol was followed with a few changes. We soaked the seeds in wet paper towels for 24 hours before planting in the same manner as the previous seeds; one seed was planted in the middle of each pot on March 23, 2014. Labels included plant species, parent number, and the date that we planted the seeds. The seeds were then hand watered until germination to ensure no drying out occurred. After germination, the plants were watered via water seeping through the bottom of the pot. Due to time constraints, only eight experimental plants and eight control plants were available for the second phase of the

experiment. On April 15, 2014, the eight treatment plants were infested with ten aphids each as done previously. All sixteen plants ranged in developmental stage. Some had only open cotyledon leaves while others also had true leaf development; however, all sixteen plants used were past the open cotyledon leaf threshold.

2.3 Covers

We created individual plant covers to prevent movement of winged aphids among plants, especially since both experimental and control groups were kept in the same area of the greenhouse. There were two main criteria that needed to be kept in mind when constructing the covers: the covers had to keep aphids in and/or out and the covers needed to be permeable to light so the plant inside could grow properly. The cylindrical covers were constructed with two embroidery rings (17.78 cm in diameter) as the base and top. A rectangular piece of chicken wire was wrapped around and attached to the two embroidery rings with hot glue to create the sides of the cover, resulting in a total cover height of approximately 36.8 cm. A cloth cover sheet was then wrapped around and attached to the outside of the embroidery rings. The cloth cover material used was called “Summerweight Garden Fabric” from Gardeners Supply Company; the material advertises that it allows 85% of light to pass through to the plants underneath; however, our covers allowed approximately 63% of the light to pass through. Using a light reader that measured the light in candles, the percentage of light under the covers was determined by measuring the light allowed through a cover and dividing it by the light reading outside of the cover; both readings were taken during sunny conditions within five minutes of each other. The appropriate size of the embroidery rings, and therefore the diameter of the whole cover, was originally determined by the overall size available for all of the plants in the experimental greenhouse. Figure 2 shows a finished cover.

Figure 2: A Finished Plant Cover

2.4 Experiment

Before we started the experiment, we randomly assigned the plants to the treatment or control groups. We used an online random number generator (http://www.random.org/coins/) for flipping quarters to assign the plants to experimental or control groups; the random generator was set to have twenty in each group. The results of the randomization of location and group assignment can be seen in Figure 3. We started the actual experiment on December 16, 2013. Ten aphids of varying sizes were placed on each of the experimental plants. To do this, we brought the experimental plants out of the greenhouse on a cart and to the prep room where the aphid stock was kept. The infestation occurred in the prep room before we brought the plants back into the greenhouse on the same cart. This move to the prep room and back was done so that the control plants would not be contaminated and accidentally infested. All aphids we placed on the plants were black or gray and showed no signs of becoming winged aphids. We used tweezers and small weighting trays to transfer the aphids between the stock and the experimental plants.

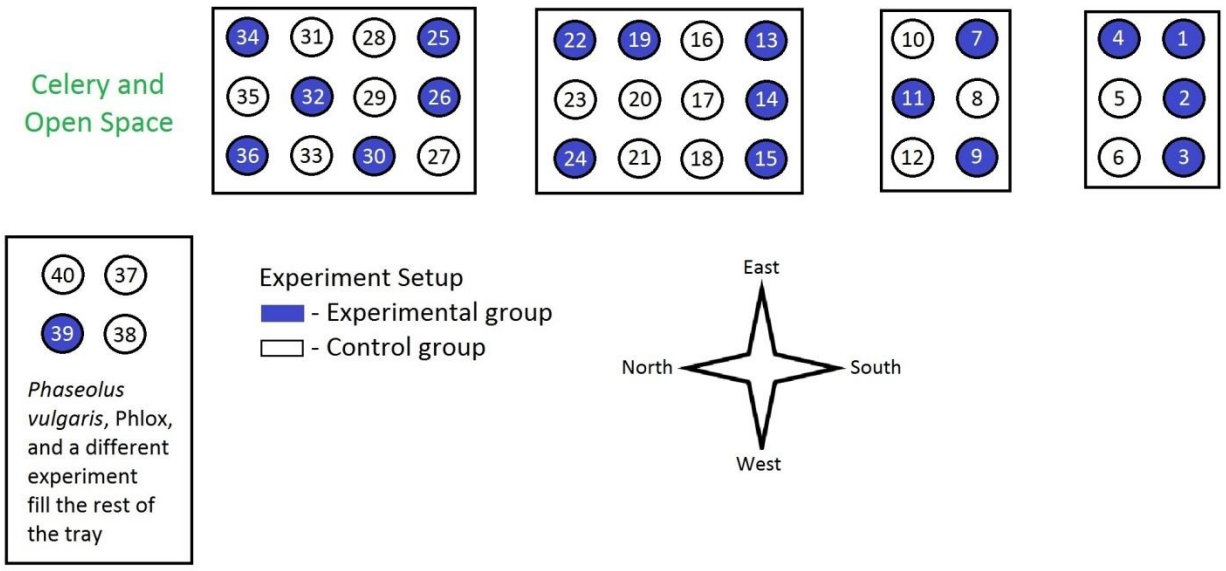

Figure 3: Diagram of Experiment Set-Up and Group Assignment for Generation 1

Data were collected every other day starting on December 20, 2013. We recorded the number of live non-winged aphids, number of live winged aphids, the soil moisture reading, number of bean pods and how many of these pods were purple, the wet root weight, and the dried weight of the seeds produced by the plants. The number of bean pods that were colored purple was noted as well because these pods were the ones that eventually produced mature seeds to plant for the second generation of plants; the pods were considered to have turned purple if over half the pod was colored purple. When distinguishing between live and dead aphids, live aphids showed movement of a leg or antenna when gently poked. Dead aphids did not move. A live aphid only counted if they were black or grey; a white 'aphid' was considered to be a molted exoskeleton and not an actual aphid. Because we also noted a minor infestation of small flies in the greenhouse, any winged aphid was closely examined to make sure it was not actually a fly. For each plant in the experimental group, we eventually recorded no live aphids remaining on the plant. We stopped counting aphids for an individual plant if, after the data from the three previous collections were examined, no live aphids had been counted.

Both experimental and control groups eventually reached a point for harvesting the matured seeds for the next generation and weighing the seeds and roots. The mature seeds were harvested when the previously purple pods became dry and appeared shriveled; the pods felt

wrinkled, hard, and rough to the touch. The seeds were stored in containers labeled with the number of the parent plant; the containers were also labeled with number of seeds and date of harvest. We kept the containers in a dry, dark area of the greenhouse prep room. The roots were then taken out of the pot by loosening the soil from the sides of the pot, sliding the soil out of the pot, and carefully crumbling the soil away from the roots so as to keep most of the root structure intact. The stem was trimmed off where it entered the soil, indicated by a change from green to white, and the roots were gently washed and patted dry before weighing was done.

When the second generation experiment began on April 15, 2014, the experiment and control groups had plants in the same location as in the first experiment. The same experiment/control group location layout was used for the second generation (Figure 4, see Figure 3 for first generation). No second generation plant was in the same place as its parent in order to minimize the effect of location on plant growth.

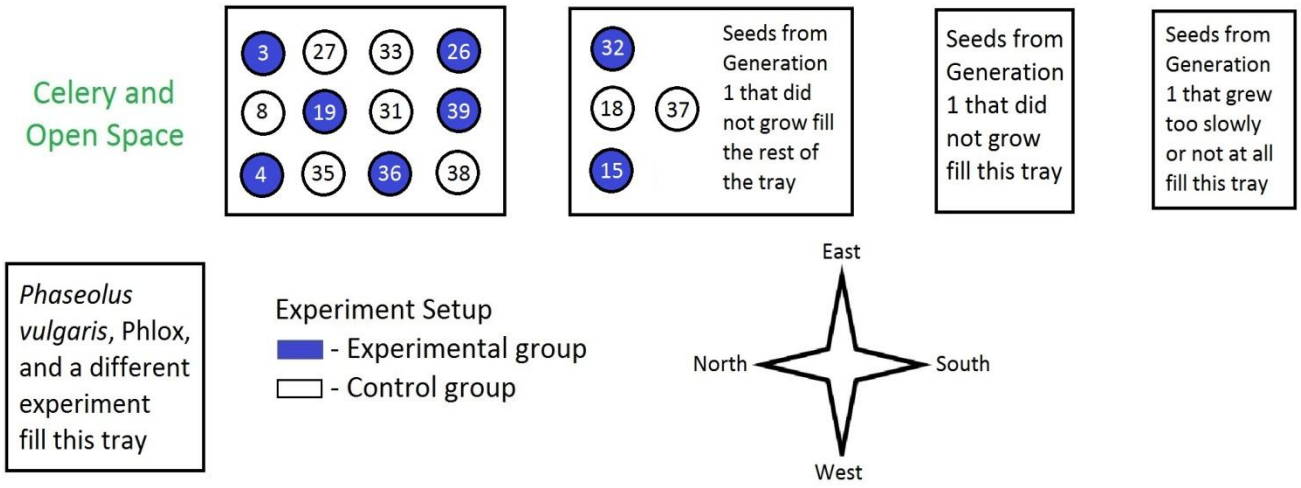

Figure 4: Diagram of Experiment Set-Up and Group Assignment for Second Generation

2.5 Data Analysis

In order to determine what test to use to analyze our experimental data, we first had to determine whether to use a parametric or non-parametric test. Parametric tests make assumptions about the distribution of the parameters and about the population distribution from which one's

data are drawn. Specifically, parametric tests assume that parameters adhere to the normal distribution and that variances are equal between groups. Non-parametric tests make less rigorous assumptions about the distributions and variances of the parameters. We chose non-parametric tests because our small sample size of only 40 plants in the parent generation (20 control and 20 experimental) make it difficult to test for deviations from normality and equality of variance.

We used a Mann-Whitney-U test to analyze within-generation data to compare certain characteristics between the control group and the experimental group. The Mann-Whitney-U test is a nonparametric alternative to the independent-sample t-test. The Mann-Whitney-U test is used to compare differences between two independent groups when the dependent variable is not normally distributed and cannot be transformed into a normal distribution (McDonald, 2009). In our case, the two independent groups are the control and experimental groups, the experimental group being the one infested with aphids. We compared three dependent variables: total number of pods produced, number of mature seeds, and individual dry seed weight. Our Mann-Whitney-U tests were conducted using IBM's SPSS software, which originally stood for Statistical Package for the Social Sciences, though the abbreviation is now used as the analytical software's title without the original connotation; specifically, IBM SPSS Statistics 19 was used for our statistical analysis (Lund, 2013).

After the ten days of the second generation live aphid population trend data were collected, comparison between the parent and offspring plants could be compared. To do so, we created a graph containing both parent and offspring aphid population trends for the first ten days of both experiments. Each parent/offspring pair was displayed in the same color but different line styles. Inferences were drawn from visually comparing a parent plant aphid population trend to the offspring plant aphid population trend; visual comparison also allowed for inferences by comparing the eight parent/offspring pairs to the other pairs.

Results

Forty plants were separated into experimental and control groups and allowed to grow until bean pods and seeds were fully matured and ready to be harvested. On average, the plants took 102 days to mature from planting to harvest; the days to harvest ranged from 93 to 114. From the forty plants in the experiment, eighteen experimental plants and nineteen control plants had at least one harvestable seed; one experimental group plant died half way through the experiment, and one experimental and one control plant did not develop enough and grew too slowly to have harvestable bean pods or seeds. Fourteen experimental plants and ten control plants had multiple seeds harvested. After the bean pod and seed harvest was complete, p-values (significance at $p = 0.05$), averages, and standard deviations were calculated using SPSS and the Mann-Whitney-U test to compare the number of pods, number of seeds, and the dry weight of seeds between the two groups of the first generation (Table 1).

Table 1: Mean and standard deviation for three plant characteristics for experimental and control groups, and results of Mann-Whitney U tests

	Sample Size	p-Value	Experimental Group	Control Group	Mann-Whitney U Test Statistic
# of pods per plant	40 plants	0.73	1.65 (0.51)	1.7 (0.73)	188.5
# of seeds per plant	40 plants	0.87	1.75 (0.85)	1.7 (1.08)	184.5
Individual Seed Dry Weight (grams)	70 seeds	0.63	0.146 (0.061)	0.138 (0.06)	571.5

Mann-Whitney U tests showed no significant difference between experimental and control groups in the number of pods per plant, number of seeds per plant, or individual seed weight (Table 1). Averages and standard deviation were very similar for the two groups, but both groups had substantial variation from plant to plant. While dry seed weight had a low standard deviation, the p-value was still not significant.

One of the most important aspects of data collection in this experiment was to track the number of live black bean aphids on the experimental *P. vulgaris* plants. Data was taken every

other day after initial infestation with ten aphids on December 16th. Figure 5 shows the change in aphid population size per plant during the 66 days of data collection. After those 66 days, no more live aphids remained.

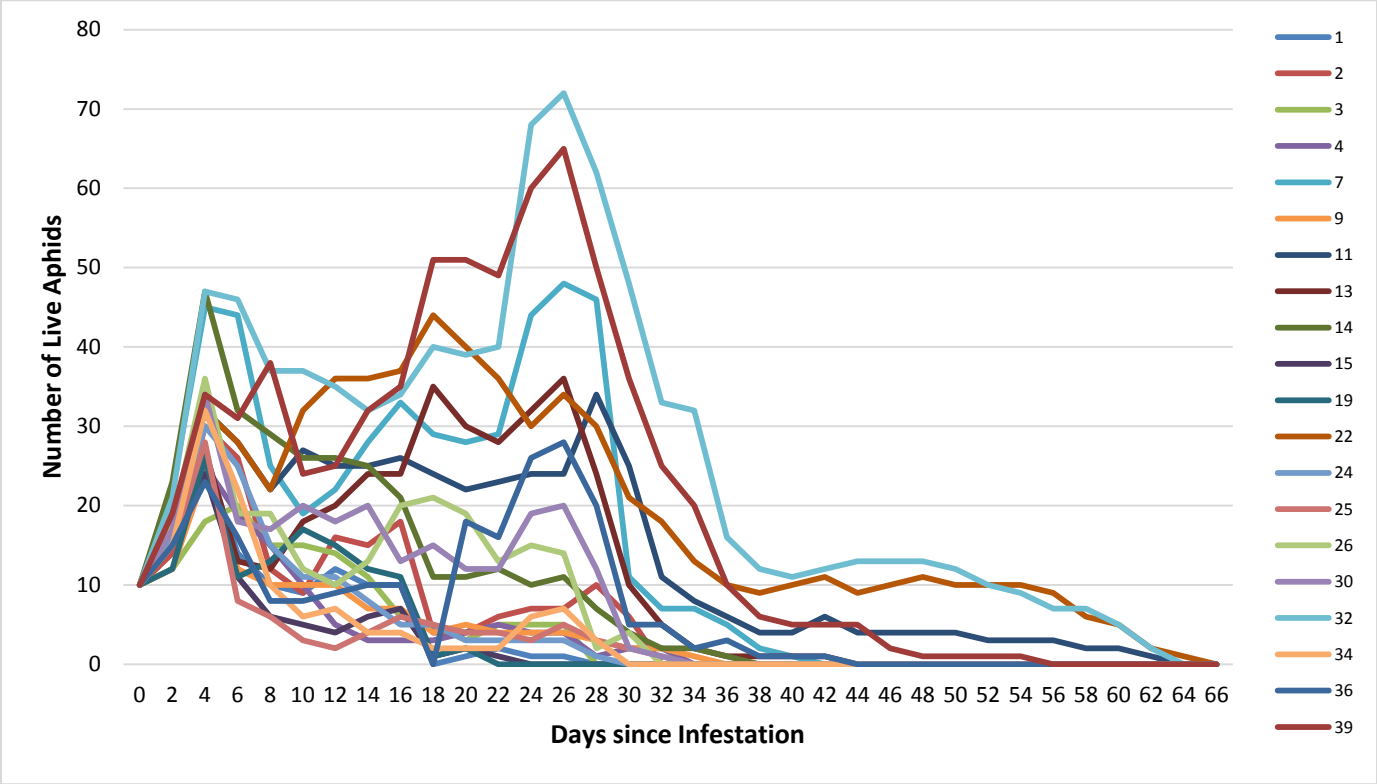

Figure 5: Black Bean Aphid Population on P. vulgaris *(20 plants). Each line corresponds to an individual plant.*

As can be seen in the Figure 5 above, the aphid population started at 10 aphids for each of the experimental plants. The graph shows a large amount of variability among plants and also over time in the number of aphids. However, some general patterns do seem to be repeated on many or most plants. The aphid population hit its first peak from days 2 to 4. From days 6 to 22, the aphid population decreased or stayed around the same. The second, and the largest, peak occurred during days 22-28. There was a sharp decline from day 28-32. After day 32, the aphid population started to decline. Beginning at day 42, experimental plants started to have no live aphids. This decline continued until day 66, when all the live aphids on all of the experimental plants had died.

Next, the seeds from the first generation were used to plant the second generation. There were 16 viable seeds from the first generation, 8 from control plants and 8 from experimental plants. These plants were treated the same way as the first generation; they were planted and then infested with 10 wingless aphids when cotyledon leaves were fully developed. We collected live aphid data as for the first generation. However, due to time constraints, there was only time to collect data on the first 8 days after infestation. The data collected and graph can be seen below in Figure 6.

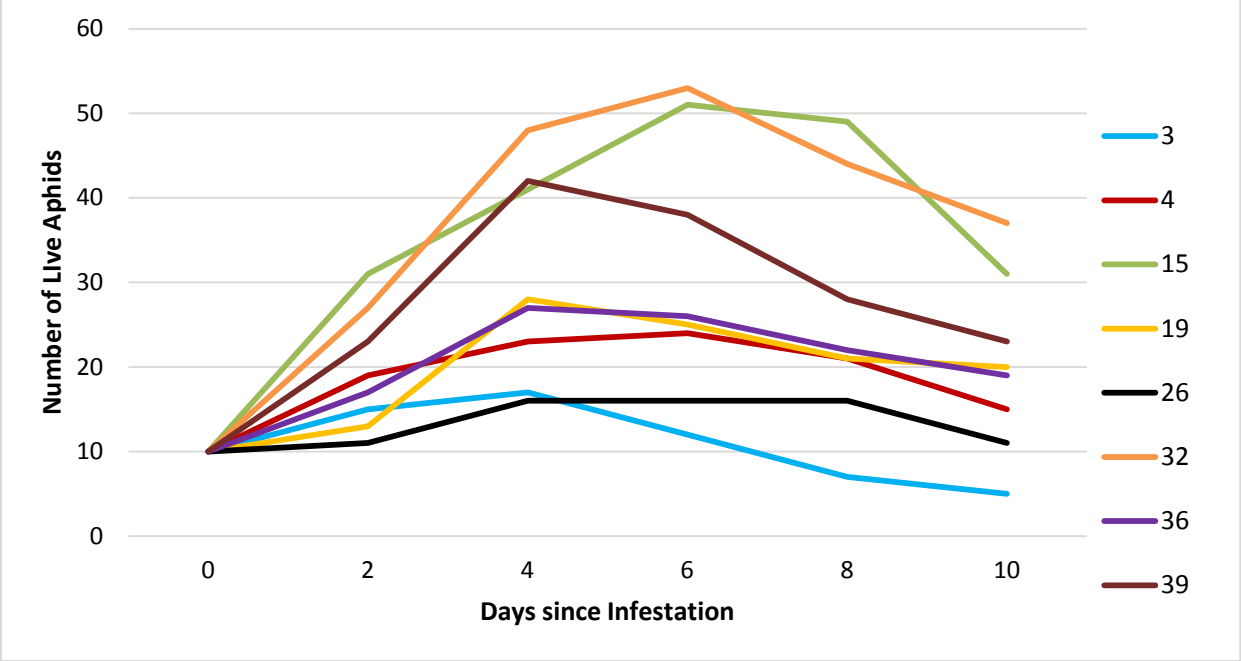

Figure 6: Black Bean Aphid Population on P. vulgaris *- 2nd Generation. Each line corresponds to an individual plant, with colors indicating parent-offspring relationships with plants shown in Figure 4 above. .*

As the graph of black bean aphid population on the second generation shows, there is an initial increase beginning from infestation of 10 aphids. This increase continues for several days, specifically until 6 days after infestation. After the sixth day, the aphid population starts to decrease.

In order to clearly see the pattern between the first generation and the second generation, a separate graph with both of the generations for the first ten days of live aphid population was created. Figure 7 below shows the eight plants in the second generation in the same color as

same plants from the first generation; the first generation plants are dotted lines whereas the second generation plants are solid lines.

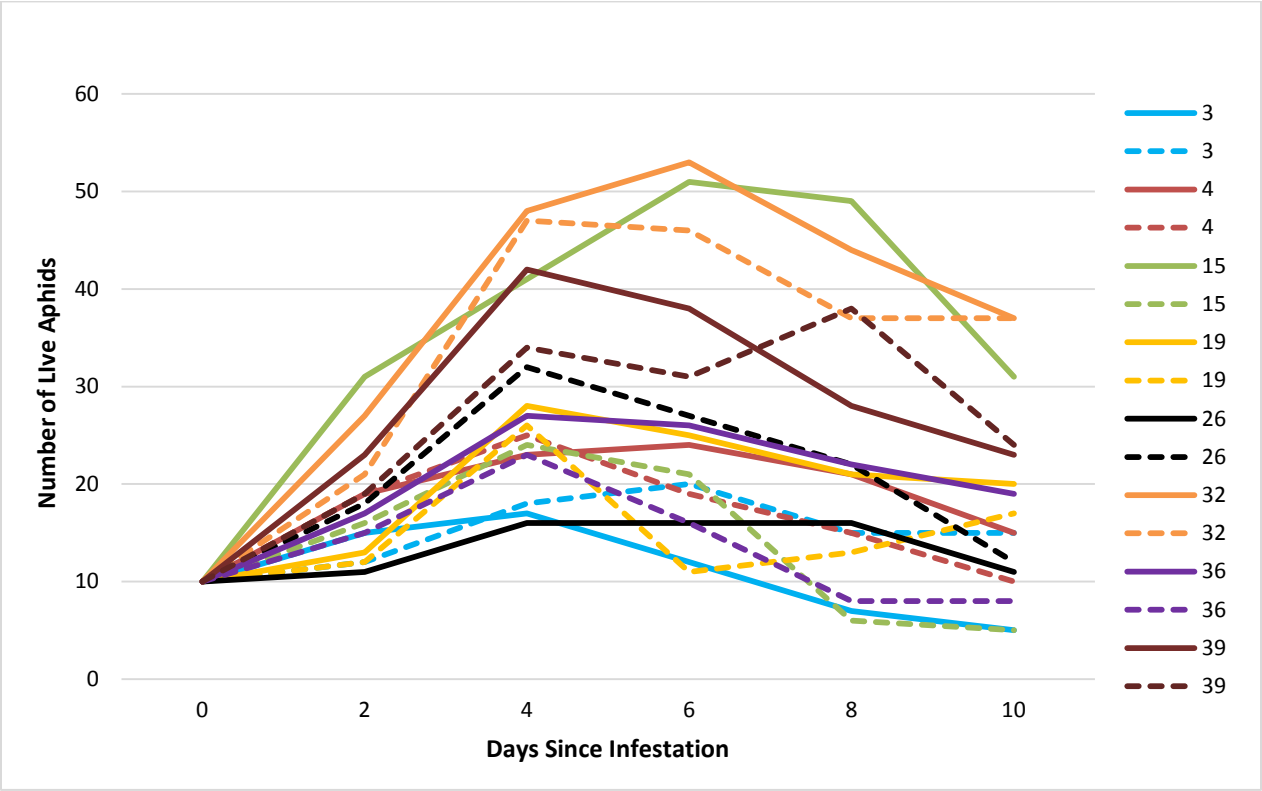

Figure 7: Black Bean Aphid Population on P. vulgaris *– broken lines show individual plants in the 1st generation and solid lines of the same color show their offspring in the 2nd generation.*

A lot of variation between parent and offspring plant can be observed. For example, parent/offspring pair 15, shown in green, has a lot of disparity; the offspring has a much higher live aphid count than the parent, differing by up to forty aphids. On the other hand, parent/offspring pair 32 has a mostly consistent trend. Additionally, while parent/offspring pair 26 starts off very different, by day ten the live aphid counts have become about equal. Similar variation can be seen in the rest of the parent/offspring pairs as well. Variation in a single day between unrelated generation one plants ranges from nine aphids to thirty-five plants, or varies by up to 26 aphids; variation in a single day between unrelated generation two plants ranges from twenty aphids to 42 aphids, or varies by up to 22 aphids. Overall, the general trends for the parent/offspring pairs are similar.

Discussion

This study aimed to test the hypothesis that tolerance to infestation by the black bean aphid in the common bean is a genetically heritable trait, allowing the plant to reproduce successfully. We predicted that live aphid numbers would be similar at a given point in time for the first and second generations. We also predicted that individual second generation plants would respond to aphid infestation in a similar manner to their parents in terms of bean pod and seed development; however, because the plants grew slower than we expected, we did not reach a point in the experiment where second generation data for number of bean pods or seeds or dry seed weight was collectable. The second generation experiment plants had infestation data collected for ten days before the project ended; the second generation plants were still growing and had not reached a reproductive age since only pink or purple buds had developed; no flowers or further bean pod development occurred. Though data pertaining to the reproductive ability of the second generation was unavailable, live aphid population data were available (for 10 days post-infestation) to make comparisons between the first and second generations. Thus, these logistical limitations mean that we cannot make complete conclusions about the heritability of aphid tolerance, but we can make preliminary inferences into resistance heritability based on the live aphid trends.

When the parent experimental and control groups were analyzed, no significant difference was found between the two for the number of bean pods and seeds produced or for dry seed weight. There are many potential reasons why no physiological response was observed. First, *P. vulgaris* Velour could be a cultivar that is very tolerant to the black bean aphid. The black bean aphid prefers *Vicia faba* above all other beans even though the black bean aphid is polyphagous and is able to feed on a variety of bean plants. Additionally, the number of pods and seeds produced along with the dry seed weight may not be the best plant characteristics to use when investigating tolerance; a measure of plant size or weight could be better characteristics to measure if they are affected by aphid infestation. Thirdly, the greenhouse environment during the majority of the experiment may not have been ideal; temperatures that were best for plants and other experiments may not have been the ideal for our bean plants, and the experiment was also run during the winter. Lastly, no observable physiological response could be due to slight differences in the starting developmental stages or different growth rates. While all plants in the

experiment had open cotyledon leaves at the start of the experiment, there were variations on how developed any true leaves were. The different stages of growth have the possibility to affect the success of aphid use of the plant host (Hein, 1992; Kieckhefer and Gellner, 1988; Petitt and Smilowitz, 1982).

Observing the growth and decay in aphid population on *P. vulgaris* is essential in understanding what affects aphid growth and death. Aphid growth can be affected by many factors such as how much direct sunlight the plant receives, how much water the plant receives, and the overall health of the plant. These factors affect how successfully aphids can feed on the plant due to nutrient availability. For example, four days after infestation, the aphid population on the first generation plants on average quadrupled, and this could be due to the aphids being able to feed on fresh, young plants that could provide nutrition to the aphid population. The slight decrease in population during days six to eighteen could be due to the aphid population increasing exponentially while the plants were still young and unable to sustain a large number of aphids; as the aphid population grows and depletes the plant of nutrients, the aphids cannot sustain a large population and many will die. The high peak from days 18-28 corresponds to an increase in plant growth and therefore the aphid population increased on the large, healthy plants. The sudden drop in aphid population during days 28-34 of the first generation experiment could be the result of two reasons: the high aphid population slowly deteriorated the plants and aphids could not properly sustain themselves, and/or the bean plants responded to the attack of the aphids via phenotypic plasticity by thickening their stems or producing toxins and directly preventing the aphids from feeding and obtaining proper nutrition to survive. After day 34, there was a slow decrease in aphid population in addition to the plants starting to reach senescence.

Additionally, a tentative conclusion can be made about the impact of aphid infestation on the eventual viability of any seeds produced. While only eight experiment plants grew soon enough to be used in the experiment, only eight control plants grew as well. Some germination of other plants was observed after the second experiment started; however, an approximately equal number of control and experiment plant offspring germinated overall since eleven control group seeds and ten experimental group seeds germinated. Nine parent plants had at least one seed germinate from the control group, and eight parent plants from the experimental group had at least one seed germinate. One experimental parent plant had two seeds germinate; one control

group plant had two seeds germinate and another had three seeds germinate. Overall, many control and experimental offspring were not viable since forty-nine seeds did not germinate. A lack of viable offspring in addition to the very similar number of viable offspring across the two groups indicates that factors other than the aphid infestation were also important to determining viability; of note is that no fertilizer was used at any point in the experiment. A degree of tolerance to infestation in the parent generation is implied since the same number of seeds germinated and grew from both experiment groups, but this possibility would require future investigation.

Though time did not allow for collection of bean pod and seed production data from 2nd generation plants, preliminary inferences about the heritability of aphid resistance based on the live aphid trends can be made. A lot of variation is displayed in figure 6; the parent/offspring pairs 32 or 3 follow the same general trend of live aphid population, but the live aphid population trends of parent/offspring pair 15 follow no similar patterns. The difference for parent/offspring pair 15 ranges from fifteen aphids to over forty aphids, whereas the differences for the parent/offspring pairs 32 or 3 only go up to approximately eight aphids. Overall, parent/offspring pairs follow generally similar trends in figure 6 for live aphid population; however, there is clear, constant disparity between the parent/offspring pair 15, and many other pairs also are dissimilar heavily in either the beginning or end of the ten day period. Additionally, variation between unrelated first generation plants and unrelated second generation plants is very similar since the two variations differ by only four aphids, implying that environmental conditions did not play a role in the expression of aphid resistance. Tentatively the conclusion that resistance to aphid infestation is somewhat heritable can be made, but at least ten more days of data collection for this experiment as well as more future experiments would be needed to make definitive conclusions.

This investigation was limited by the short time available for the second generation, which limited our aphid count data and prevented us from gathering any data on plant characteristics (e.g., seed number). If the second experiment had started sooner, then more data would be available for decisive conclusions. However, the three months between infestation and complete harvest of the first experiment was much longer than expected. The main time frame for the first experiment was during the winter; day length was short and while temperature was

controllable in the greenhouse, some fluctuation did happen as a result of periodic very cold temperatures outside. These non-ideal conditions are likely the cause of the forty-two day difference between the average experimental days to mature seed harvest time and the manufacturer's estimate. The length of the first experiment was affected by circumstances beyond the study's control, and the same circumstances were not present for the start of the second experiment. Spring began and outside temperatures were warmer, allowing for longer light availability and less temperature fluctuation inside. We observed that the second generation plants appeared to grow faster once germination occurred. We suggest for future studies to start the set of experiments over the summer so that up to three generations of data could be collected and compared. If the experiments are not able to start over the summer, starting within the first week of class would also allow for more data collection and more time since the experiments will still need to run during the winter months.

In addition to the time limitations, the investigation was limited by the extremely varied growth rate. When observations are taken into account, the growth on one half of the experimental setup was faster; flowers and pods developed sooner, and senescence and harvest occurred sooner. A range of up to two weeks between different stages occurring was observable on either half of the experiment room in the greenhouse. When time from planting to seed harvest is considered, the range was twenty-one days; the minimum time to harvest for the experiment was ninety-three days, a full forty-two days longer than the manufacturer estimate of fifty-one days. The range in growth rate is likely due to light availability and/or temperature differences. Spatial randomization was used to avoid light being a confounding variable; however, we noted during many data collections that light was highest in the northeast corner because of the surrounding buildings' shadows and the direction that the greenhouse faces. This corner contained the left half of the experiment where the fastest growth and development was observed. To account for the light differences that occur naturally, we suggest a reorganization of the plants in future studies. This reorganization could include distributing the plants around the northeast corner of the benches or the addition of artificial light fixtures around the entirety of the experiment. To determine that the average amount of light is equal throughout the experimental setup, we also suggest using a light meter to measure light before the start of future experiments.

Along with the issues of time and plant growth constraints, the very small sample size for control and experimental groups also proved to be a problematic variable. Small sample size was a result of a lack of space in the greenhouse. Without covers, over double the number of plants could be used. However, the covers were necessary to prevent winged aphids from leaving the original experimental plant and skewing live aphid numbers on another experimental plant or starting an aphid colony on a control plant. Not much can be done to increase the number of plants in each group due to space constraints, and the small sample size hampered our ability to analyze the collected data and make concrete inferences from our data.

To expand the scope of this investigation of coevolutionary relationships, aphid success on *P. vulgaris* could be explored in future experiments. To do so, aphids that have developed over a few generations, such as the aphids present on plant 32 between days twenty-four and twenty-eight, could be collected; from these collected aphids, males and sexual female morphs would need to be obtained on the black bean specific primary host to allow for growth of a new genetically varied colony. The new genetically varied colony that originated from the originally successful but genetically identical aphids could be used to test the ability these aphids to develop into a thriving population. The population trends of the aphids in the new experiment could theoretically show that success on *P. vulgaris* can improve over time. Such an improvement would be measurable because of the many generations of aphids possible over a short time. A response on behalf of the plant to any change in aphid ability to use the host plant would not be as evident because the plant population would take longer to display any discernible improved traits. If an experiment using aphids to explore the coevolutionary relationship between the black bean aphid and *P. vulgaris* were to be carried out, more insight into the relationship between host and parasite could be developed to inform future conclusions.

In summary, we are able to preliminarily accept the hypothesis that tolerance to infestation by the black bean aphid in the common bean is a genetically heritable trait because the plant can successfully reproduce. Overall, the eight offspring that were in the second experiment for ten days followed the same general pattern for aphid infestation as the parent plant in the first experiment. The majority of seeds harvested from either experimental or control parent plants did not germinate however, and along with this lack of germination, the second experiment was not able to be completed. A small sample was used originally in the first

experiment as well. Therefore, though evidence from the aphid population trend comparison indicates that resistance could be heritable, definitive conclusions cannot be made about the heritability of aphid resistance or tolerance until further experimentation is performed.

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