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Costs of resistance in a mustard plant (Arabidopsis thaliana) associated with two specialist insects| Crucifer flea beetles (Phyllotreta cruciferae) and diamondback moths (Plutella xylostella)

Bridget Barker
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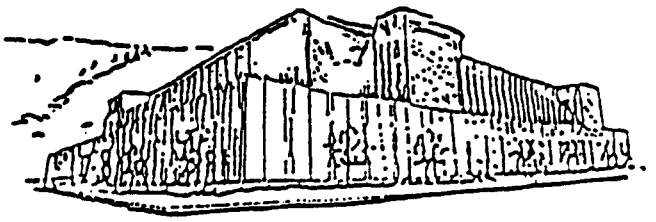

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COSTS OF RESISTANCE IN A MUSTARD PLANT (*ARABIDOPSIS THALIANA*) ASSOCIATED WITH TWO SPECIALIST INSECTS: CRUCIFER FLEA BEETLES (*PHYLLOTRETA CRUCIFERAE*) AND DIAMONDBACK MOTHS (*PLUTELLA XYLOSTELLA*)

By

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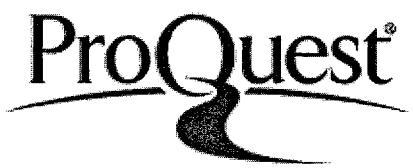

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Costs of Resistance in a Mustard Plant (*Arabidopsis thaliana*) Associated with Two Specialist Insects: Crucifer Flea Beetles (*Phyllotreta cruciferae*) And Diamondback Moths (*Plutella xylostella*) (60pp.)

Director: Thomas Mitchell-Olds TM-O by DAC

Herbivory by insects removes plant biomass that could be used for growth and reproduction. Although plants employ a wide range of defense traits against herbivory, insects may cause substantial damage in natural plant populations. Within populations of the same species, there is wide variation in both plant resistance characteristics and susceptibility to herbivory. If herbivory is detrimental to plant fitness, then why are resistant genotypes not the most common in a population? Possibly, a plant's ability to defend itself against insect herbivory may involve costs or tradeoffs. Plants that are well defended against a particular insect may show reduced defenses against other insects or pathogens and thus incur an ecological cost. In addition, plants that defend against herbivory may divert resources from growth or reproduction, so the plant may incur an energetic cost. However, few studies have shown any cost of resistance.

I examined two potential costs for *Arabidopsis thaliana* against two specialist insects: Diamondback moth larvae (*Plutella xylostella*) and crucifer flea beetles (*Phyllotreta cruciferae*). 1) Ecological costs were measured by comparing damage from two herbivores and determining if resistance to one insect confers susceptibility to the other, indicating an ecological cost. There were no ecological costs for this system. 2) Energetic costs were measured by comparing growth rate and resistance levels of 48 ecotypes. Results show that although there is a trend for an energetic cost of resistance to both insect herbivores for *Arabidopsis,* it is not significant. This suggests that either cost may not be as important as previously assumed in maintaining genetic variation for resistance traits, or that there is selection for the minimization of cost.

Dedication

This work is dedicated to the memory of
my grandmother, Maxine Dorothea Keup,
who passed away September 27, 1999.
She always encouraged me to pursue my dreams,
and taught me that education is
the most valuable gift we can give to ourselves.

Acknowledgements

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INTRODUCTION

All plants require light, water, carbon dioxide, and nutrients to grow, reproduce and defend themselves. One or more of these resources are generally limited for any given plant. Herbivores exert selective pressure by increasing mortality and removing biomass. Herbivores consume approximately 10% of plant productivity in natural communities, which is greater than the average allocation to reproduction (Coley et al. 1985). Insect herbivores reduce plant fitness by reducing seed production (Ehrlen 1995, Louda and Potvin 1995, Root 1996). Herbivory can also affect competitive interactions between plants and plant community structure (Louda et al. 1990, Burger and Louda 1995). For these reasons, we expect herbivory to be detrimental to plant fitness (Marquis 1992).

Because herbivory is detrimental, it is no surprise that plants defend themselves. Plant resistance to insect herbivory is influenced by a complex set of interactions that includes both a host plant's response to herbivory, and an insect's recognition of the plant as a potential host (Rausher 1996). Plants can respond to herbivory by changing investment in defensive traits, or by altering their predictability of occurrence in locations that herbivores cannot effectively find (Feeny 1976). Plants can escape herbivory by increasing defensive chemistry or other defensive traits to which herbivores may be unable to adapt. Alternatively, they can reduce apparency or predictability and then change chemistry or other traits to which herbivores cannot adjust (Chew and Courtney 1991).

Insects can respond to plant resistance traits through gradual or rapid adaptive change, developing the ability to digest secondary metabolites, avoid trichomes, digest waxes, change phenology in response to plant defense challenges. Some insects will remain unchanged, finding a host plant unsuitable, and move on to find new host plants. In such ways, a dynamic equilibrium is maintained between two changing systems, the plant and the insect (Dethier 1952).

Fraenkel (1959) observed that an insect species cannot feed on all plants, and is usually restricted to a few related host species. He connected plant resistance to herbivores and the presence of secondary metabolites, and argued that secondary metabolites are so variable within and among plant species that they are probably not essential for basic plant metabolism. The link between metabolites and plant resistance was extended by Ehrlich and Raven (1964) who noted that secondary metabolites evolved early in angiosperm evolution at a time roughly coinciding with major evolutionary radiation of phytophagous insects. Finally, Rhoades and Cates (1976) argued that secondary metabolites must have some positive effect on plant fitness that offsets the energy required to produce them. Because of this, most recent work has focused on secondary metabolism as an important cause of herbivore deterrence (Cates 1975, Berenbaum et al. 1986, Mauricio 1998). Indeed, many plants produce chemicals that are toxic to most herbivores (Berenbaum 1986).

However, we know that insects can be affected by a number of plant traits besides chemistry, including morphology, color, and phenology (Painter 1951). Insects can disperse to a new area and be attracted or repelled by certain plants based on olfactory, visual, or tactile cues. When an insect encounters a potential host plant, there is a series of chemical and behavioral events leading to recognition of a suitable host plant by the insect (Papaj and Rausher 1983). The insect may be repelled if the plant produces a chemical that is unpleasant to the insect (Zangerl et al. 1996). Thick wax layers or dense trichomes may physically prevent the insect from eating the nutritious tissue inside the plant and spiny projections may injure soft-bodied insects (Ågren and Schemske 1992). Insects can be attracted to a plant by color or pattern recognition of leaves or flowers. Phenology also plays a role in host plant use. The plant must be available to the insect during its period of active growth or its reproductive cycle. Plants may employ additional, unusual means to deter herbivory, such as by hyperaccumulation of heavy metals (Boyd and Martens 1994). Based on one or more of these factors, the insect either accepts or rejects the plant as a suitable host.

Clearly, many plant traits might affect an insect's preference for a particular plant. Consequently, focusing on a single defense trait might miss the *overall* resistance or susceptibility of the plant. Therefore, my research examines overall

levels of damage and resistance to herbivores, and considers the trade-off between overall resistance and growth rate, regardless of the mechanism of plant defense.

Although many defense strategies are effective at deterring herbivory, there is a high level of variation within and among plant species for defenses to herbivory. Many studies have found genetic variation for resistance to herbivory in natural and agricultural populations (Ågren 1993, Ågren and Schemske 1992). However, since there are obvious benefits of resistance, why do we find susceptible genotypes in plant populations? This apparent contradiction has lead ecologists to suggest that there are costs associated with resistance (Rausher and Simms 1989, Ågren 1993). If resistance is expensive, then high levels of resistance are favorable only when herbivores are present in a system. In the absence of herbivory, faster growing plants without defenses would be more competitive (Louda et al. 1990). The balance between costs and benefits of resistance could cause persistence of genetic variation for resistance in populations. Cost of defense is an important concept for life history evolution, because it imposes constraints on an organism's ability to respond to selection. In this study, I examine two types of costs: energetic and ecological.

Energetic costs can arise from the biosynthesis, maintenance, and turnover of resistance traits (Mooney et al. 1983). Many defenses use nitrogen, sulfur, and carbon in their construction, thereby redirecting limited nutrients that could

otherwise go to biomass and reproduction. If plants redirect essential nutrients from growth to reproduction, then a cost of resistance is detected as a negative correlation between resistance and growth rate, or between resistance and reproduction. However, plants can differ in their allocation of resources to growth, defense and reproduction (Bazzaz et al. 1987). Thus, to avoid complications associated with variable allocation of resources to reproduction, one may quantify cost and benefit in terms of growth rate before the onset of flowering, when allocation will be divided between defense, reproduction and growth.

Ecological costs occur when mechanisms causing resistance to one herbivore also influence susceptibility to other herbivores. Not all insects respond in the same way to the same resistance traits. Defensive traits that are effective against a certain herbivore may have no effect on another herbivore. It is even possible that a factor that negatively influences one herbivore will positively affect another. Thus, when considering plant resistance to herbivory, it is important to consider ecological interactions of different insects on the same host plant (Pilson 1996).

Observations show that host plants attract specific fauna, with anywhere from 20 to 300 species of insects found on single plant species (Hodkinson and Hughes 1982). Therefore, selection for many resistance traits may occur simultaneously (Pilson 1996). For example, an increase in a particular secondary

metabolite may reduce herbivory by one insect, but might actually increase herbivory by another species (Giamoustaris and Mithen 1995). If selection is independent or pairwise, meaning that plant response to a particular herbivore does not confer resistance to another insect, then there are generally no genetic correlations among levels of resistance to different pests, and no ecological interactions (Hougen-Eitzman and Rausher 1994). Alternatively, if selection is diffuse, or if a host plant's response to one herbivore confers either resistance or susceptibility to another, selection by one herbivore would affect the outcome for others, and genetic correlations and ecological interactions would be expected.

For example, increased investment in defensive traits could deter most non-specialist insects. In general, herbivores tend to be either "specialists" or "generalists." Generalists feed on many unrelated plant hosts. Specialist insects are able to detoxify or tolerate plant defenses. However, since they are generally only able to circumvent one or a few types of related defensive chemicals, these insects are restricted to only one or a few closely related species. Specialist insect herbivores are often stimulated to feed or oviposit by the very same secondary chemicals that are produced by their host plants and that serve as effective deterrents to generalist insects (Chew 1988). Thus, an increase in defensive chemicals may help defend a plant against generalist herbivores, but this defense may have an ecological cost because it attracts specialist herbivores. This cost is predicted to be especially acute in plant species that are fed on primarily by

specialist herbivores, because it would likely increase the number of specialist herbivores.

Alternatively, plants can increase or decrease the types and quantities of secondary chemicals produced. If specialists are unable to find the plant because of this reduction, the plant can escape herbivory. Of course, a reduction of secondary chemicals could increase the plant's susceptibility to generalist herbivores. This might imply that plants that are fed on by mostly specialist insects would decrease investment to secondary chemicals rendering those plants less chemically apparent and plants fed on by mostly generalists would increase defensive traits.

Although it is widely believed that costs of resistance provide the trade-offs responsible for maintaining genetic variation, it has been difficult to actually measure costs of resistance in plants (Simms and Rausher 1989, Simms 1992). Only a handful of studies has documented costs (Rehr et al. 1973, Cates 1975, Simms and Rausher 1987, Castro et al. 1988, Bergelson 1994, Zangerl et al. 1997). There are several reasons why costs might not be found in empirical studies of plant resistance.

First, focusing on a particular defense trait may miss the influence of other traits and fail to detect the combined cost of all traits (Bergelson 1994). Alternatively, measuring one trait might overestimate cost if correlated traits lack a defensive role (Zangerl et al. 1997).

Second, measuring growth rate after the plant has stopped actively growing, and begins shunting resources to reproduction, may underestimate the actual energetic cost (Fagerström 1989). If one measures growth rate after a plant has begun to flower, but does not measure number of flowers, seeds, or offspring produced, one may underestimate the importance of growth rate.

Third, low heritabilities may hinder experimental measurement of costs of resistance (Bergelson and Purrington 1998). Understanding the underlying relatedness of individuals of a plant population of would allow for partitioning costs to various genotypes.

My work focuses on three main questions. 1) Is there genetic variation for insect resistance in *Arabidopsis thaliana*? For many plant species, resistance to herbivory is a heritable, quantitative trait (Courtney and Chew 1991, Mithen et al. 1995), and we expect a similar pattern for *Arabidopsis*. 2) Is there a correlation between resistance to two specialist herbivores, *Plutella xylostella* and *Phyllotreta cruciferae*? To varying degrees, both insects are specialists of Brassicaceae, but feed on leaves during different life stages, so there is no clear expectation for correlations between resistance to these two insect herbivores. 3) Is there a cost of resistance such that resistant plants grow more slowly? We expect that plant genotypes that are well defended against herbivory will grow slower due to the physiological cost of constitutive defense traits. By examining costs associated

with resistance to insect herbivores, we can assess the importance of costs in maintaining genetic variation for resistance among ecotypes of *Arabidopsis*.

Materials and Methods

Biology of the Species

Arabidopsis thaliana is a member of the family Brassicaceae. It has a broad temperate distribution and there are many ecotypes available for study. The life cycle of the plant is approximately six weeks. *Arabidopsis* exhibits a small, rosette growth-form, typically producing 9 to 15 trichome-covered leaves before bolting. In many ecotypes, bolting occurs within 3 weeks of planting, and the plant may continue to flower for many weeks before senescence. Flowers are about two millimeters long, with four green sepals, four white petals, six stamens and a central gynoecium, and are largely self-pollinating. Each plant produces thousands of small seeds (0.5 mm long) within long slender siliques. *Arabidopsis* has simple roots with no nitrogen fixing bacteria or mycorrhizal associations. Natural pathogens include numerous viruses, herbivores, bacteria and fungi (Meinke, et al 1998). *Arabidopsis* also has the most extensively studied genome of any plant, with >50% of its genome sequenced. Its physiology and ecology is less well described, and much about its secondary metabolism is unclear (Chapple 1994).

Arabidopsis thaliana has many useful characteristics for studies of insect resistance. Plants in the Brassicaceae produce secondary metabolites, termed glucosinolates, which are known to influence insect behavior. These plants are self-pollinating and largely homozygous (Lister and Dean 1993, Bergelson et al.

1998). Therefore, with proper replication and randomization, differences observed between ecotypes grown in the same environment are due mainly to genetic differences. Seeds used in the present experiments are from collections of *Arabidopsis* ecotypes maintained by the *Arabidopsis* seed stock center at Ohio State University, providing an excellent starting point for screening many homozygous lines for resistance to insect herbivory.

Plutella xylostella, or the Diamondback moth, is a member of the order Lepidoptera and the family Plutellidae. Diamondbacks are distributed throughout the world and are a common pest for *Brassica* farmers, causing a great deal of damage. Females lay eggs on the leaves of the host plant which hatch within 4 to 8 days. Larvae go through four instars and consume a large amount of food, eating all leaf tissue except veins and upper epidermis, thus creating a "window pane" effect. The first instar mines leaf tissue, whereas subsequent instars are surface feeders. The larval stage usually lasts 15 to 18 days. When fully grown the larvae construct a fine open-network cocoon on the underside of the leaves of the host plant or in a protected nook. Pupae emerge as adults within 10 to 15 days. Adults are weak flyers, readily carried by wind currents. They are relatively inactive during the day, ovipositing at dusk for a few hours, then are inactive until the following evening. Adults usually feed on nectar from cruciferous weeds near large agricultural plots (Harcourt 1957). In the north temperate region, diamondback moths have 4 to 6 generations during a single northern growing

season, but do not survive extended freezing temperatures. Therefore, northern populations migrate from the 36th parallel every year. Studies of migration patterns suggest that the first generation of moths develop primarily on crucifer weeds, and following generations damage crops. Diamondback moths (DBM) used in these experiments were purchased as eggs from New York State Agricultural Experiment Station and raised on an artificial wheat-germ based diet (see appendix B) for use in experiments. Mass rearing of DBM is recommended for host plant resistance studies (Shelton et al. 1991).

Phyllotreta cruciferae, or the crucifer flea beetle, is a member of the order Coleoptera in the family Chrysomelidae. These insects are distributed throughout the northern temperate region and are another common pest for *Brassica* farmers. Flea beetle adults chew small holes in the leaves of host plants and larvae feed on the roots of plants. Common food choices are *Brassica rapa* and *Brassica napus*. Flea beetles produce 1-3 generations in a season, depending on weather conditions. In warmer years with mild winters, beetles will reproduce in greater numbers. Adults can tolerate colder conditions and often survive late into fall, however they generally will not lay eggs at temperatures below 17° C (Kinoshita et al. 1979). Adults lay eggs in the soil near the base of a host plant in late spring through summer, and eggs take from 5 to 20 days to hatch. Larval development is divided into three stages, lasting from 10 to 25 days. The pupal stage lasts from 8

to 18 days. Flea beetles were collected from agricultural plots in Missoula and Ravalli counties in Montana.

Greenhouse and Planting methods

Arabidopsis plantings were placed in a 4° C cooler for two weeks to facilitate germination. The flats were then placed in the growth chamber under fluorescent lights (Gro-Lux and Cool White) with 18-hour days, at 23° C, and 60% humidity. New trays and pots were used for all experiments. Pots were filled with Scott's Peat-lite growing mix, a mixture of peat moss and vermiculite. Two tablespoons of Osmocote, a time release fertilizer, were added to each flat. Flats were rotated every day, to ensure even growth in all flats. I used eight-inch by sixteen-inch 96-well flats and a computer-generated randomized complete block design for each experiment. This design was used because we expect some environmental differences between flats. Only one seed was planted in each of the 1/2" x 1" x 1" wells. Wells that had more than one germinating plant were deleted. Plants that had any sign of damage were also removed to avoid confounding factors.

Heritability of Resistance to Crucifer Flea Beetles

Three experiments were performed to examine genetic variation for resistance, heritability of resistance, and ecological interactions between different herbivores in *Arabidopsis.* To determine if there is heritable variation for insect resistance, I screened fourteen homozygous ecotypes for the most resistant and the most susceptible to herbivory. The fourteen ecotypes were planted in four flats (14 ecotypes x 6 reps per flat x 4 flats = 336 plants). Germination dates were recorded and the plants grown for 4 weeks before exposure to herbivory. The flats were then placed in boxes with mesh sides and tops. Approximately 50 *Phyllotreta cruciferae* were placed in each cage. The insects fed on the plants for 48 hours, after which the insects were removed, and the plants were scored for size and damage. Size was estimated by measuring width across the widest part of the rosette and the height of the flowering stalk. Damage was measured by counting the number of holes chewed in the leaves.

Analysis of these data showed that there was genetic variation for resistance among ecotypes, and a resistant parent and a susceptible parent were chosen to cross-pollinate to use for genetic analysis in the next experiment. I chose the ecotypes that show the greatest difference in resistance to maximize genetic variation in the subsequent offspring. To form the recombinant inbred lines, two parental plants were cross-pollinated by first removing the sepals, petals and stamens from an unfertilized flower of one parent plant, and pollinating with

anthers that were removed from the other parent. The fertilized ovary was allowed to mature and the silique was collected when ripe. These seeds represent the F1 generation. These few seeds were planted and when they germinated only one plant was kept to produce seeds for the next generation. I chose the healthiest plant in order to obtain the greatest amount of seed for future experiments. Young leaves were gathered from this F1 plant and both parental ecotypes for DNA extraction using the Kunkel prep (Appendix A). PCR (Polymerase Chain Reaction) was performed using CAPS (Cloned Amplified Polymorphic Sequence) primers known to be polymorphic between the parents to verify if the cross was successful. The PCR product was digested with restriction enzymes, and then run on an agarose gel. The F1 plant possessed both parental bands, and was deemed a successful cross.

This F1 plant generated thousands of seeds, which represent the F2 generation. These F2 seeds were randomly selected from a tube, planted, germinated, and seed from 72 F2 individuals was collected separately. This formed the F3 seed stock that was used in our experiment. Each generation of each line is continued by a single self-fertilized plant and thus reduces homozygosity by 50% in each generation. This is because there is a 50% chance the genes will be heterozygous and 50% chance they will be homozygous at each locus for each recombination event (Hartl and Clark 1989, Lister and Dean 1993).

To determine the heritability of resistance to herbivory by flea beetles, F3 generation seed families were planted and the plants exposed to herbivory by *Phyllotreta cruciferae*. 72 F3 lines, 12 F2 and 6 of each parental ecotype were planted in sixteen 96 well flats (1 representative from each line x 72 lines + 12 F2 seeds + 6 resistant parent seeds + 6 susceptible parent = 96 plants per flat x 16 flats = 1536 plants). The date of germination was recorded, and the plants were grown for 3 weeks before exposure to herbivory for 48 hours by *Phyllotreta cruciferae*. Approximately 50 insects were placed in each box along with one flat of plants. Damage was scored by counting the number of holes chewed in the leaves of the plants and size was estimated by measuring across the widest part of the rosette.

Ecological Interactions

The third experiment was designed to examine possible ecological costs of resistance. 16 flats of plants with 72 F3 lines, 12 F2 and 6 of each parental ecotype flats (1 representative from each line x 72 lines + 12 F2 seeds + 6 resistant parent seeds + 6 susceptible parent = 96 plants per flat x 16 flats = 1536 plants), were planted. Eight of these flats had to be discarded due to damage, so data was collected on the remaining eight flats. Size was estimated by measuring rosette diameter before herbivory. The flats were placed in mesh bug boxes, and plants were exposed to herbivory by placing five eggs of *Plutella xylostella* on each plant. The eggs hatched and larvae fed on the plants for one week. Damage was scored using a scale from 0 (no damage) to 5 (severe damage).

Ecological interaction was determined by comparing the results of the flea beetle and diamondback moth experiments. By plotting the average resistance of an ecotype to diamondback moths and flea beetles, the genetic correlation of resistance levels can be determined. A positive correlation would show that resistance to one herbivore means a plant is resistant to another. A negative correlation would show that resistance to one herbivore means an ecotype is susceptible to the other, indicating an ecological trade-off. If no significant correlation is found, there is a lack of predictive value: we cannot say anything about resistance to different herbivores based on the observation of only one

herbivore. I would expect that specialist herbivores respond to similar traits in *Brassicaceae* because of the specialized chemistry found in this family, which would result in a positive correlation. However, since these herbivores are in different orders and feed on the plant during different life stages, these insects may not respond to the same defensive traits in the same way, which would result in a non-significant or negative correlation. A negative correlation would suggest that there are ecological trade-offs for *Arabidopsis*. In being resistant to flea beetles, the plant is susceptible to attack by diamondback moths and vice versa.

Measuring the Cost of Resistance

Resistance to Diamondback Moths in 48 Ecotypes

It was noted from the previous flea beetle experiments that *Arabidopsis* ecotypes with high versus low levels of flea beetle resistance appeared to differ in size: resistant ecotypes seemed to grow more slowly (personal observation). To further investigate this apparent trend three experiments were performed. First, 48 ecotypes from the *Arabidopsis* seed stock center were planted in eight flats (48 ecotypes x 2 reps per flat x 8 flats = 768 plants). Flats were placed in the growth chamber and germination dates were recorded. Plants were grown for four weeks and exposed to herbivory by DBM first instar larvae at a density of one larva per plant. Rosette diameter was measured across the widest part of the rosette before herbivory. Larvae fed on the plants for four days inside Plexiglas boxes with one mesh side for air circulation. Boxes were kept under fluorescent lights with 12-hour day-length. Herbivory was scored from 0 (no damage) to 10 (100% damage, plant totally eaten).

Resistance to Flea Beetles in 48 Ecotypes

For the second experiment, 48 ecotypes of *Arabidopsis thaliana* were planted in eight flats, in the same manner as the previous experiment. Germination dates were recorded and the plants grown for three weeks before exposure to herbivory. Size was measured in millimeters across the widest part of the rosette.

The flats were then placed in Plexiglas boxes with one mesh side for ventilation. Twenty-five *Phyllotreta cruciferae* were placed in each cage. Boxes were kept under fluorescent lights with 12-hour day-length. The insects fed on the plants for four days and were then removed, and the plants were scored for damage. Damage was measured by counting the number of holes chewed in the leaves.

Growth Rate

Growth rate was measured in absence of herbivory. Forty-eight ecotypes of *Arabidopsis thaliana* were planted in eight flats (48 ecotypes x 2 reps per flat x 8 flats = 768 plants) and germination dates recorded as before. All plants were weighed (fresh weight) when the first plant began bolting. Rosette diameter was measured across the widest part of the rosette and the number of leaves was counted. I measured growth rate at the same time for all plants because I would expect that the cost of resistance would be highest while the plant is actively growing, before the onset of flowering. Also, flowering times differed greatly among these ecotypes, and waiting for each individual to flower before harvesting might bias my results.

We expect that plants that are more resistant to herbivory will grow more slowly because of the nitrogen component of the glucosinolate molecule. Nitrogen is one of the most limited resources for most plant species (Mooney et al. 1983). Glucosinolates are also a constitutive defense, and thus must be constructed before damage from herbivory occurs.

Statistics and Quantitative Genetics

Flats, populations and families were considered random effects in the analyses of variance (ANOVA) and analyses of covariance (ANCOVA) as a randomized complete block design with flats as blocks was used in all experiments. This means that each seed was placed randomly within each flat and each flat had at least one representative from each family or ecotype group. Dependent variables were either damage or growth rate.

Heritability was calculated by determining the coefficient of determination attributable to F3 genotypes. This was calculated by determining the R^2 value for the ANOVA with F3 families included in the model (Table 3) and then subtracting the R^2 value from the ANOVA without the F3 families included (Hartl and Clark 1989, Falconer and MacKay 1996).

Pearson's correlation was used to determine the correlation of size and damage to determine if there is a trade-off between growth and resistance, as well as correlation of damage by the two herbivores to determine if there is an ecological cost for resistance, by using mean values calculated from least square means. I used SPSS version 7.0 to analyze all data.

Results

Heritability of Resistance to Crucifer Flea Beetles

There was significant genetic variation among 14 ecotypes exposed to herbivory by *Phyllotreta cruciferae*. The ANCOVA for flea beetle damage indicates that there is significant genetic variation ($p < 0.0005$, Table 1) for resistance to flea beetles. A resistant and a susceptible genotype were identified: Sei-O from Italy and Tac from Washington, USA (Table 2). Sei-O and Tacoma plants were chosen for further exploration of the heritability of resistance. Table 2 also shows the mean number of holes chewed by flea beetles, the standard error and the number of individuals for each ecotype examined. These plants were cross-pollinated, and self-fertilized progeny raised to the F3 generation (see methods). When F3 plants were exposed to herbivory by *P. cruciferae*, there were significant differences in resistance levels and evidence of genetic variation among F3 lines (ANOVA, $p < 0.005$, Table 3).

The heritability of flea beetle resistance was approximately 11%. Heritability is the proportion of the variance attributable to the family factor in the ANOVA. While this may seem a low heritability value, it is acceptable for a quantitative trait. This value attributes 11 percent of the variation in insect resistance to the effect of F3 family. Other variation, such as variation in the insect population, would influence resistance as well.

The same planting design was used and plants were exposed to herbivory by *Plutella xylostella*. Analysis again showed significant differences among F3 lines for resistance to diamondback moth larvae, and thus there is genetic variation for resistance ($p < 0.0005$, Table 4). The heritability of resistance to diamondback moths was calculated in the same manner as for flea beetles. Heritability of diamondback moth resistance is approximately 25%. This value is more than double the results for flea beetles, possibly because lab reared insects would exhibit less variation than field collected insects.

Energetic Costs – Grow or Defend?

To address the potential trade-off between resistance and growth in a direct way, I performed three separate experiments to quantify resistance to flea beetles and diamondback moths and growth rate of 48 ecotypes. Resistance to flea beetles among 45 ecotypes of Arabidopsis shows that there is a lack of evidence of genetic variation for flea beetle resistance among these ecotypes ($p > 0.10$, Table 5). Indeed, the effects of flat and size are the more significant values ($p < 0.001$). As the results for this insect herbivore are not significant, further interactions, such as growth trade-offs or ecological interactions are not predicted to be significant for this insect.

In a comparison of resistance to herbivory by *P. xylostella* among 48 ecotypes using ANCOVA, there were significant differences in resistance among ecotypes and evidence of genetic variation for resistance to diamondback moth larvae ($p < 0.001$, Table 6). The model explains 43% of the variation in resistance to diamondback moths.

Finally, there were significant genetic differences in size among the 48 ecotypes. Ecotypes show significant genetic variation for growth rate ($p < 0.001$, Table 7). This allows us to compare diamondback moth resistance with growth rate to determine if there is a cost of resistance for Arabidopsis. Correlation shows that plants that are more resistant to diamondback moths tended to grow slower,

but this trend was not significant at the 0.05 level ($p < 0.10$, Table 9). A two-tail Pearson's correlation shows that there is insufficient evidence of a trade-off of growth for plants that are resistant to Diamondback moths. This means that there is a trend in this experiment of a cost of resistance for *Arabidopsis*.

Calculations for fleabeetle resistance versus growth rate are not predicted to be significant, because the results for resistance to flea beetles were not significant. A two-tail Pearson correlation shows that these results are not significant ($p < 0.2$, Table 10) and we can assume that there are no costs associated with resistance to flea beetles for *Arabidopsis*. A summary of insect damage ordered by ecotypes and average weight of each is listed in Table 12.

Ecological Interactions

I used Pearson correlation analysis to determine ecological interactions between resistance to contrasting herbivores. A positive correlation would show that resistance to one insect herbivore confers resistance to the other herbivore, whereas a negative correlation would indicate an ecological trade-off because resistance to one insect would confer susceptibility to another.

In the F3 experiment, resistance to *P. cruciferae* and *P. xylostella* was genetically uncorrelated in this segregating population when comparing F3 families (Fig. 1 and Table 5). Although there is a weak negative correlation ($r = -0.009$), it is not statistically significant ($p = 0.941$), and this suggests that the genes segregating in this cross do not cause ecological trade-offs for resistance. Because the results for fleabeetles in the ecotype experiment were not significant, a correlation analysis would not be predicted to show significant results. A significant positive correlation was found, however, and this suggests that there are no ecological costs associated with resistance to these two insect herbivores ($r= 0.356$, $p < 0.05$, Table 11 and Fig. 2). Thus, these results appear to be in agreement with each other, there are no ecological costs associated with resistance to these insect herbivores.

Discussion

Herbivory by insects removes host plant biomass. Plants have the ability to reduce herbivory with many defense strategies. Likewise, insects can adapt to plant defenses, or move to other host plants within a population or community. Most insects have a relatively narrow host range, and are usually restricted to a few related species. Secondary metabolites, which may be induced by herbivory or show constitutive expression, have been widely studied. In addition, trichomes and other plant traits affect insect behavior. Given the variety and effectiveness of plant defenses, and the deleterious consequences of herbivory, it is surprising that plants display genetic variation for resistance. This suggests that there are physiological costs of resistance: allocation of resources to defense may reduce resources for growth, and thus reduce the competitive ability of a plant within a population. Ecological costs may also play a part in maintaining genetic variation within a population. Ecological costs occur if herbivores respond in opposite ways to plant traits: an increase in a particular chemical may deter one herbivore, but attract another. In many studies, measuring cost has been somewhat problematic. In this study, I examine ecological and allocation costs to resistance in a model plant species, *Arabidopsis thaliana*.

My findings indicate that plant resistance to herbivory is a quantitative and heritable trait in *Arabidopsis*. This is consistent with other studies of plant resistance (Ågren 1993). Quantitative traits are those phenotypic traits influenced

by genetic segregation of more than one gene. Genetic and environmental effects influence quantitative variation. In the research reported here, the environmental component was controlled by using recombinant inbred lines or homozygous ecotypes, which allows for quantification of genetic variation among lines (Falconer and Mackay 1994). Therefore, the differences among families or ecotypes have mainly a genetic component.

Heritability of genetic variation is responsible for the resemblance between relatives. Heritability is measured as either broad or narrow sense heritability. Broad sense heritability is the proportion of total phenotypic variance that is attributable to genetic variance, including additive genetic variation, epistatic and dominance effects. Narrow sense heritability measures only additive genetic variation (Falconer and Mackay 1994), and is the primary contributor to evolutionary change. In this study, broad sense heritability for resistance to flea beetles among ecotypes was approximately 11 percent. Although this may seem low, it is a reasonable figure for a complex quantitative trait such as insect resistance. Heritability for resistance to diamondback moths was approximately 25%, much higher than the heritability for flea beetle resistance, likely due to the lab rearing of diamondback moths which decreases environmental variation within the population.

In the F3 experiments there was no significant genetic correlation between diamondback moth and flea beetle feeding. To investigate this further, I looked at

a larger number of ecotypes. It appears that there is a significant positive correlation: plants resistant to flea beetles were also likely to be resistant to diamond back moths, and thus there is no evidence of an ecological trade-off. However, as the results for flea beetle resistance among ecotypes was not significant, the information on ecological cost may be subject to statistical limitations. In conclusion, there is no evidence of any ecological cost of resistance for *Arabidopsis* for these two insect species.

To examine the allocation cost of resistance, I looked at resistance among many ecotypes to flea beetles and diamondback moth larvae and measured growth rate in three separate experiments. Theory suggests that in many plant species, larger plants typically have greater fecundity (Mitchell-Olds, 1992) and thus a smaller plant may sacrifice fecundity to be well defended. In *Arabidopsis*, as well as other plants, genes that regulate resource allocation are the suggested cause of trade-offs (Herms and Mattson 1992, Mitchell-Olds 1996). If there is a cost of resistance for *Arabidopsis*, we would expect to detect it with these experiments. However, I did not find a significant cost of resistance for flea beetles, and a slight trend for a cost of resistance to diamondback moths

What could explain the lack of evidence for a genetic basis of resistance for flea beetles among ecotypes? For one thing, flea beetles were collected from wild populations for each experiment, and thus environmental variables influencing populations would change. This could easily increase uncontrolled variation in

these experiments, such that the results are not reliable. Future work with flea beetles would require a system for lab rearing. Second, there is little evidence for flea beetles feeding on *Arabidopsis* in the wild. Perhaps there is no genetic variation among Arabidopsis genotypes because these plants are maximally defended against flea beetles.

Genetic variation for resistance to diamondback moths was detected in both the F3 experiment and the ecotype experiment. Heritability values were also much higher for diamondback moth resistance than flea beetle resistance. This suggests that lab rearing of insects results in lower levels of environmental variation among insects. Additionally, personal field observations also indicate that diamondbacks can complete their life cycle on *Arabidopsis*. Literature also suggests that as diamondbacks migrate north, their initial hosts are weedy mustards, on which they complete at least one life cycle before they attack Brassica crop species, such as cabbage and kale (Harcourt, 1957).

Because no genetic variation for flea beetles was found, a significant correlation between growth rate and flea beetle resistance would not be predicted. This is indeed the case. Additionally, I found a lack evidence for a cost of resistance for diamond back moths. Costs may be expected if we look at one of the most commonly researched chemicals in the Brassicaceae, glucosinolates.

Glucosinolates are biologically active secondary metabolites found in the Brassicaceae (Magrath et al. 1994, Renwick 1996). Glucosinolates, constitutive

defenses found in all members of the Brassica family, are composed of glucose, nitrogen, and sulfur. Therefore, cruciferous plants invest important resources in these compounds before damage to the plant occurs (Bones and Rossiter 1996). Glucosinolates are contained in cellular vacuoles, and corresponding myrosinase hydrolytic enzymes are contained in extracellular compartments (Van Etten and Tookey 1979). In *Arabidopsis*, a small gene family encodes myrosinase, and the number of myrosinase loci is less than other Brassicaceae (Chadchawan et al. 1993). Upon damage to the leaf, these products come into contact and isothiocyanates are released - the characteristic mustard oils that are unpleasant to many organisms. The overall complexity of the system indicates an important role for crucifer plants (Van Etten and Tookey, 1979). Additionally, indole glucosinolates may be physiologically related to indole acetic acid, a growth hormone in plants, which would further indicate that Arabidopsis sacrifices growth for resistance (Chapple 1994).

However, it is important to remember that secondary metabolites may have other functions. Pathogen defense, attraction of pollinators, protection from UV light, structural support, temporary nutrient storage, phytohormone regulators, drought resistance, facilitation of nutrient uptake, protection of roots, and mediators of soil microbe interactions are some of the possibilities (Herms and Mattson, 1992). Some of these could correlate with insect resistance traits and reduce the cost of resistance (Siemens and Mitchell-Olds 1996).

Trichomes, spiny projections from leaves, are thought to be a structural defense and have been shown to be genetically variable and heritable in *Brassica* and *Arabidopsis* (Ågren and Schemske 1992, Mauricio 1998). They are composed of carbon, which may represent a costly investment of limited resources for plants. Trichomes have other functions such as increasing boundary layers, decreasing UV radiation, and their defensive role is less clear.

Arabidopsis employs other means of defense against herbivory, in addition to glucosinolates and trichomes. In recent work, jasmonate has been implicated in insect defense (McConn et al. 1997). Other Brassicaceae are also tolerant of herbivory (Brandt and Lamb 1993, Stowe 1998) and thus maintain lower levels of defensive compounds. *Arabidopsis* also is a very small plant that grows quickly. Small plants provide little food for herbivores, and may cause insect herbivores to reduce the egg-loads placed on each plant, to mature early, and to exhibit a grazing habit of moving from plant to plant (Thompson 1983). Fast growing plants are less apparent and may not attract generalist herbivores (Mithen et al. 1995). In addition, *Arabidopsis* grows in very dense patches, which would increase the grazing habit of insects. Clearly, many possible components of the plant-insect interaction remain for future exploration.

The results of my study indicate that it is not energetically costly to defend against two insect herbivores. Additionally, these specialist insects appear to be

responding to similar plant features, and thus resistance is not ecologically costly for *Arabidopsis*.

Further investigation into the actual genes involved in resistance might elucidate the mechanisms of defense in *Arabidopsis*. I have raised approximately 300 F6 RI lines from the cross of flea beetle resistant and susceptible ecotypes. I have also crossed ecotypes that are resistant and susceptible to diamondback moths and have several thousand F2 seeds. These crosses can be used in QTL mapping to identify loci correlated with resistance. In another 2 years, the entire *Arabidopsis* genome will be sequenced. This would allow for further fine scale mapping to identify genes that correlate with resistance. Once these genes are identified, their function can be determined. I predict that some of these will be glucosinolate and myrosinase genes. Others may lead to some interesting surprises. Finally, when genes are determined, the next step would be to look to other closely related species, such as *Arabis lyrata*, to determine the universality of my results within the Brassicaceae.

Figure 1: Correlation of Insect Damage in 72 F3 RI Families

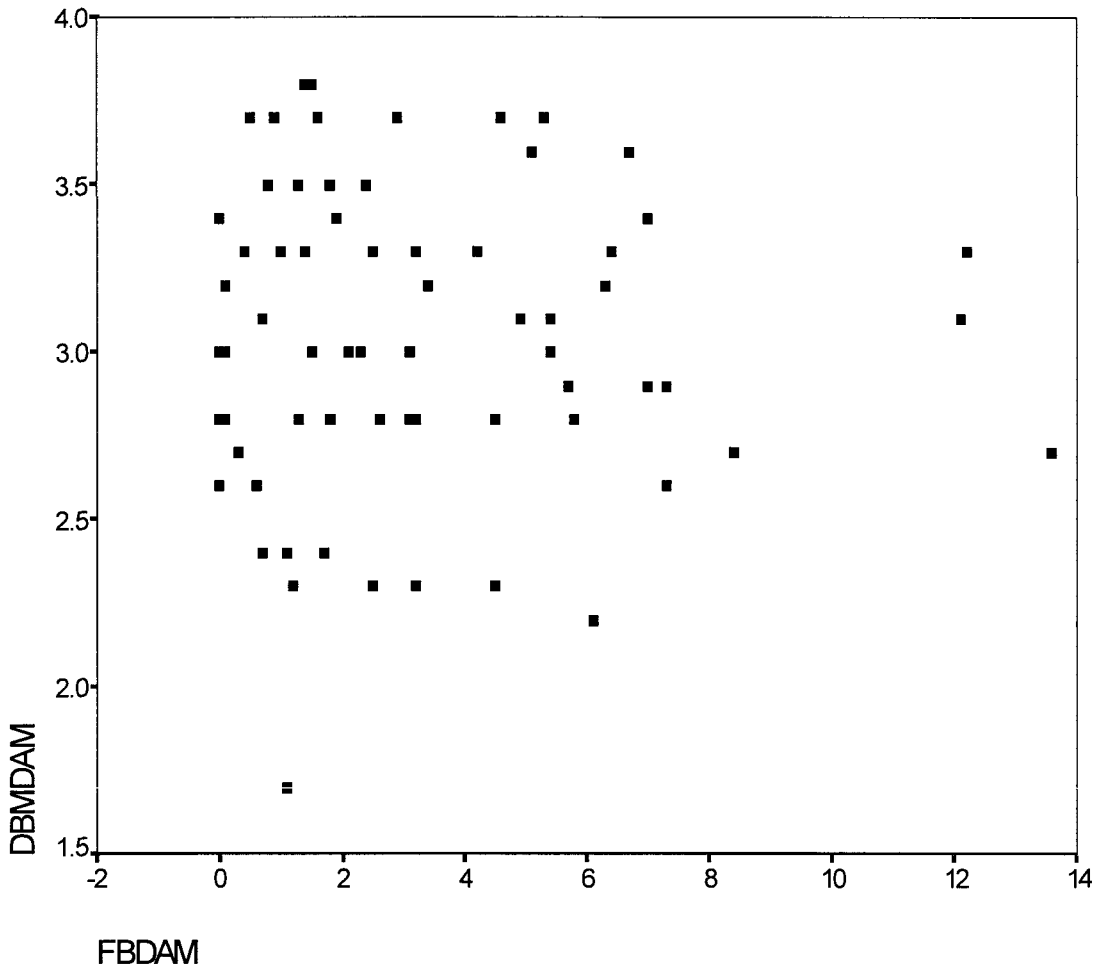


Figure 2: Correlation of insect damage for 36 ecotypes of *Arabidopsis*

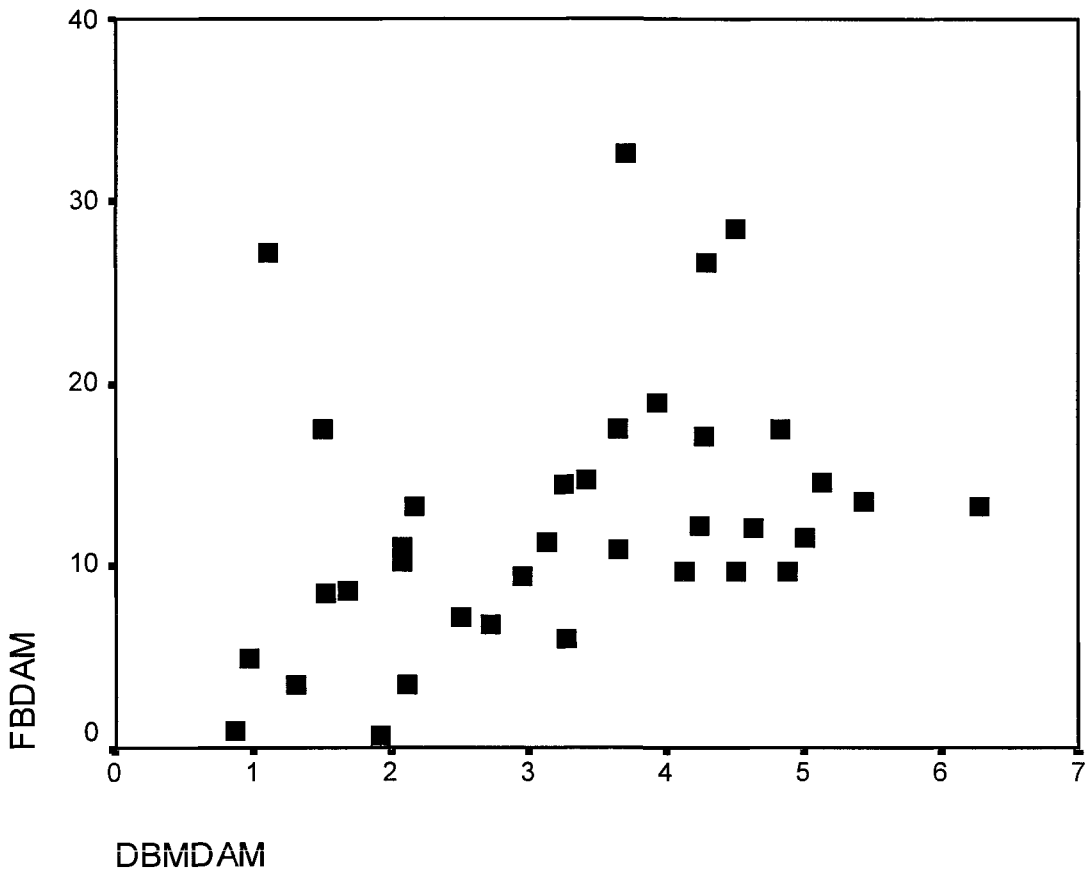


Figure 3: Correlation of growth rate and Diamondback Moth damage

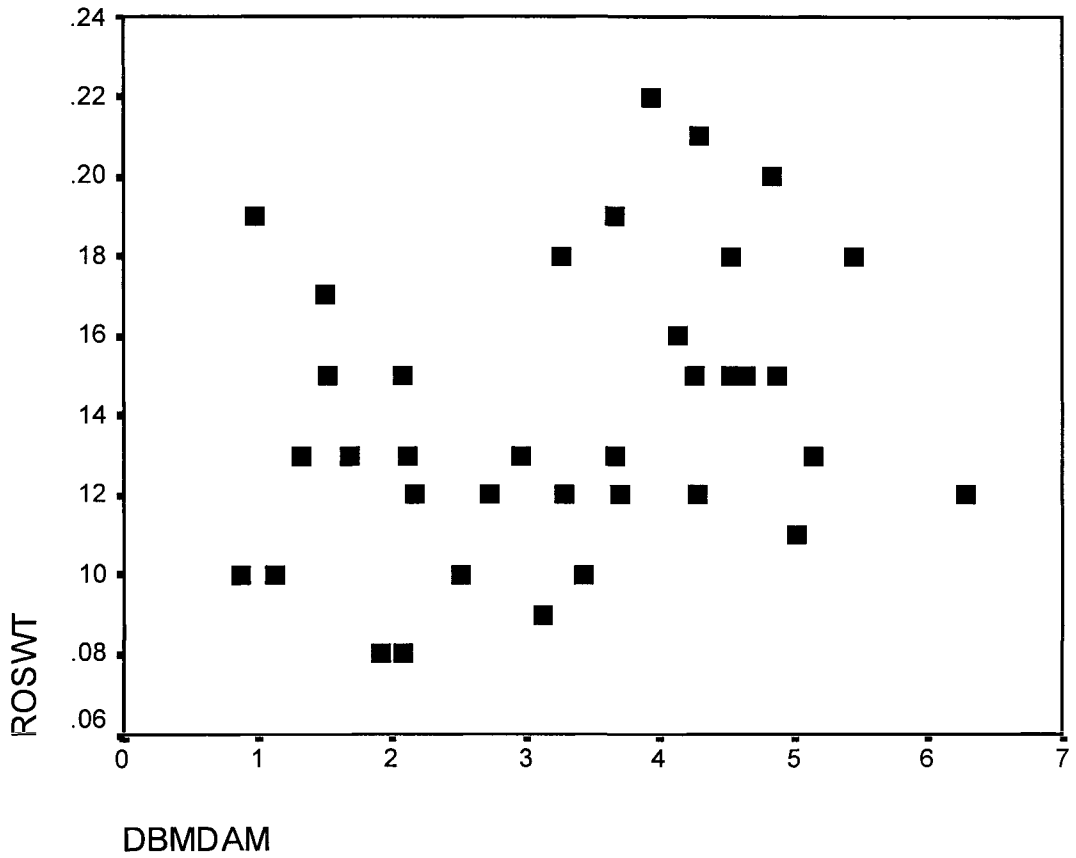
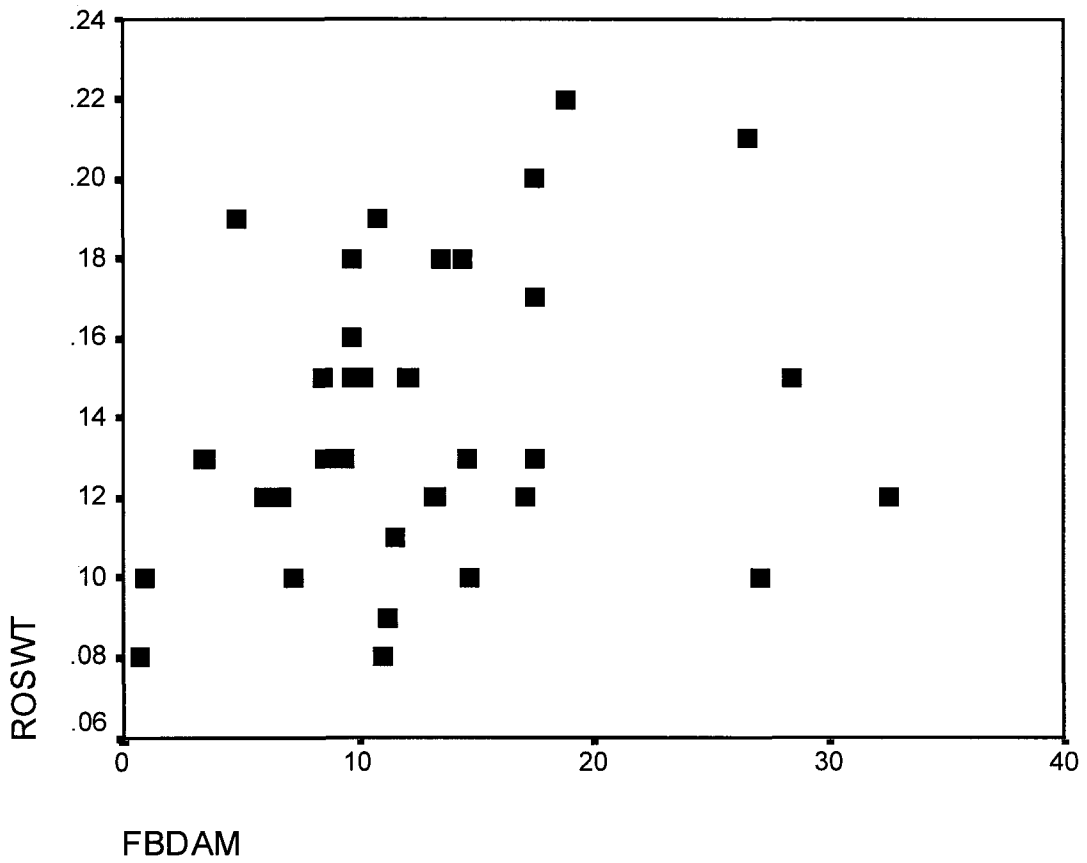


Figure 4: Correlation of growth rate and flea beetle damage



Tables

Table 1: ANCOVA for resistance to *P. cruciferae* of 14 *A. thaliana* ecotypes

Source	Degrees of Freedom	Mean Square	F-ratio	p-value
Flat	3	1693.084	4.184	0.007
Ecotype	13	1383.239	3.419	**0.000**
Flat*Ecotype	39	404.556	0.761	0.845
Rosette width	1	27378.475	51.492	0.000
Rosette height	1	2410.512	4.534	0.034
Error	206	531.704		

Dependent variable: number of holes N= 264 Multiple R^2: 0.532

Table 2: Damage to *Arabidopsis* by *P. cruciferae* in experiment 1

Ecotype	**Mean number of holes**	**Standard Error**	**N**
Sei-O	10.5	6.99	15
NI	11.1	5.65	19
Nd-O	22.7	6.59	13
La-O	23.8	5.72	16
MT-O	24.5	5.26	20
Perm-1	25.6	6.14	14
Su-O	25.7	5.33	23
Kil-O	28.3	4.66	24
d-Ler	31.7	5.20	20
Cal	33.4	5.38	23
NO-0	34.4	5.50	19
Tacoma	42.4	5.24	19
Kas-1	44.9	4.88	24

Table 3: ANOVA for resistance to *P. cruciferae* in 67 F3 families

Source	D.F.	Mean Square	F-ratio	p-value
Covariates (combined)	2	1227.32	18.45	0.000
Germination date	1	52.54	0.79	0.374
Rosette diameter	1	1673.34	25.16	0.000
Main Effects (combined)	83	99.29	1.49	0.004
Flat	15	88.62	1.33	0.176
ID	68	102.78	1.55	**0.004**
Model	85	134.46	2.02	0.000
Residual	751	66.52		
Total	836	73.43		

Dependent variable: number of holes N=837 Multiple R^2: 0.186

Table 4: ANOVA for resistance to *P. xylostella* in 60 F3 families

Source	D.F.	Mean Square	F-ratio	p-value
Covariates (combined)	2	5.75	8.49	0.000
Germination date	1	1.61	2.38	0.124
Rosette diameter	1	6.75	9.96	0.002
Main Effects (combined)	68	1.49	2.19	0.000
Flat	7	2.08	3.07	0.004
ID	61	1.41	2.08	**0.000**
Model	70	1.67	2.46	0.000
Residual	328	0.68		
Total	398	0.85		

Dependent variable: relative damage N=398 Multiple R^2: 0.344

Table 5: Pearson correlation of *P. xylostella* (DBM) and *P. cruciferae* (FB) damage for 66 F3 families

		DBM DAM	FB DAM
Pearson correlation	**DBM DAM**	1	-.009
	FB DAM	-.009	1
Sig. (2-tailed)	**DBM DAM**	.	0.941
	FB DAM	0.941	.

Table 6: ANCOVA for *P. cruciferae* resistance among 45 ecotypes of *A. thaliana*

Source	df	Mean Square	F	p-value
Covariates (combined)	2	2704.76	5.26	0.005
Germination date	1	81.51	0.16	0.691
Rosette diameter	1	4841.23	9.42	0.002
Main Effects (combined)	51	1077.31	2.10	0.000
ID	44	664.94	1.29	**0.103**
Flat	7	3704.72	7.21	0.000
Model	53	1364.70	2.66	0.000
Residual	534	513.79		
Total	587	590.61		

Dependent variable: damage N = 587 $R^2 = 0.21$

Table 7: ANCOVA for *P. xylostella* resistance among 47 ecotypes of *A. thaliana*

Source	df	Mean Square	F	p-value
Covariates (combined)	2	89.24	18.15	0.000
Germination date	1	132.76	27.00	0.000
Rosette diameter	1	3.27	0.67	0.415
Main Effects (combined)	53	27.94	5.68	0.000
Flat	7	55.94	11.38	0.000
ID	46	23.36	4.75	**0.000**
Model	55	35.02	7.12	0.000
Residual	510	4.92		
Total	565	7.85		

Dependent variable: number of holes N=566 Multiple R^2: 0.43

Table 8: ANCOVA of growth rate for *Arabidopsis* ecotypes

Source	df	Mean Square	F	p-value
Covariates (combined)	2	0.867	826.1	0.000
Germination date	1	1.355	1291.4	0.000
Rosette diameter	1	0.002	18.2	0.000
Main Effects (combined)	50	0.0005	4.86	0.000
Flat	7	0.0005	4.87	0.000
ID	43	0.0005	4.75	**0.000**
Model	52	0.006	57.61	0.000
Residual	488	0.0001		
Total	540	0.00068		

Dependent variable: Rosette weight N = 541 $R^2 = 0.86$

Table 9: Pearson correlation of *P. xylostella* damage and rosette weight for 36 ecotypes of *Arabidopsis*

		DBM DAM	ROSETTE
Pearson correlation	**DBM DAM**	1	0.285
	ROSETTE	0.285	1
Sig. (2-tailed)	**DBM DAM**	.	0.083
	ROSETTE	**0.083**	.

Table 10: Pearson correlation of *P. cruciferae* damage and rosette weight for 36 ecotypes of *Arabidopsis*

		FB DAM	ROSETTE
Pearson correlation	**FB DAM**	1	0.243
	ROSETTE	0.243	1
Sig. (2-tailed)	**FB DAM**	.	0.153
	ROSETTE	**0.153**	.

Table 11: Pearson correlation of *P. cruciferae* and *P. xylostella* damage for 36 ecotypes of *Arabidopsis*

		DBM DAM	FB DAM
Pearson correlation	**DBM DAM**	1	0.356
	FB DAM	0.356	1
Sig. (2-tailed)	**DBM DAM**	.	0.033
	FB DAM	**0.033**	.

Table 12: Ecotypes - Growth rate and damage

Ecotype	P. cruciferae damage Ave. number of holes	P. xylostella damage Ave. relative damage	Rosette Weight Ave. fresh weight	Number of observations
aa-o	10.2	2.07	0.15	16,13,11
abd-o	14.6	5.14	0.13	15,16,11
ba-I	17.5	3.66	0.13	12,7,11
bch-3	5.96	3.28	0.12	9,13,7
col	11.2	3.13	0.09	14,12,12
condara	12.1	4.64	0.15	16,14,16
cvi-o	14.7	3.42	0.10	13,12,10
da(1)12	0.88	0.86	0.10	14,11,10
db-I	6.72	2.72	0.12	13,13,14
di-g	13.2	2.16	0.12	9,16,13
edi-o	18.9	3.94	0.22	16,16,15
ei-2	13.3	6.28	0.12	12,13,14
ema-I	32.6	3.70	0.12	15,14,15
est	17.5	1.50	0.17	9,16,10
hodja	9.65	4.88	0.15	16,13,15
jl-3	8.56	1.68	0.13	13,14,14
ka-o	11.0	2.08	0.08	16,11,16
kas-I	10.8	3.66	0.19	12,14,14
ler	0.68	1.92	0.08	15,6,13
lip-o	26.6	4.29	0.21	16,9,16
mrk-o	12.2	4.25	0.15	16,14,14
ms-o	9.64	4.13	0.16	13,12,14
nd-I	3.47	1.31	0.13	15,14,14
oy-I	17.1	4.28	0.12	6,16,11
petergof	11.5	5.01	0.11	12,15,14
rd-o	3.45	2.11	0.13	15,15,8
rsch-o	9.35	2.96	0.13	11,15,15
sei-o	27.1	1.11	0.10	16,16,14
shahdara	7.20	2.51	0.10	13,14,14
sorbo	17.5	4.84	0.20	16,12,16
ta-o	9.68	4.52	0.18	11,16,15
tsu-I	28.5	4.52	0.15	9,16,12
wei-o	4.86	0.97	0.19	14,16,15
wl-o	14.4	3.26	0.18	15,13,14
ws-3	8.50	1.52	0.15	9,13,12
yo-o	13.5	5.45	0.18	14,11,12

Appendix A

Kunkel Extraction Buffer

In a 25 ml tube mix:

50 mM Tris-HCl, pH 8.0 (1 ml of 1M solution)
20 mM EDTA, pH 8.0 (.8 ml of .5M solution)
0.3 M NaCl (1.2 ml of 5 M solution)
2% Sarkosyl (2 ml of 20% solution - must be autoclaved)
5 M Urea (6g)
0.5% SDS (1mL of 10% solution)
5% Buffered Phenol, pH 8.0 (1 ml)

Fill to 20 ml with triple distilled water
Buffer must be kept from direct light and used within a few days.

Kunkel Mini-prep

1) Put 2-3 leaves in 1.5-ml tube and put on dry ice.

2) Dip the tube in liquid nitrogen and fill 1/2 full. Dip pestle in liquid nitrogen and grind until liquid. Tips should be changed between each sample and placed in beaker with a weak acid solution.

3) Add 650 ul Kunkel Buffer and vortex until all leaf material is in solution. If leaves are not ground enough, they can be ground more.

4) Add 650 ul 1:1 phenol:chloroform, mix by inverting tube 30-40 times.

5) Centrifuge @ 14,000 for 10 minutes.

6) Remove top layer, avoiding getting any green liquid. Place liquid in new tube and add 650 ul 1:1 phenol: chloroform. Mix and centrifuge as before.

7) Remove top layer and put in new tube. Add 2/3 of this volume isopropyl. (i.e. if you pipette 600 ul top layer, add 400 ul of isopropyl) Place sample in -20 freezer for AT LEAST 30 min.

8) Centrifuge @ 14,000 in cold for 10 minutes.

9) Pour off isopropyl, this can be saved for more DNA, either freeze or centrifuge immediately.

10) Add 500ul 70% ethanol and centrifuge for 4 minutes.

11) Pour off ethanol, pipette any excess, and air dry for 1-2 hours.

12) Add 10-15 ul triple distilled water and 1 ul RNAse.

Appendix B

Diamondback Moth Artificial Diet and rearing methods

Clean counter with bleach, acetone or ethanol before starting.

Ingredients	Amount
Mix and bring to a boil	
Water	375ml
Agar	12g
Cool and put in blender	
Add to blender and mix for 1 minute	
Casein	15.75g
Sucrose	16.88g
Raw wheat germ, finely ground	21.88g
Add to blender and mix for 1 minute	
Wess salt mix	4.50g
Potassium sorbate	0.50g
Cellulose	3.13g
Methylparaben	0.68g
Add to blender and mix for 2 minutes	
Raw linseed oil	3.25ml
Lepidopteran vitamin mix	4.50g
Aueromycin	0.50g
Propyl gallate	0.10g
KOH 45%	0.588g in 1.125ml water
Formaldehyde	0.75ml

This recipe will fill 6 cups. All supplies were ordered from BioServe.

1. Pour mix into Styrofoam food cups ½ inch deep. Cover and let cool for one hour. Place egg sheets in cups with approximately 300 eggs per cup. Seal cups tightly with lids to prevent larvae from escaping. Keep cups at 27° C with 12 hours of light exposure. Larvae will pupate at the top of the container. Cut bottoms off cups and place tops in mesh cages. The cages should be kept at 50% relative humidity.

2. Adults are fed a 10% sucrose solution with yellow food coloring added. Sucrose solution is poured in a 100-ml Erlenmeyer flask with dental wicking inserted. Wrap the mouth of the flask with Parafilm to prevent adults from drowning.

3. Placing aluminum foil strips soaked in cabbage juice in the cages attracts females to oviposit on them. Grooves should be made in the foil to simulate leaf veins. Blend 60g of cabbage leaves and 500ml of distilled water to make the cabbage juice, which should be autoclaved. Care should be taken to avoid pesticide residue on cabbage. Egg sheets are collected every 24 hours, sterilized in a 10% formaldehyde solution for ten minutes, and rinsed in tap water for one hour. The sheets should air dry for one hour before use or storage. Eggs can be stored in plastic bags for a maximum of 4 weeks at 5° C until needed.

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