CHAPTER FOUR

CORRELATED RESPONSES TO DIVERGENT ARTIFICIAL SELECTION IN VEGETATIVE ANTHOCYANIN EXPRESSION

4.1 ABSTRACT

Flavonoids exert important plant functions, such as protection from UV radiation, and mediate biotic interactions, including herbivory and the induction of mutualistic mycorrhizal associations. Anthocyanin pigments are conspicuous products of the flavonoid biosynthetic pathway are that contribute to a variety of plant functions, including flower and fruit color and mediating interactions with pollinators and fruit dispersers. Their role in plant defense against herbivores and plant pathogens is less clear.

In this study, I investigated the role of anthocyanins in resistance against insect herbivores and a plant pathogen. I artificially selected lines of rapid-cycling *Brassica rapa* for divergent expression of anthocyanin pigments in the hypocotyls, or embryonic stem. Expression of anthocyanins in the stem of fully developed plants differed between selected lines and the base population, but I detected no significant difference in anthocyanins in the leaves. Selected lines did not incur a fitness cost, relative to the base population, in terms of seed production.

There were significant correlated effects of anthocyanin expression on the level of damage and infection. Lines expressing higher levels of anthocyanins were more susceptible to *P.rapae* and *P. cruciferae* and less susceptible to *T. ni* and *A. brassicicola*. Feeding by *S. exigua* and colony size of *B. brassicae* did not differ among lines producing extreme anthocyanin contents. No direct role of anthocyanins on resistance

was demonstrated, but these results strongly suggest that selection on the vegetative expression of anthocyanins may affect its interactions with herbivores and pathogens. Given the multiple functions with which they are directly and indirectly associated, anthocyanins constitute a set of traits that may be subject to conflicting selection pressures.

4.2 INTRODUCTION

Plants face a diversity of stresses that may impose natural selection. Their environment may be limited in amount of nutrients, water, and sunlight. In addition, plants are attacked by a variety of enemies, including vertebrate and invertebrate herbivores, and bacterial, viral, and fungal pathogens (Mooney et al. 1991). Plants are commonly a engaged in mutualistic interactions with mycorrhizal fungi, pollinators and seed dispersers. All of these impinging selection forces are combined and, together with population genetic constraints, shape its evolutionary response.

In this study, I artificially selected rapid-cycling *Brassica rapa* (Brassicaceae) for higher and lower expression of anthocyanins. Anthocyanins and the larger group of secondary metabolites to which they belong, the flavonoids, have been implicated to play a role in responses to both biotic and abiotic stresses (Bohm 1998). I compared the resulting populations, under laboratory conditions, in terms of their seed production, photosynthetic rates, glucosinolate profile, and interactions with a number of natural enemies. The natural enemies encompassed both a fungal pathogen and several insect herbivores, including specialists and generalists, larval and adult stages, and leaf and

phloem feeders. The results suggest anthocyanins have an important impact upon disease and herbivory levels.

Flavonoids are the product of the phenylpropanoid pathway, and include flavones, isoflavonoids, flavonols, tannins, and anthocyanins. Strong evidence suggests they play a role in protection against damage from UV-B radiation and free radicals (Shirley 1996), and avoiding photooxidation though the attenuation of visible light and consequent reduction of excitation pressure (Steyn et al. 2002). Studies with flavonoid mutants have found a correlated effect upon male fertility (Shirley 1996). Flavonoids have been implicated in the induction of nodulation by symbiotic *Rhizobium* species and as anti-microbial and anti-viral compounds (Dixon and Steele 1999). They have been reported as herbivore feeding deterrents and attractants (Onyilagha et al. 2004), and as insect oviposition stimulants (Bohm 1998). For instance, QTL mapping has identified a locus controlling several flavonoid pathway genes as explaining some of the variation in maize resistance to the corn earworm, *Helicoverpa zea* (Yencho et al. 2000). In contrast, bacteria are known to induce the biosynthesis of crucifer-specific phytoalexins, a group of flavonoids of small molecular weight having antimicrobial effect. These play a role in oviposition preference of *Delia* flies (Baur et al. 1998). Flavonoids are also sequestered by some insect species and used to generate color patterns relevant in mate-recognition (Geuder et al. 1997; Wiesen et al. 1994).

Among the flavonoids, anthocyanins have received particular attention because they play an important role in producing flower and fruit color and thus in attracting pollinators and fruit dispersers (Bohm 1998). Likewise, they are responsible for the red coloration in young and aging leaves, which has also been implicated in the attraction of

several insects to plants. *Pieris rapae* is less attracted to the red foliage of the Rubine variety of Brussels spouts than to the green varities (Dunn and Kempton 1976), and red cabbages were less selected by alate aphids of *Brevicoryne brassicae* (Radcliffe and Chapman 1966).

It has been suggested that anthocyanins protect expanding leaves from disease (Coley and Aide 1989; Coley and Barone 1996) and herbivory (Coley and Kursar 1996). For instance, cyanidin-3-glucoside has been shown to have an inhibitory effect upon larval growth of tobacco budworm *Heliothis viriscens* (Delgado-Vargas et al. 2000), and cotton germplasm expressing a red leaf color phenotype showed less foliar damage than genotypes with normal green leaf color (Jones et al. 2000). While there are some claims in the agronomic literature that anthocyanins provide resistance to flea beetles (SeedQuest 2002), experiments have hardly been conclusive either way (M. Gruber, personal comm.). Recent studies have looked at links between flower color mutants and their impact on herbivory (Fineblum and Rausher 1997; Irwin et al. 2003; Ritchey 1999) with mixed results. Fineblum and Rausher (1997) detected no differences in herbivory between white-flowered and pigmented genotypes of the morning glory, *Ipomoea purpurea*, while Irwin et al. (2003) and Ritchey (1999) detected differences in preference or performance of insect herbivores to wild radish, *Raphanus sativus,* and birdfoot violet, *Viola pedata*, respectively.

However, there seems to be little supportive evidence that anthocyanins play a direct role in resistance (Close and Beadle 2003). Plants accumulate anthocyanin in response to wounding (Gonzales et al. 2002) and pathogen infection (Kangatharalingam et al. 2002). Anthocyanins accumulate in regions surrounding infection or wounding, but

tend to occur after infection has been repressed and thus may not play a direct role in resistance (Hipskind et al. 1996). They may instead reduce photoinhibition (Close and Beadle 2003; Close et al. 2002).

Changes in anthocyanin expression, in reproductive or vegetative tissues, may impact enemies via direct or indirect pathways (Fineblum and Rausher 1997). A population may change its anthocyanin expression by up-regulating the whole flavonoid pathway, thus leading to an increase in concentrations of many flavonoids, or up-regulating further downstream of the pathway, leading to an increase in concentration of only a subset of flavonoids. Increase in anthocyanin expression may also occur by down-regulation of side branches of the flavonoid pathway, leading to a decrease in the products that are downstream of that side branch. Genotypes of *Ipomoea purpurea* with nonfunctional copies of chalcone synthase (CHS), the first enzyme in the anthocyanin biosynthetic pathway, received greater herbivore damage and twice the intensity of infection by the fungal pathogen *Rhizoctonia solani* than the wildtype (Zufall and Rausher 2001). Anthocyanin production may tradeoff with the induction of other more biologically active antifungal flavonoids (Lo and Nicholson 1998). Studies with genetic mutants have also found that anthocyanin pathway genes are correlated with trichome densities (Kubo et al. 1999), a potential mechanism of plant herbivore resistance.

Given the variety of mechanisms by which anthocyanin expression may be changed, the effects of anthocyanin expression on the outcome of biotic interactions may best be studied using a number of replicate populations that, while appearing phenotypically similar, may vary in critical underlying processes. Furthermore, insect herbivores, and plant enemies in general, are known to respond in very idiosyncratic

ways to plant characteristics. In order to conclusively demonstrate the effects of any plant characteristic upon the outcome of biotic interactions, a variety of enemies must be employed against the same plant populations.

Artificial selection provides an ideal tool to study biotic interactions. It provides a means of detecting genetic variation and estimating genetic correlations between the trait that is the target of selection and other traits. The possibility of establishing multiple replicates within treatments allows one to account for sampling variance among founding populations, and to assess repeatability of results. Additionally, the products of selection constitute populations that can be subject to repeated analysis. In the case of plants, seeds from the terminal generation can be stored and used for a number of different studies.

In order to investigate the influence of anthocyanins on insect herbivores and fungal pathogens in *B. rapa*, I used artificial selection to generate populations expressing divergent levels of anthocyanins in the hypocotyl during an early stage of development. I assayed the relative resistance of these divergent populations to a variety of enemies, including larvae of the specialist butterfly *Pieris rapae* (Pieridae), larvae of the generalists moths *Trichoplusia ni* (Noctuidae) and *Spodoptera exigua*(Noctuidae), adults of the flea beetle *Phyllotreta cruciferae* (Chrysomelidae), colonies of the aphid *Brevicoryne brassicae* (Aphididae), and infection by the specialist fungal pathogen *Alternaria brassicicola* (Deuteromycetes). This approach allowed me to address the following questions: (1) Is there a response to selection for hypocotyl anthocyanin expression? (2) Is there a correlated response in leaf anthocyanin content and leaf photosynthetic rates? (3) Is there a correlated response in realized levels of defense

against herbivores and/or pathogens? (4) Is there any pattern to the response of a variety of enemies to changes in anthocyanin expression?

4.3 MATERIAL AND METHODS

Brassica rapa L. (syn *B. campestris*: Brassicaceae) is an out-crossing annual plant, native to Eurasia. Rapid cycling *B. rapa* is a variety established by artificial selection for early flowering, small plant size, absence of seed dormancy, rapid seed development, and high female fecundity at high densities in controlled laboratory conditions (Williams and Hill 1986). Seed of this variety was obtained from the Crucifer Genetics Cooperative at the University of Wisconsin, Madison (CRCG stock #1-1, Aaa). This stock contains substantial allozyme variation (Williams and Hill 1986) and displayed rapid responses to artificial selection for several traits involved in plant defense, e.g., trichome density (Ågren and Schemske 1994), glucosinolate content (Stowe 1998), and disease resistance (Mitchell-Olds and Bradley 1996). Plants were grown individually in 2.5 x 2.5 x 4 cm plug tray cells filled with commercial potting soil (ProMix), watered with a subirrigation system ad libitum, fortified with Peter's 15-15-15 fertilizer during the first two weeks at 50 ppM of nitrogen, and grown under 24hrs of fluorescent light (200 μmol m^{-2} s^{-1}).

4.3.1 Selection design

Four days after sowing, when cotyledons were fully expanded, hypocotyl color was scored visually according to a categorical scale (1= green; 3= very light purple; 5=intermediate; 7= purple; 9=dark purple). From an initial population of 600 individuals, sixty individuals of each extreme of the color scale were selected and assigned to two directional selection treatments, toward a *Green* and toward a *Purple* extreme. Each of

these groups was further randomly subdivided into three lines, consisting of twenty individuals per line. Plants were mass-pollinated within line, with the use of bee-sticks (Williams 1980), seed was collected, mixed within line and used to sow the subsequent generation. Each generation, approximately 200 plants per line were color-scored, and twenty plants belonging to the corresponding extreme color score of their treatment were chosen and mass-pollinated. This process of divergent artificial selection was repeated for a total of 6 generations of selection. Unsown seed from the original base population was refrigerated and used in subsequent comparisons.

After 6 generations of selection, in order to evaluate response to selection, 112 plants from each line and 200 plants from the base population were grown simultaneously and their hypocotyl color was scored. To test divergence of selected populations, the distribution of color scores of each line was compared with that of the base population in separate permuted analysis of variance.

4.3.2 Physiological and phytochemical measurements

A reasonable concern in artificial selection experiments is that inbreeding depression, brought about by reduced population sizes, might affect the variables of interest. To assess the possibility of inbreeding depression, after six generations of selection, a random sample of forty plants per selection line and sixty plants from the base population were grown under common conditions. Following the initial flowering period, fourteen days after sowing seed, plants were mass-pollinated within population every other day, for twelve days, encompassing the peak flowering period. Plants were allowed to develop fully and the number of seeds per seed pod was counted. To compare

plant fitness, as expressed by number of seeds produced (square root transformed), selection lines and the base population were compared in a nested ANOVA.

A separate set of twenty plants per line was grown to quantify anthocyanin content of stem and leaf tissue by spectrophotometric assay. Equal mass of stem and leaf tissue (150mg) from 10 individuals chosen at random from each line was macerated in 1ml of acidified methanol (1 part concentrated HCL:99 parts methanol). I used plants eleven days after sowing. Although this stage is much later than the ontogenetic window during which selection took place, this stage was the most relevant to assess with regard to interaction with herbivores and the fungal pathogen. Spectrophotometric analysis of the extract solution was performed on a Beckman DU-640 spectrophotometer. The absorbance of the extract solution was measured at 529 nm, the peak of anthocyanin absorbance. The A_{529} due to anthocyanin absorbance was corrected by subtracting 28.8% of A_{650}, the red peak absorbance for degraded chlorophyll (Murry and Hackett 1991; Sims and Gamon 2002). The corrected absorbance was assumed to be directly proportional to the concentration per mass of anthocyanins in the plant tissues. I used a MANOVA to compare anthocyanin content between treatments in both leaf and stem tissue, with selection treatment as a fixed effect and line nested within treatment as a random effect. A significant MANOVA was followed by individual nested ANOVA. The overall correlation between leaf and stem anthocyanin content was tested using a Pearson product-moment correlation.

Selection on hypocotyl color might have resulted in changes in other pigments, particularly chlorophyll. To examine whether these changes or correlated effects of altered anthocyanin expression affected photosynthetic rates, I examined light response

curves on two randomly selected plants from each line and the base population. Measurements were made using a Licor 6400, on one fully expanded leaf, twelve days after sowing, at 28ºC and at internal CO_2 concentrations of 330 ±15 ppm.

I assessed whether response to selection for anthocyanin expression was correlated with changes in glucosinolate content. Plants from each treatment line were grown simultaneously and, 14 days after sowing, seven plants of equivalent size and stage of development from each line were chosen for analysis. I removed the first pair of true leaves, freeze-dried them, noted leaf dry weight and sent samples to Jonathan Gershenzon's lab in the Max Planck Institute for Chemical Ecology, Jena, Germany, where they performed the extraction, purification, separation and identification of leaf glucosinolates.

The extraction and purification of glucosinolates followed the basic sephadex/sulfatase protocol (Hogge et al. 1988). Lyophilized samples (ca 20mg) were ground in the "Paintshaker" and immersed in 80% methanol (4 ml) plus 0.05 ml of internal standard (4-hydroxybenzyl glucosinolate, 1mM). After 10 min gentle shaking at room temperature, samples were centrifuged at 4300*g* for 10 min and the supernatant fraction loaded onto a small (100 mg) column of DEAE Sephadex A25. The column was rinsed with 67 % (aqueous) MeOH and deionized water and, after capping, was treated with 50 µl sulfatase solution and incubated overnight. The resulting desulfoglucosinolates were eluted from the column with 2 x 0.8 ml 60 % (aqueous) MeOH, and the combined eluent was evaporated to dryness under a stream of nitrogen. Desulfoglucosinolates were reconstituted in 0.4 ml of water.

Separation of desulfoglucosinolates was achieved on a Hewlett Packard HP 1100 Series system with autosampler and diode-array detector. The procedure employed a C-18, fully-endcapped, reversed phase column (LiChrospher RP-18, 250 x 4.6 mm i.d., 5 μm particle size, Chrompack) operated at 1 ml min^{-1} and 25 °C. The system was equipped with a C-18 LiChrospher (75 x 4.6 mm i.d., 5 μm particle size, Chrompack) reverse phase guard column. Injection volume was 40 μl. Elution was accomplished with a gradient (solvent A: H_2O, solvent B: MeCN) of 1.5-5 % B (6 min), 5-7 % B (2 min), 7-21 % B (10 min), 21-29%B (5min), and 29-43 % B (7 min), followed by a cleaning cycle (43-100 % B in 0.5 min, 2.5 min hold, 100 to 1.5 % B in 0.1 min, 5 min hold). Eluting compounds were monitored at 229 nm, and peaks were identified by match of retention time and UV spectrum with those of standards of desulfoglucosinolates. Concentrations of glucosinolates were calculated in relation to the internal standard applying the response factors established for single desulfoglucosinolates (Brown et al. 2003).

I compared the total glucosinolate content, expressed as μmol g dry weight $^{-1}$ of leaf material, between treatments using a mixed-model nested ANOVA, with selection treatment as a fixed effect, and line nested within treatment as random effect. Differences in glucosinolate profile, using each individual glucosinolate as a separate response variable, were compared in a MANOVA with the same factorial design, followed by univariate analyses for each compound. Glucosinolates present in small quantities and entirely absent in some plants, were square root transformed.

4.3.3 Bioassays

To measure the relative resistance of divergent selection lines to a natural enemy, in each bioassay individuals from all selected lines were randomly planted in three blocks

of 20 x 10 individual plants. Within each block, plants were immediately adjacent to one another. Each block had an independent subirrgation system. The outermost rank and file of each block was treated along with innermost plants but excluded from subsequent analysis to avoid border effects, leaving a total of 144 experimental plants per block. Thus, for each enemy the experimental design consisted of 3 blocks, for a grand total of 432 experimental plants and 72 per population.

4.3.3.1 Lepidoptera

The imported cabbageworm, *Pieris rapae* L. (Pieridae), is a native of Europe. Its larval stages are specialized upon Brassicaceae, although occasionally found on members of the Capparidaceae, which also produce isothiocyanates. The cabbage looper, *Trichoplusia ni* (Hübner) (Noctuidae), is a polyphagous folivore in its larval stages. While favoring Brassicaceae, it is known to cause leaf damage to over one hundred species in 29 plant famlies (Sutherland 1965). Laboratory colonies of both species were established in July 1998 from eggs provided by the New York State Agricultural Experiment Station, in Geneva, NY. The beet armyworm, *Spodoptera exigua* (Noctuidae), feeds as larvae on leaves and fruits of a wide range of species, including cruciferous crops. Eggs of *S. exigua* were obtained from AgriPest (Zebulon, North Carolina).

Eleven days after sowing seed, I traced the outline of the first true leaf and estimated its area using tpsdig (version 1.31, Rohlf 2001). Two neonate larvae of *P. rapae*, *T. ni*, or of *S. exigua* were placed on each plant, including border plants. First instar larvae suffer high mortality rates, are more susceptible to plant chemical defenses, and plant morphological traits (e.g., trichomes, leaf waxes). They tend to be more

selective among plant genotypes and constitute a critical life stage for establishment on a host plant (Zalucki et al. 2002). Plants within a block are in close proximity, allowing larvae to easily move between them. Leaf area damaged after 48hrs of *T. ni* exposure and 72 hrs in case of *P.rapae* and *S. exigua* was measured with a transparent grid (4 mm^2 grid squares). To test whether selection treatment varied in number of grid squares (square root transformed) eaten by either species, I used mixed-model nested ANOVA, with selection treatment as a fixed effect, and both block and line nested within treatment as random effects. The unit of replication for testing for a treatment effect is the number of lines within a treatment, whereas the number of plants is the unit of replication for the line within treatment. The area of the first true leaf, measured immediately prior to larvae placement, was used as a covariate to account for differences in initial size among plants.

4.3.3.2 Flea Beetles

Cabbage flea beetles, *Phyllotreta cruciferae* Goeze (Coleoptera: Chrysomelidae: Alticinae) feed primarily on leaves of Brassicaceae hosts, although they are also known to feed upon other hosts that produce mustard oils (i.e., allyl isothiocyanate). Beetles were collected in September 2002 at the Long Island Horticultural Research Station in Riverhead, New York. Eleven days after sowing, the first true leaf of each plant was traced and the three blocks of experimental plants were placed side by side within a fine mesh enclosure (1mm^2). Approximately 250 adult beetles were released at one extreme of the enclosure, closest to one of the experimental blocks, and dispersed rapidly within it. After 72 hrs, leaf area damaged was measured with a transparent grid (4 mm^2 grid squares). To test whether selection treatments varied in number of grid squares (square root transformed), I used mixed-model nested ANOVA, with selection treatment as a

fixed effect, and both block and line nested within treatment as random effects. The area of the first true leaf was used as a covariate to account for differences in initial size among plants.

4.3.3.3 Cabbage Aphid

The cabbage aphid, *Brevicoryne brassicae* L. (Homoptera: Aphididae), is a specialist on Brassicaceae, particularly the genus *Brassica*. Aphids penetrate leaves and stems, and feed on plant phloem sap. They can cause generalized stunted plant growth and are important virus vectors. Several aphid colonies were collected in September 2003 from two neighboring *Raphanus sativus* plants growing on the margins of a cabbage farm in Eastern Long Island, New York. They were transplanted onto rapid cycling *B.rapa* and maintained on this host under laboratory conditions for two months before being used experimentally. The bioassay consisted of two blocks of experimental plants, as described above. Eight days after sowing, the first true leaf of each plant was traced and two first instar nymphs were placed on each plant. After a period of nine days, the total number of aphids on each plant was tallied. The aphid colony size was assumed to be complementary to the resistance of a plant to aphid establishment, growth, and reproduction. To test whether selection treatments varied in colony size (square root transformed), I used mixed-model nested ANOVA, with selection treatment as a fixed effect, and both block and line nested within treatment as random effects. The area of the first true leaf was used as a covariate to account for differences in initial size among plants.

4.3.3.4 Leaf Spot Infection

Cabbage Leaf Spot (*Alternaria brassicicola* (Schwein.) Wiltshire, Deuteromycetes, Dematiaceae) is a necrotrophic pathogen causing disease in a wide range of Brassicaceae hosts. Infection manifests itself as dark, circular spots on leaves (Kucharek 1994). Spores were collected from a diseased *Brassica oleracea*, from Riverhead, New York and cultured on a $39gL^{-1}$ potato dextrose agar (ICN Biomedicals, Inc.) medium, at 25ºC, with 200 µmol m^{-2} s^{-1} fluorescent illumination on a 14h-light/8h-dark photocycle.

Eight days after sowing, *A. brassicicola* spores produced on PDA petri dishes were suspended in 1.5% gelatin (Sigma) solution (Dhingra and Sinclair 1995; Rangel 1945). Spore concentration was determined with an hemacytometer and adjusted to 10^6 spores mL^{-1}. All three blocks of plants were misted with the spore solution and kept under conditions of high relative humidity (RH=90%) for the next 72 hours. Fourteen days after sowing, plants were submitted to a second 72h period of high humidity, after which disease severity was scored, using a categorical scale (Williams 1985). Each planted was attributed a numerical category to indicate the degree of expression of disease (0=disease absent; 1=very low expression, a low number of small foci of disease; 3=low expression, affecting <20% of leaf area; 5=intermediate conspicuous expression, affecting 20-75% of leaf tissue; 8= high expression, covering 75-95% of tissue; 9= very high expression, affecting nearly 100% of plant). Disease severity score (square root transformed) was analyzed by separate mixed-model nested ANOVA, with treatment as a fixed effect, and both block and line nested within treatment as random effects.

4.3.4 Correlations among populations

Whether plant resistance to different enemies is correlated bears on our understanding of potential ecological constraints acting on the evolution of plant defense and on the nature of coevolution (see Chapter 1). In this chapter, I describe artificial selection for a plant trait and measurement of biotic interactions in separate bioassays. Correlations between unselected traits, i.e. effect of different enemies between selection treatments, do not provide a quantitative estimate of quantitative genetic parameters, e.g., genetic correlations (Conner 2003). I cannot estimate phenotypic correlations as I did not measure more than one plant trait, biotic or physiologic, on the same individual plants. However, I can calculate correlations among population means measured for different traits separately, as an indication of possible association among traits.

In order to remove the variation due to experimental block and leaf size from each response variable, i.e., leaf area damage, colony size, and disease severity, for each bioassay dataset, I performed an analysis of variance of these variables using only those block and leaf size as factors in the model. The residuals of this analysis correspond to variation unexplained by differences in block and leaf size, and thus more closely approximates differences due to population and line within population (as well as unexplained error variance). For each pair of bioassays, I calculated Spearman correlation coefficients (ρ) between the all six line residual means. In addition, I calculated Spearman correlations between these residual means and estimates of line mean leaf and stem anthocyanin content. Spearman's ρ uses ranks of the population means rather than the data values themselves. A high positive value of ρ indicates that populations have similar ranking in their mean residual in a given pair of bioassays.

All statistical analyses were performed on JMP v3.2.2 (SAS Institute, 1998) and JMP v.5.0.1 (SAS Institute, 2001).

4.4 RESULTS

4.4.1 Selection design

Hypocotyl color score responded rapidly to artificial selection (Fig. 1). After six rounds of selection, all selection lines had significantly different hypocotyl color distribution from that of the base population (Fig. 2, $P<0.0001$ for all pair-wise comparisons).

Total seed set of selection treatment populations did not differ from that of the base population ($F_{1,4} = 1.2221$, $P=0.3920$, Fig. 3, Table 1), suggesting the absence of inbreeding depression as a result of artificial selection and of a fitness cost to the alteration in anthocyanin expression.

4.4.2 Physiological and phytochemical measurements

Anthocyanin content differed significantly among selection treatments (MANOVA: $F_{2,62} = 39.2357$, $P<0.0001$). Although, leaf anthocyanin content did not differ among selection treatments ($F_{1,4} = 0.1882$, $P=0.6867$), stem anthocyanin content did differ significantly between treatments ($F_{1,4} = 56.6706$, $P=0.0019$), with *Purple* selection lines expressing three times that of *Green* selection lines (Fig. 4). The correlation between stem and leaf anthocyanin content was low and non-significant ($r=0.2223$, $P=0.0664$).

Physiologically, selection treatments did not differ in any consistent manner in their response to light availability (Fig. 5). While *Purple* lines have a fairly homogenous response pattern, *Green* lines varied substantially, spanning the range of the sample. However, overall differences among lines are not attributable to selection treatment.

There were also no significant differences in total glucosinolates among treatments ($F_{1,4} = 0.6589$, $P = 0.4617$, Table 2, Fig. 6). Separation into individual compounds revealed the presence of six different glucosinolates: 3-Butenyl (3-But), Methylpropyl (MeProp), 4-Pentenyl (4-pent), Indol-3-yl-methyl (I3M), 4-Methoxy-Indol-3-yl-methyl (4MOI3M), and 1-Methoxy-Indol-3-yl-methyl glucosinolate (1MOI3M) (Fig. 7). 3-Butenyl was the most abundant glucosinolate in all samples (average of 96% of total glucosinolates in both selection treatments). Other compounds were detected in varying amounts and concentrations close to detection limit (Table 3). Multivariate analysis revealed no significant differences in glucosinolate profile (MANOVA: $F_{6,26} = 0.8810$, $P=0.5225$). Univariate analyses of each glucosinolate also reveal no significant differences among treatments in any of the individual compounds.

4.4.3 Bioassays

4.4.3.1 Lepidoptera

There were significant differences among selection treatments in amount of damage by both *P. rapae* ($F_{1,4} = 15.438$, $P=0.0166$) and *T.ni* ($F_{1,4} = 45.661$, $P=0.019$) (Table 5). The specialist *P. rapae* inflicted more damage on *Purple* selection lines than on *Green* lines (Fig. 8), whereas the generalist *T. ni* fed more upon plants from the *Green* selection lines (Fig. 9, Table 4). Similar bioassays performed on plants after only three generations of selection produced parallel results, albeit with statistically non-significant

differences between treatments: *P. rapae* $F_{1,4} = 1.3401$, P=0.3108; $area\_consumed_{Purple}$ = 33.57 ± 2.48 ($\bar{x}$ mm^2 ± 1 SE), n=201, vs. $area\_consumed_{Green}$ = 24.975 ± 2.05, n=203); *T.ni* $F_{1,4} = 0.288$, P=0.6185; $area\_consumed_{Purple}$ = 40.884 ± 2.16, n=215, vs. $area\_consumed_{Green}$ = 42.302 ± 1.64, n=216).

There were no significant differences among treatments in damage inflicted by the generalist *S. exigua* ($F_{1,4} = 0.0905$, P=0.779) (Fig. 10, Table 5). The covariate, area of first true leaf, had no significant effect on leaf area damage by *P. rapae* ($F_{1,419} = 1.926$, P=0.659) or *T. ni* ($F_{1,414} = 0.0213$, P=0.884), but did have a significant effect in the case of *S. exigua* ($F_{1,414} = 36.1305$, P<0.001), which imposed greater damage on plants with smaller first leaves (Pearson's corr. coef. = -0.935). There were no significant differences among lines within selection treatments for herbivore damage (*P. rapae*: $F_{4,419} = 0.2551$, P=0.9065; *T. ni*: $F_{4,414} = 0.8651$, P=0.4848; *S. exigua*: $F_{4,414} = 0.5532$, P=0.6968).

4.4.3.2 Flea Beetles

There were significant differences between selection treatments in amount of damage by *P. cruciferae* ($F_{1,4} = 14.8166$, P=0.017) (Fig. 11, Table 5). The *P. cruciferae* specialist inflicted more damage on *Purple* selection lines than on *Green* lines (Table 4). The covariate, area of first true leaf, had no significant effect upon leaf area damage ($F_{1,413} = 0.0019$, P=0.9652). There were no significant differences among lines within selection treatments ($F_{4,413} = 1.0788$, P=0.3665), but there were significant differences among blocks of experimental plants ($F_{2,413} = 58.2156$, P<0.001), probably reflecting proximity to location of beetle placement within the enclosure.

4.4.3.3 Cabbage Aphid

There were no significant differences between selection treatments in colony size of *B. brassicae* ($F_{1,4}$ = 0.400, n.s., Fig. 12, Table 5). The covariate, area of first true leaf, was significantly related to final colony size ($F_{1,275}$ = 13.8270, P<0.001), with plants with initial greater leaf area possessing larger final aphid colonies. In addition, there was a significant block effect ($F_{1,275}$ = 27.4481, P<0.001) and significant variation among lines within treatment ($F_{4,275}$ = 3.4195, P=0.0095).

4.4.3.4 Leaf Spot Infection

Variation between selection treatment in disease severity score inflicted by *A. brassicicola* was statistically significant ($F_{1,4}$ = 14.8166, P=0.017, Table 6, Fig.13). Whereas 44.4% of plants from pooled *Purple* lines incurred no to nearly no signs of infection and 28% expressed intermediate to high levels of disease expression, the numbers for pooled *Green* lines were respectively 31.3% and 38.1% (Fig. 14). A bioassay performed on plants after only three generations of selection revealed similar results: $F_{1,4}$ = 7.561, P=0.0514; disease severity $_{Green}$ = 3.30640.884 ± 0.15 (mean score ± 1 SE) , n=216 vs. disease severity score$_{Purple}$ = 2.728 ± 0.14, n=216.

4.4.4 Correlations among populations

Most pairwise Spearman ρ tests were nonsignificant, indicating that across bioassays line populations were not consistent in their ranking of leaf damage, aphid colony size and disease severity (Table 7). But lines had significantly similar rankings in leaf damage incurred by *P. rapae* and *P. cruciferae* (Rho = 0.8286, P = 0.0416) and a significant negative correlation between rankings in leaf damage by *P. rapae* and disease severity incurred *A. brassicicola* (Rho = - 0.9429, P = 0.0048) Rankings in mean line

amount of anthocyanin expressed in either leaf or stem were uncorrelated with rankings in any bioassay.

4.5 DISCUSSION

Selection resulted in rapid differentiation of populations, demonstrating significant quantitative genetic variation in hypocotyl color. Ideally, in artificial selection experiments one should establish control populations that are not subject to any artificial selection pressures yet are exposed to laboratory selection and are kept at population sizes similar to that of the selected populations. This was not done here for logistic reasons, however seed from the base population was preserved allowing for some comparisons (Fry 2003). Significant differences in color scores between each selected population and the base population suggests that divergence among treatments was due to successful artificial selection in opposing directions. Furthermore, as there were no significant differences in seed set between the base and selected populations, I was able to assuage whether selection resulted in inbreeding depression.

Artificial selection acting on color at an early stage of development (hypocotyl) was strongly associated with changes in stem coloration at later stages. Using spectrophotometric methods, I demonstrated that changes in color are largely due to changes in anthocyanin concentrations. Eleven-day old plants exhibited a significant difference among treatments in anthocyanin concentration in the base of the stem, but no significant difference in leaf anthocyanins. Populations selected for purple hypocotyls

occasionally exhibited visible purple pigmentation in the leaves (particularly line *Purple*1), but I did not detect a statistical differences among selection treatments in leaf anthocyanin content, perhaps due to lack of sensitivity of the methods used.

There are numerous cases of flower color polymorphisms involving anthocyanin mutations, e.g., *Lotus corniculatus* (Jewell et al. 1994), *Ipomoea purpurea* (Fry and Rausher 1997), *Linaria canadensis* (Wolfe and Sellers 1997), *Mimulus* sp. (Bradshaw and Schemske 2003; Schemske and Bradshaw 1999), *Raphanus sativus* (Irwin et al. 2003), and *Viola* sp. (Farzad et al. 2002; Ritchey 1999). There were no readily observable differences between selection treatments in flower color, although flower color was not examined quantitatively,

Seed color is also determined partly by anthocyanin expression, e.g. in soybean (Lindstrom and Vodkin 1991) . In rice, a mutant has been identified that inhibits anthocyanin accumulation in leaves while enhancing proanthocyanidins in the pericarp (Reddy et al. 1995). Again, I did not quantitatively examine changes in seed coat color, but did find that, after six generations of selection, a few plants in one of the *Green* lines generated only seeds with white seed coats.

Comment: Procurar mais referencias

There were no consistent differences among treatments in light response curves, i.e. photosynthetic rates, suggesting that changes in color were not due to significant changes in chlorophyll content, that changes in anthocyanins did not affect rates of photosynthesis, and that it is unlikely that differences in amount of leaf photosynthesis account for differences in herbivory or disease.

Irwin et al. (2003) compared indole glucosinolate production between two flower color morphs of *Raphanus sativus* (Brassicaceae). Although they found no effect of

anthocyanin morph on indole glucosinolate concentration, among plants damaged by *P. rapae* larvae induction of these compounds was greater in anthocyanin-dominant morphs than in anthocyanin-recessive morphs. This suggested a link between anthocyanin expression and expression of glucosinolates, a family of secondary compounds characteristic of a few plant families, including Brassicaceae, that have documented biotic effects (Louda and Mole 1991; VanEtten and Tookey 1979). Furthermore, there is some evidence of combinatorial effects of glucosinolates and flavonoids upon insect behaviour, even among crucifer-specialists such as the diamondback moth, *Plutella xylostella* (van Loon et al. 2002). The populations selected for higher and lower anthocyanin content, however, did not differ in leaf total glucosinolate content or leaf glucosinolate profile.

Selection treatments did not differ in terms of damage imposed by the lepidopteran *S. exigua* or colony growth rate of *B. brassicae*. However, they differed significantly in the amount of damage incurred by several insect herbivores, particularly the lepidopterans *P. rapae* and *T. ni*, and the flea beetle, *P. cruciferae*. This implicates that changes in anthocyanin expression are correlated with plant resistance to herbivory. The direction of this effect was dependent on the herbivore. Whereas *Purple* populations, expressing high stem anthocyanin content, were less fed upon than *Green* populations by the generalist *T. ni* (or conversely were found to be more resistant to this herbivore), *Purple* populations were more fed upon by the specialists *P. rapae* and *P. cruciferae*.

Irwin et al. (2003) examined preference and performance of a variety of folivores between two natural flower color variants of *R. sativus*, in particular *P.rapae*, *S. exigua*, *B. brassicae*, the western flower thrips, *Frankliniella occidentalis*, and the gray garden

slug, *Agriolimax reticulatus*. All herbivores except *B. brassicae* and *F. occidentalis* preferred the anthocyanin-recessive, yellow and white colored morph over the anthocyanin-dominant, pink and bronze flowered morph. In addition, while most herbivores performed better on the anthocyanin-recessive morphs, *P.rapae* and *S. exigua* larvae exhibited higher performance on the anthocyanin-dominant morphs. I likewise observed higher feeding performance of *P. rapae* on high anthocyanin populations, but in contrast *S. exigua* and *B. brassicae* did not differ in their response to selection treatments.

Selection treatment also affected the disease severity imposed by *A. brassicicola*: *Purple* populations exhibited lower incidence of disease severity. A number of flavonoids have been demonstrated to have antimicrobial and antifungal properties (Arima et al. 2002; Arima and Danno 2002; Pandey et al. 2002). This may indicate that selection increased the allocation to stem anthocyanins was correlated increased to concentration of other flavonoids in the leaves.

While it is interesting to observe that changes due to selection resulted in differences in biological activity against a fungal pathogen and some insect herbivores, no clear pattern emerged as to whether plant defense towards enemies in these two kingdoms can be generalized to be positive or negatively correlated. Rather, it appears to be quite dependent on the choice of particular species.

Likewise, I found no general pattern of effect of selection treatment on specialist versus generalist species. It has been suggested that secondary compounds that are effective plant defenses have become ineffective towards specialist insects that have become adapted to their restricted host range (Blau et al. 1978; Carroll and Hoffman 1980). In my experiment, *P. rapae* and *P. cruciferae*, both *Brassica* specialist herbivores,

responded similarly, i.e. both fed more on high stem-anthocyanin lines. However, anthocyanins were correlated with increased resistance to the specialist pathogen *A. brassicicola*, but the specialist aphid *B. brassicae* did not exhibit differences in colony growth rate among selection treatments. Among the generalist species, *S. exigua* did not distinguish among treatments (Irwin et al. 2003), while *T. ni* fed more on low anthocyanin lines. In fairness, the specialist/generalist dichotomy in relation to secondary compounds was intended to apply to compounds that are more family-specific, and not to compounds as taxonomically universal among higher plants as anthocyanins.

These results indicate that selection on hypocotyl color directly led to changes in expression of stem anthocyanin content, but no detectable, consistent change in anthocyanin expression in other plant tissues suggests a highly modular expression of this pathway. Despite no detectable differences in leaf anthocyanin content, treatments differed significantly in the amount of leaf fed upon by a number of folivores and in the degree of *A. brassicicola* disease on leaves. As no direct causal effect of anthocyanins on differences in natural enemy performance seems likely, this strongly suggests that changes in stem anthocyanin content were correlated with changes in the expression of other biologically active leaf characters.

The different biotic effects among selection treatments could be mediated by changes in other plant characters that are affected by anthocyanins. For example, anthocyanin absorbance impacts light energy available for photosynthesis and selection might have acted on other pigment pathways, such as production of chlorophyll. However, I detected no differences among selection treatments in photosynthetic rates.

Selection may also have acted on genes with pleiotropic effects. Anthocyanins are products of the flavonoid biosynthetic pathway, along with compounds of known biological activity, e.g., lignins, tannins, flavones, and flavonols. An increase in anthocyanin expression due to changes in the pathway may be accompanied by an increase or decrease of the expression of these compounds, depending on where modification of the flavonoid pathway occurs (Fineblum and Rausher 1997). A generalized allocation of resources to the entire pathway would increase concentrations of a number of other flavonoids, which unlike anthocyanins might be expressed in the leaves. A change further downstream in the pathway might increase allocation to anthocyanin production while reducing the production of other compounds. Pleiotropy might also have occurred between apparently disparate traits, i.e., not linked via biosynthetic pathways. For instance, it appears that trichome density is correlated with anthocyanin expression (Kubo et al. 1999), and as mentioned earlier Irwin et al. (2003) detected correlated changes in anthocyanin and glucosinolate inducibility. I investigated two potential factors that could mediate such indirect effects: photosynthetic rates and glucosinolates. However, selection treatments did not vary significantly in either factor.

Overall, across bioassays there was no consistent correspondence between levels of damage incurred by a specific line, e.g., lines that incurred least damage by *P. cruciferae* were not consistently the most extreme ranking line in other bioassays. An overview of Spearman Rhos of rankings among bioassays does reveal two statistically significant and high in absolute value correlation coefficients (one positive between *P. rapae* and *P. cruciferae* and one negative between *P. rapae* and *A. brassicicola*) and a few marginally significant correlation coefficients (P=0.0724). More dramatically,

bioassay rankings of lines were uncorrelated with levels of leaf and stem anthocyanin content. This does not undermine the overall analysis of variance of each bioassay indicating an effect of selection. But it does suggest that although lines responded to selection similarly (most bioassays have non-significant line within treatment effects) enemies are interacting with different plant characters; or, that lines have responded to selection along different pathways. Lines within treatment may be expressing similar phenotypes due to changes at different underlying loci (Abouheif 1997), perhaps causing different changes in the flavonoid pathways or in other aspects leading to differences in the interaction with specific insects.

In addition, in some bioassays it is clear that comparisons among certain pairs of lines from each treatment would not have revealed a significant treatment effect. This emphasizes the importance of population replication (Fry 2003), particularly with regard to the study of biotic interactions. The need for population replication applies both to selection experiments and to lab and field experiments, which tend to use seed collected from a single source. Natural plant populations have different histories of interaction with natural enemies, have independent evolutionary histories, and different standing genetic variation, thus the use of seed collected from multiple populations allows for more robust conclusions regarding the genetics of biotic interactions (Thompson 1994).

The results of a bioassay also depend on how the enemy responds to plant characteristics and, as with plants, enemy populations may differ genetically in traits relevant to interactions with plants, e.g., host preferences. The logistical nightmare implied by an ideal design involving a spectrum of plant populations and a number of different enemies and enemy populations should humble us when attempting conclusions

or generalizations from experiments involving only a limited source of plant populations or a limited number of enemies.

These complexities highlight the importance of using lines of contrasting phenotypes and exposing these populations to a variety of enemies from different taxonomic categories, feeding guilds, and levels of specialization. Only by performing a number of such studies will we be able to transcend the idiosyncrasies of any plant-enemy interaction and begin to glimpse a pattern of how plants evolve to multiple enemies.

Source	d.f.	SS	F
Treatment	2	36.582	1.222 n.s
Line[Treatment]	4	51.804	4.020 ***
Error	285	918.100	

Table 4.1 Nested, mixed-model analysis of variance of seed set per plant (square root transformed) among select
population. Lines nested within selection treatment was considered a random effect and treatment a fixed factor.
P<0.01; *** P<0.005)

Source	d.f.	SS	F
Treatment	1	89.857	0.6589
Line[Treatment]	4	544.432	0.7862
Error	31	5366.904	

Table 4.2 Nested, mixed-model analysis of variance of total glucosinolate concentration (μmol/g leaf dry weight)
lines. Lines nested within selection treatment was considered a random effect and treatment a fixed factor.

	Green Lines	*Purple* Lines
Total glucosinolates	20.683 ± 3.02	23.627 ± 3.02
3-But	19.856 ± 2.94	22.700 ± 2.90
MeProp	0.153 ± 0.05	0.153 ± 0.05
4-Pent	0.444 ± 0.05	0.514 ± 0.07
I3M	0.094 ± 0.02	0.106 ± 0.02
4MOI3M	0.061 ± 0.01	0.093 ± 0.02
1MOI3M	0.069 ± 0.01	0.062 ± 0.01

Table 4.3 Mean foliar glucosinolate content of first pair of true leaves fourteen days after sowing seed, in µmol g dry weight ($\bar{x}$ ± 1 SE) for lines selected for high and low anthocyanin expression.

Population	*P. rapae*	N	*T. ni*	n	*S. exigua*	n	*P. cruciferae*	n	*B. brassicae*	N
Green	54.714 ± 2.36	213	50.413 ± 2.04	211	32.024 ± 2.05	214	11.848 ± 1.82	211	55.599 ± 3.47	1
Purple	62.056 ± 2.35	215	41.703 ± 2.12	213	33.066 ± 2.31	209	23.621 ± 2.36	211	57.439 ± 2.63	1

Table 4.4 Average leaf area damage, among all lines of each selection treatment, by larvae of *P.rapae*, *T.ni*, *S.ex* *P.cruciferae* ($\bar{x}$ mm^2 ± 1 SE); mean colony size of *B. brassicae* ($\bar{x}$ individuals ± 1 SE); and mean disease score infl *brassicicola* ($\bar{x}$ ± 1 SE) on lines selected for decreased (*Green*) and increased (*Purple*) anthocyanin expression.

	P. rapae			*T. ni*			*S. exigua*			*P. cruciferae*			*B.*
Source	d.f.	SS	F	d.f.	SS	F	d.f.	SS	F	d.f.	SS	F	d.f
Block	2	5.345	1.714	2	13.039	5.489	2	9.037	2.922	2	213.631	58.216**	1
Treatment	1	6.486	**15.44***	1	15.917	**14.66***	1	0.076	0.091	1	14.129	**20.835***	1
Line[Treatment]	4	1.593	0.255	4	4.3483	0.865	4	3.422	0.553	4	7.918	1.0789	4
Area 1st Leaf	1	3.008	1.926	1	0.0268	0.021	1	55.88	36.131**	1	0.0035	0.002	1
Error	419	654.28		414	520.20		414	640.31		413	757.78		27

Table 4.5 Nested, mixed-model analyses of variance of leaf area damaged (square root transformed) by larvae of *S.exigua*, and adults of *P.cruciferae*; and of mean colony size (square root transformed) of *B. brassicae.* Lines ne treatment and experimental block were considered random effects. The area of the first true leaf was included as for differences in total plant size. A significant treatment effect refers to differences between populations artificia and higher hypocotyl anthocyanin expression. (* P<0.05; ** P<0.01; *** P<0.005)

Source	d.f.	SS	F
Block	2	1.94886	1.94595
Treatment	1	5.77704	**14.8166***
LineTreatment]	4	1.55349	0.7770
Error	420	209.93376	

Table 4.6 Nested, mixed-model analysis of variance of disease score (square root transformed) inflicted by *A. br*
nested within selection treatment and experimental block were considered random effects. A significant treatmen
differences between populations artificially selected for lower and higher hypocotyl anthocyanin expression. (* F
*** P<0.005)

	P. rapae	*T. ni*	*S. exigua*	*P. cruciferae*	*B. brassicae*	*A. brassicicola*	Leaf Anth.	
P. rapae	—	- 0.6000	0.3714	**0.8286***	0.2571	**- 0.9429*****	0.3714	
T. ni	0.2080	—	- 0.4286	- 0.7714	- 0.4857	0.6571	0.0857	
S. exigua	0.4685	0.3965	—	0.3714	0.7714	- 0.2571	- 0.3714	
P. cruciferae	0.0416	0.0724	0.4685	—	0.0857	- 0.3143	0.0286	
B. brassicae	0.6228	0.3287	0.0724	0.8717	—	- 0.3143	- 0.2571	
A. brassicicola	0.0048	0.1562	0.6228	0.0724	0.5441	—	- 0.2571	
Leaf Anthocyanin	0.4685	0.8717	0.4685	0.9572	0.6228	0.6228	—	
Stem Anthocyanin	0.0724	0.1108	0.6228	0.2080	0.4685	0.1108	0.2080	

Table 4.7 Numbers above the diagonal correspond to Spearman Rank Correlations among all six selected popula
damage inflicted by each enemy and estimated leaf and stem anthocyanin content. For each enemy, I used residu
only block and leaf area (when applicable) in order to extract variation due to these factors. Spearman rank corre
calculated between mean residuals for each population. Numbers below the diagonal correspond to p-values for
coefficient.

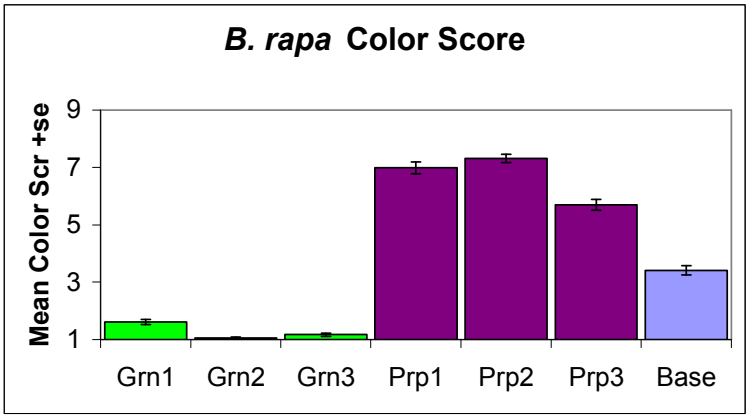

Fig. 4.1 Mean hypocotyl color score of base population and each selected lines after six generations of selection (

permuted ANOVAS indicate significant differences of all selected lines from base population ($P < 0.0001$).

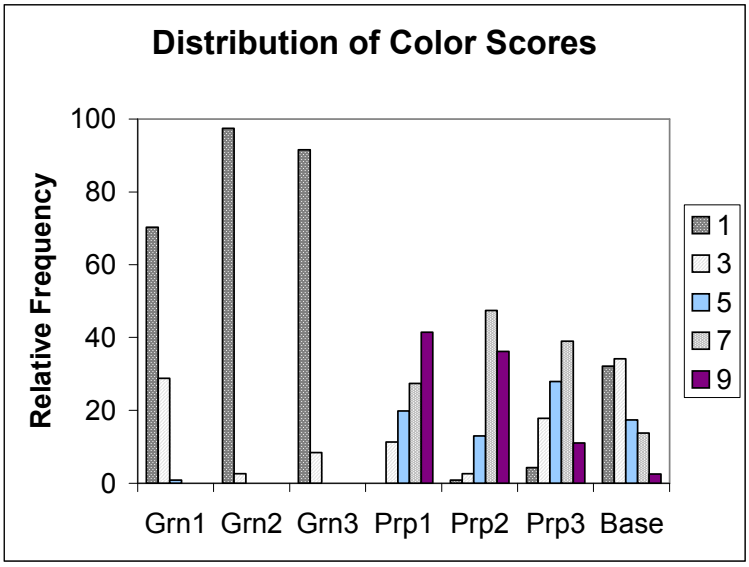

Fig. 4.2 Relative frequency of each color score category for each selection line and base population, after six gen

Hypocotyl color was scored visually four days after sowing, according to a categorical scale (1= green; 3= very l

5=intermediate; 7= purple; 9=dark purple).

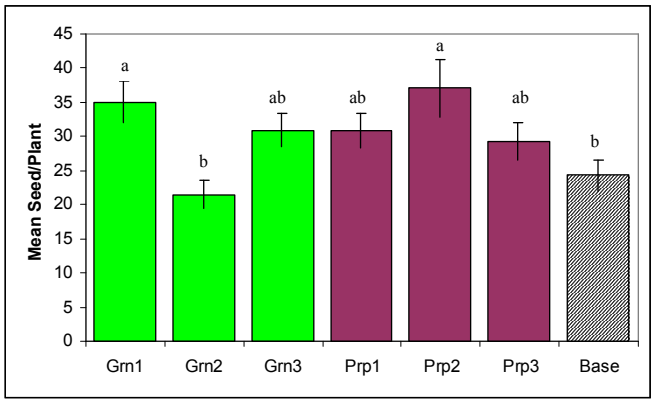

Fig. 4.3 Average seed production of plants from all base population and selection lines ($\bar{x}$ ± 1 SE).

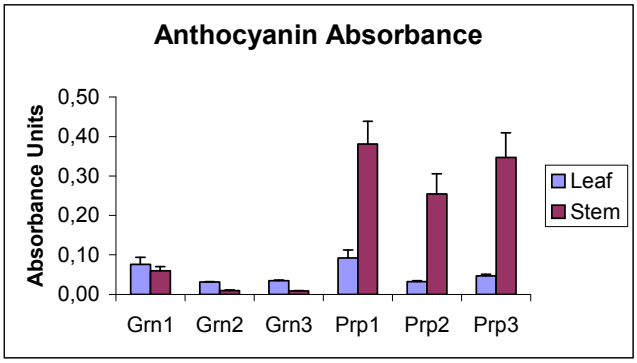

Fig. 4.4 Average corrected absorbance of acidified methanol leaf extract, proportional to the concentration per m
in the plant tissues ($\bar{x}$ ± 1 SE). Selection differed in anthocyanin content (MANOVA, P<.0001). In univariate anal
anthocyanin content did not differ significantly among selection treatments, stem anthocyanin content did differ
treatments ($F_{1,4}$ = 56.6706, P=0.0019),

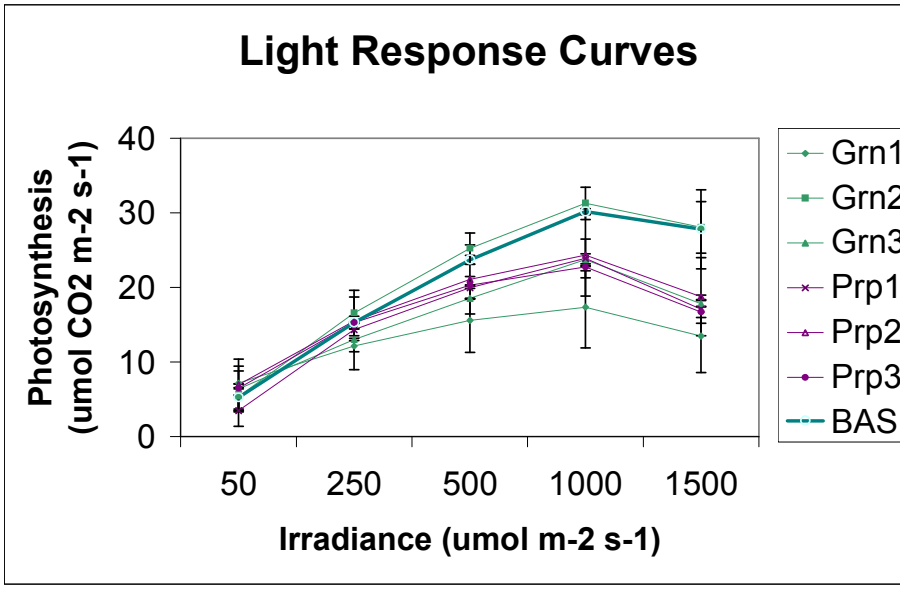

Fig. 4.5. Photosynthetic rate estimates at different levels of irradiance ($\bar{x}$ ± 1 SE), measured using a Licor 6400, on leaf, twelve days after sowing, at 28ºC and at internal CO2 concentrations of 330 ±15ppm. Light response curves among treatment.

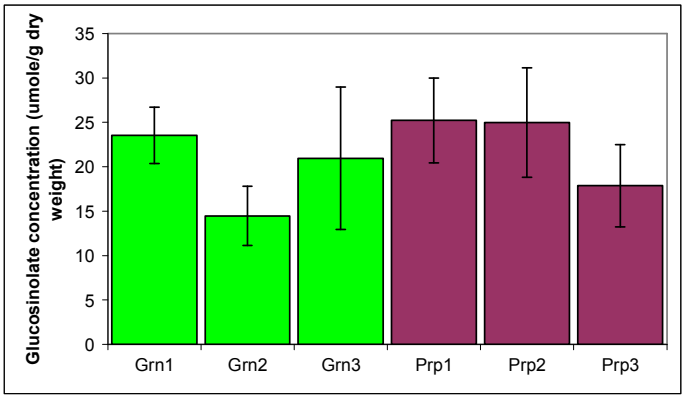

Fig. 4.6 Total glucosinolate content of each selection line ($\bar{x}$ ± 1 SE). Analysis of variance revealed no significant selection treatments.

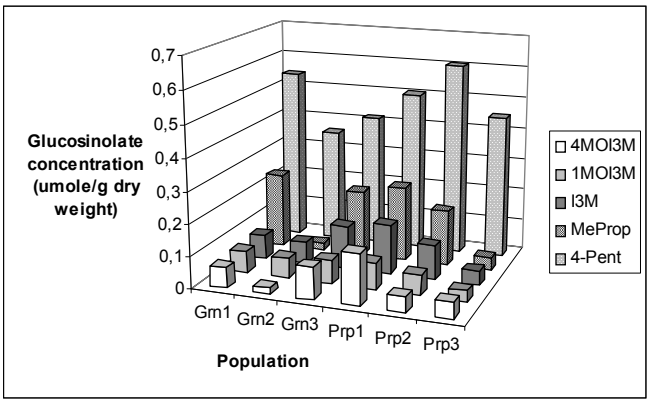

Fig.4.7 Concentration of Methylpropyl (MeProp), 4-Pentenyl (4-pent), Indol-3-yl-methyl (I3M), 4-Methoxy-Indo
(4MOI3M), and 1-Methoxy-Indol-3-yl-methyl glucosinolate (1MOI3M). A sixth identified glucosinolate, 3-Bute
corresponded to 96% of total glucosinolate content, and its pattern of expression is closely reflected in Fig. 6. A l
no difference between selection treatment in glucosinolate profile. Univariate analyses exhibited no differences b
any of the individual glucosinolate. [I'm thinking this graph might be unnecessary. That I could present this data
of showing the treatment means and se in a table, show those as a graph that would be easier to read that this one

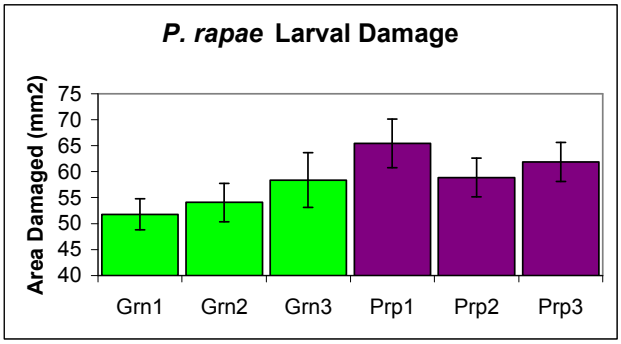

Fig. 4.8 Average leaf area damaged by first instar larvae of *Pieris rapae* ($\bar{x}$ ± 1 SE). *Purple* lines incurred signific

(ANOVA, P = 0.0193)

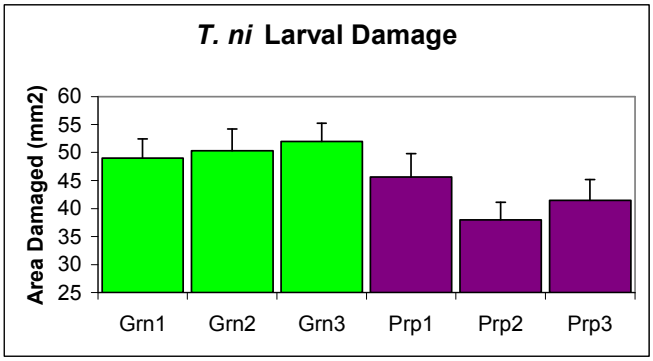

Fig. 4.9 Average leaf area damaged by first instar larvae of *Trichoplusia ni* ($\bar{x}$ ± 1 SE). *Green* lines incurred signi

(ANOVA, P = 0.0193)

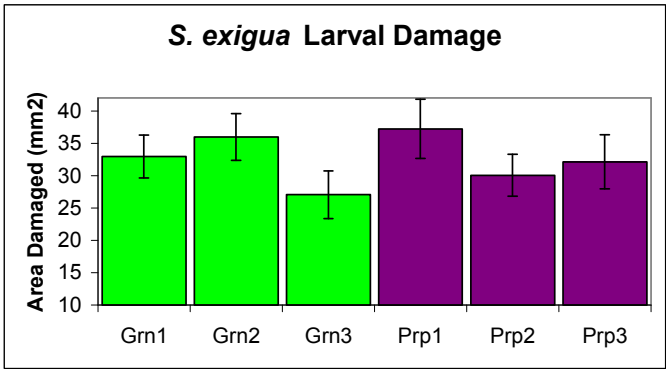

Fig. 4.10 Average leaf area damaged by first instar larvae of *Spodoptera exigua* ($\bar{x}$ ± 1 SE). Selection treatments

significantly.

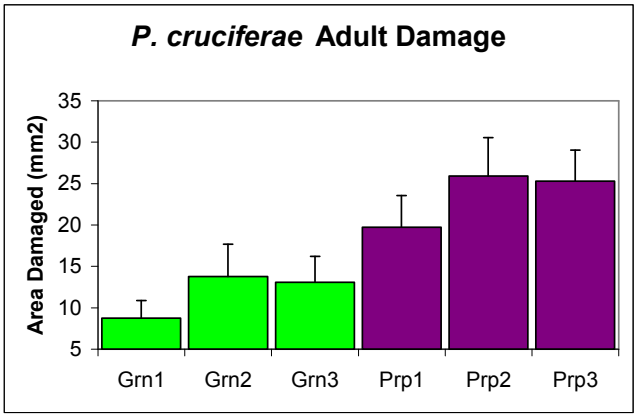

Fig. 4.11 Average leaf area damaged by adults of *Phyllotreta cruciferae* ($\bar{x}$ ± 1 SE). *Purple* lines incurred signific

(ANOVA, P = 0.0087).

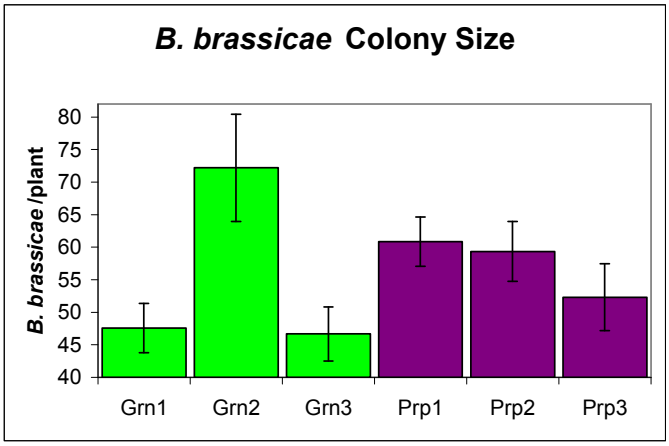

Fig. 4.12 Average colony size of *Brevicoryne brassicae* ($\bar{x}$ ± 1 SE). Each plant was "seeded" with two early instar colony size per plant was counted after nine days. Selection treatments did not differ significantly in final colony

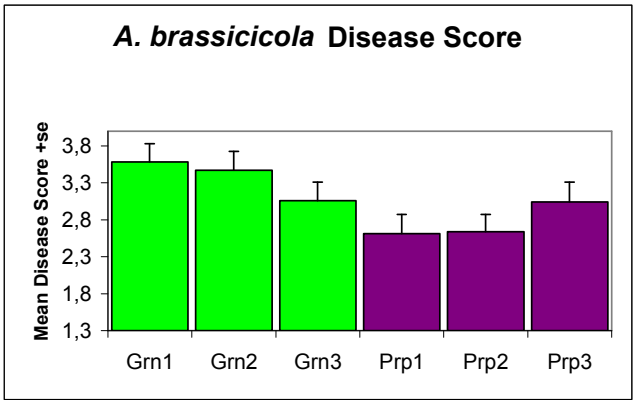

Fig. 4.13 Average disease severity score caused by *Alternaria brassicicola* ($\bar{x}$ ± 1 SE). *Purple* lines were significa

disease (ANOVA, P = 0.0171).

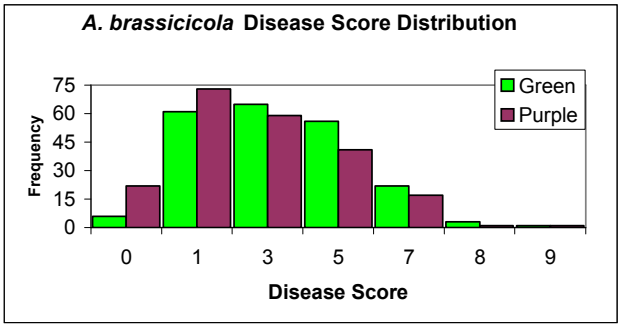

Fig. 4.14 Distribution of disease severity scores caused by *Alternaria brassicicola*. (0=disease absent; 1=very lov
number of small foci of disease; 3=low expression, affecting <20% of leaf area; 5=intermediate conspicuous exp
75% of leaf tissue; 8= high expression, covering 75-95% of tissue; 9= very high expression, affecting nearly 100

4. 6 REFERENCES

Abouheif, E. 1997. Developmental genetics and homology: a hierarchical approach. Trends in Ecology & Evolution 12:405-408.

Ågren, J., and D. W. Schemske. 1994. Evolution of trichome number in a naturalized population of *Brassica rapa*. American Naturalist 143:1-13.

Arima, H., H. Ashida, and G. Danno. 2002. Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. Bioscience Biotechnology & Biochemistry 66:1009-1014.

Arima, H., and G. Danno. 2002. Isolation of antimicrobial compounds from guava (*Psidium guajava* L.) and their structural elucidation. Bioscience Biotechnology & Biochemistry 66:1727-1730.

Baur, R., E. Städler, K. Monde, and M. Takasugi. 1998. Phytoalexins from *Brassica* (Cruciferae) as oviposition stimulants for the cabbage root fly, *Delia radicum*. Chemoecology 8:163-168.

Blau, P. A., P. Feeny, L. Contardo, and D. S. Robson. 1978. Allylglucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. Science 200:1296-1298.

Bohm, B. A. 1998. Introduction to Flavonoids. Chapter 7: Flavonoid Functions in Nature *in* B. Ravindranath, ed. Chemistry and Biochemistry of Organic Natural Products. Harwood Academic Publishing, Amsterdam, Netherlands.

Bradshaw, H. D., and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature 426:176-178.

Brown, P. D., J. G. Tokuhisa, M. Reichelt, and J. Gershenzon. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry 62:471-481.

Carroll, C. R., and C. A. Hoffman. 1980. Chemical feeding deterrent mobilized in response to insect herbivory and counteradaptation by *Epilachna tredecimnotata*. Science 209:414-416.

Close, D. C., and C. L. Beadle. 2003. The ecophysiology of foliar anthocyanin. The Botanical Review 69:149-161.

Close, D. C., C. L. Beadle, G. K. Holz, and P. H. Brown. 2002. Effect of shadecloth tree shelters on cold-induced photoinhibition, foliar anthocyanin and growth of *Eucalyptus globulus* and *E. nitens* seedlings during establishment. Australian Journal of Botany 50:15-20.

Coley, P. D., and T. M. Aide. 1989. Red coloration of tropical young leaves - a possible antifungal defense. Journal of Tropical Ecology 5:293-300.

Coley, P. D., and J. A. Barone. 1996. Herbivory and plant defenses in tropical forests. Annual Review of Ecology and Systematics 27:305-335.

Coley, P. D., and T. A. Kursar. 1996. Anti-herbivore defenses of young tropical leaves: Physiological constraints and ecological trade-offs. Pp. 305-335 *in* S. S. Mulkey, R. L. Chazdon and A. P. Smith, eds. *Tropical forest plant ecophysiology*. Chapman & Hall, New York.

Conner, J. K. 2003. Artificial selection: A powerful tool for ecologists. Ecology 84:1650-1660.

Delgado-Vargas, F., A. R. Jiménez, and O. Paredes-López. 2000. Natural pigments: carotenoids, anthocyanins, and betalins - characteristics, biosynthesis, processing, and statability. Critical Reviews in Food Science and Nutrition 40:173-289.

Dhingra, O. D., and J. B. Sinclair. 1995. Basic plant pathology methods. Lewis Publishers, Boca Raton.

Dixon, R. A., and C. L. Steele. 1999. Flavonoids and isoflavonoids: A gold mine for metabolic engineering. Trends in Plant Science 4:394-400.

Dunn, J. A., and D. P. H. Kempton. 1976. Varietal differences in the susceptibility of Brussels sprouts to lepidopterous pests. Annals of Applied Biology 82:11-19.

Farzad, M., R. Griesbach, and M. R. Weiss. 2002. Floral color change in *Viola cornuta* L. (Violaceae): a model system to study regulation of anthocyanin production. Plant Science 162:225-231.

Fineblum, W. L., and M. D. Rausher. 1997. Do floral pigmentation genes also influence resistance to enemies? The W locus in *Ipomoea purpurea*. Ecology 78:1646-1654.

Fry, J. D. 2003. Detecting ecological trade-offs using selection experiments. Ecology 84:1672-1678.

Fry, J. D., and M. D. Rausher. 1997. Selection on a floral color polymorphism in the tall morning glory (*Ipomoea purpurea*): Transmission success of the alleles through pollen. Evolution 51:66-78.

Geuder, M., V. Wray, K. Fiedler, and P. Proksch. 1997. Sequestration and metabolism of host-plant flavonoids by the lycaenid butterfly *Polyommatus bellargus*. Journal of Chemical Ecology 23:1361-1372.

Gonzales, W. L., C. C. Ramirez, N. Olea, and H. M. Niemeyer. 2002. Host plant changes produced by the aphid Sipha flava: consequences for aphid feeding behaviour and growth. Entomologia Experimentalis Et Applicata 103:107-113.

Hipskind, J., K. Wood, and R. L. Nicholson. 1996. Localized stimulation of anthocyanin accumulation and delineation of pathogen ingress in maize genetically resistant to *Bipolaris maydis* race O. Physiological and Molecular Plant Pathology 49:247-256.

Hogge, L. R., D. W. Reed, E. W. Underhill, and G. W. Haughn. 1988. HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid-chromatography mass-spectrometry. Journal of Chromatographic Science 26:551-556.

Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, and G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. Ecology 84:1733-1743.

Jewell, J., J. McKee, and A. Richards. 1994. The keel color polymorphism in *Lotus corniculatus* L.: Differences in internal flower temperatures. New Phytologist 128:363-368.

Jones, D., G. O. Myers, and B. R. Leonard. 2000. Effect of leaf color on growth and development of armyworms in cotton. 14th Biennial Int'l Plant Resistance to Insects Workshop, 26:

Kangatharalingam, N., M. L. Pierce, M. B. Bayles, and M. Essenberg. 2002. Epidermal anthocyanin production as an indicator of bacterial blight resistance in cotton. Physiological and Molecular Plant Pathology 61:189-195.

Kubo, H., A. J. M. Peeters, M. G. M. Aarts, A. Pereira, and M. Koornneef. 1999. ANTHOCYANINLESS2, a homeobox gene affecting anthocyanin distribution and root development in *Arabidopsis*. Plant Cell 11:1217-1226.
Kucharek, T. 1994. *Alternaria* diseases of crucifers. Plant Pathology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, Gainesville.
Lindstrom, J. T., and L. O. Vodkin. 1991. A soybean cell wall protein is affected by seed color genotype. Plant Cell 3:561-571.
Lo, S.-C. C., and R. L. Nicholson. 1998. Reduction of light-induced anthocyanin accumulation in inoculated *Sorghum mesocotyls*. Implications for a compensatory role in the defense response. Plant Physiol. 116:979-989.
Louda, S., and S. Mole. 1991. Glucosinolates: chemistry and ecology. Pp. 123-164 *in* G. A. Rosenthal and M. R. Berenbaum, eds. *Herbivores: Their Interaction with Secondary Plant Metabolites*. Academic Press, San Diego, CA.
Mitchell-Olds, T., and D. Bradley. 1996. Genetics of *Brassica rapa*. 3. Costs of disease resistance to three fungal pathogens. Evolution 50:1859-1865.
Mooney, H. A., W. E. Winner, and E. J. Pell. 1991. Response of plants to multiple stresses. Physiological Ecology Series. Academic Press.
Murry, J. R., and W. P. Hackett. 1991. Difydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase *Hedera helix* L. Plant Physiology 97:343-351.
Onyilagha, J. C., J. Lazorko, M. Y. Gruber, J. J. Soroka, and M. A. Erlandson. 2004. Effect of flavonoids on feeding preference and development of the crucifer pest *Mamestra configurata* Walker. Journal of Chemical Ecology 30:109-124.
Pandey, M. K., R. Pandey, V. P. Singh, V. B. Pandey, and U. P. Singh. 2002. Antifungal activity of 4',7-dimethoxyisoflavone against some fungi. Mycobiology 30:55-56.
Radcliffe, E. B., and R. K. Chapman. 1966. Varietal resistance to insect attack in various cruciferous crops. Journal of Economical Entomology 59:120-125.
Rangel, J. F. 1945. Two *Alternaria* diseases of cruciferous plants. Phytopathology 35:1002-1007.
Reddy, V., S. Dash, and A. Reddy. 1995. Anthocyanin pathway in rice (*Oryza sativa* L.): Identification of a mutant showing dominant inhibition of anthocyanin in leaf blade and accumulation of proanthocyanidins in the pericarp. Theoretical and Applied Genetics 91:301-312.
Ritchey, C. M. 1999. The effect of anthocyanin on herbivory in *Viola pedata* (Birdfoot Violet). MS. Truman State University, Kirksville, Missouri.
Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proceedings of the National Academy of Sciences of the United States of America 96:11910-11915.
SeedQuest. 2002. Study lays groundwork for canola varieties that deter flea beetles. Pp. http://www.seedquest.com/News/releases/2002/march/4240.htm. SeedQuest, Saskatoon, Saskatchewan.
Shirley, B. W. 1996. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends in Plant Science 1:377-382.

Sims, D. A., and J. A. Gamon. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sensing of Environment 81:337-354.
Steyn, W. J., S. J. E. Wand, D. M. Holcroft, and G. Jacobs. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist 155:349-361.
Stowe, K. A. 1998. Realized defense of artificially selected lines of *Brassica rapa*: Effects of quantitative genetic variation in foliar glucosinolate concentration. Environmental Entomology 27:1166-1174.
Sutherland, D. W. S. 1965. Biological investigations of *Trichoplusia ni* (Hubner) (Lepidoptera, Noctuidae), and related and associated species damaging cruciferous crops on Long Island, NY, 1960-63. PhD. Cornell University
Thompson, J. N. 1994. The coevolutionary process. Pp. 376. University of Chicago Press.
van Loon, J. J. A., C. Z. Wang, J. K. Nielsen, R. Gols, and Y. T. Qui. 2002. Flavonoids from cabbage are feeding stimulants for diamondback moth larvae additional to glucosinolates: Chemoreception and behaviour. Entomologia Experimentalis et Applicata 104:27-34.
VanEtten, C. H., and H. L. Tookey. 1979. Chemistry and Biological effects of Glucosinolates *in* G. A. Rosenthal and D. H. Janzen, eds. *Herbivores: Their Interaction with Secondary Plant Metabolites*. Academic Press, New York, NY.
Wiesen, B., E. Krug, K. Fiedler, V. Wray, and P. Proksch. 1994. Sequestration of host-plant-derived flavonoids by Lycaenid butterfly *Polyommatus icarus*. Journal of Chemical Ecology 20:2523-2538.
Williams, P. H. 1980. Bee-sticks, an aid in pollinating cruciferae. Hortscience 15:802-803.
Williams, P. H. 1985. Crucifer Genetics Cooperative Resource Book. University of Wisconsin, Madison, WI.
Williams, P. H., and C. B. Hill. 1986. Rapid-cycling populations of *Brassicas*. Science 232:1385-89.
Wolfe, L. M., and S. E. Sellers. 1997. Polymorphic floral traits in *Linaria canadensis* (Scrophulariaceae). American Midland Naturalist 138:134-139.
Yencho, G. C., M. B. Cohen, and P. F. Byrne. 2000. Applications of tagging and mapping insect resistance loci in plants. Annual Review of Entomology 45:393-422.
Zalucki, M. P., A. R. Clarke, and S. B. Malcolm. 2002. Ecology and behavior of first instar larval Lepidoptera. Annual Review of Entomology 47:361-393.
Zufall, R., and M. D. Rausher. 2001. Diffuse coevolution and anthocyanin production. Botany 2001: "Plants and People", Albuquerque, New Mexico