CHAPTER THREE

CORRELATED RESPONSES OF RAPID CYCLING *BRASSICA RAPA* (BRASSICACEAE) TO ARTIFICIAL SELECTION FOR RESISTANCE TO *ALTERNARIA BRASSICICOLA* (DEUTEROMYCETES)

3.1 ABSTRACT

Plants have a variety of mechanisms that provide resistance to a broad diversity of herbivores and pathogens. Whether there are common mechanisms that affect both classes of plant enemies is a question that is being addressed using different approaches. For instance, studies of induced plant response to injury and infection suggest there is substantial cross-talk among pathways induced by herbivores and pathogens. This interaction may be synergistic or antagonist, resulting in concerted response or a trade-off in defense to both enemies.

In this study, I approach plant defense as a 'black box' by artificially selecting populations of rapid-cycling *Brassica rapa* (Brassicaceae) for greater resistance to a fungal pathogen, the cabbage leaf spot *Alternaria brassicicola* (Deutoeromycetes). After seven generations, selected lines exhibited significantly greater resistance to *A. brassicicola* than unselected control lines. Selected lines did not incur a fitness cost, in terms of reduction of seed set, relative to control lines. Treatments varied in glucosinolate profile, a family of secondary compounds known to have biological effect on both pathogens and herbivores.

Lines that evolved greater resistance to *A. brassicicola* did not exhibit correlated resistance to other enemies, in particular to larvae of three lepidopterans (*Pieris rapae*, *Trichoplusia ni*, and *Spodoptera exigua*), adults of a flea beetle (*Phyllotreta cruciferae*), or to the cabbage aphid (*Brevicoryne brassicae*). This suggests the independence of resistance to fungal pathogens and a broad set of insect herbivores.

3.2 INTRODUCTION

Plants are attacked by a variety of enemies, including fungal pathogens and insect herbivores. Historically, the study of plant interactions with these groups of enemies has developed as two separate fields, conducted largely by plant pathologists, on the one hand, and ecologists and entomologists, one the other, with little interaction between the two fields, to the extent that they established different vocabulary for similar phenomena. Whereas plant pathologists focused largely on the genetic and mechanistic basis of induced resistance, of cell-cell interactions, the study of plant resistance against insect herbivores conducted whole plant studies, focusing on the role of constitutive chemical defenses. Gene-for-gene models became the paradigm of plant-pathogen interactions (Flor 1956), whereas no clear evolutionary model of plant resistance to insect herbivores has emerged (Berenbaum 1995). More recently, this divide has been bridged, particularly with the increasing research of the pathways of inducible plant responses to both pests and pathogens. Indeed, most of the recent reviews addressing the question of how plants cope with multiple enemies have focused nearly exclusively on induced resistance (Bostock et al. 2001; Maleck and Dietrich 1999; Paul et al. 2000; Walling 2000; Wittstock and Gershenzon 2002). This emphasis in the literature may be a result of the

availability of better tools, both molecular and physiological, for studying induced defenses, rather than the greater importance of this form of defense to the plant (Wittstock and Gershenzon 2002).

Plants resist pathogen infection by a variety of induced mechanisms (Hammerschmidt and Nicholson 1999; Walton 1997), including the hypersensitive response (HR; a rapid, localized cell death), the oxidative burst (the production of active oxidizing compounds and enzymes), cell wall modifications (*e.g.*, lignification), and the production of pathogenesis-related proteins (PR; *e.g.*, chitinases) and phytoalexins (a generic term for low molecular weight plant antibiotics) (Hammerschmidt 1999). These responses, largely localized to the site of infection, are accompanied by a systemic increase in the level of salicylic acid (SA) that is thought to trigger the systemic acquired resistance (SAR) pathway, which leads ultimately to the expression of PR genes throughout the plant and a generalized increase in plant resistance to pathogens.

The best characterized mechanism of response to insect feeding, or more generally tissue wounding, is that induced by jasmonic acid (JA), which initiates many plant processes, including the production of protein inhibitors (PIs) and of secondary metabolites (e.g., nicotine in *Nicotinana*, indole glucosinolates in *Arabidopsis*) (Karban and Baldwin 1997). Although, the SA- and JA- mediated pathways have generally been associated with plant responses to pathogen and insect respectively, it becoming clear that no such clear separation exists. For instance, whiteflies, aphids and other non-chewing insects appear to activate the SA-dependent pathways, *i.e.*, to induce a response more akin to that against a plant pathogen (Moran et al. 2002; Walling 2000).

Furthermore, induced responses to pathogens and herbivores are hardly independent. Infection of *Arabidopsis* with *Alternaria brassicicola* activated both SA- and JA- dependent classes of PR proteins (Penninckx et al. 1996). There is some evidence for coordinated responses against insects and pathogens, a seemingly adaptive response given that wounds generated by insect feeding can be followed by infection (Karban et al. 1987; McIntyre et al. 1981). However, our understanding of the biochemistry and genetics of the induced pathways has revealed considerable cross-talk between the two pathways (Bostock et al. 2001; Maleck and Dietrich 1999; Thaler et al. 2002a), and that interactions can be negative. For instance, salicylic acid is known to inhibit the lipoxygenase involved in jasmonate sythesis, such that a pathogen infection may inhibit a wound-response (Karban and Baldwin 1997; Thaler et al. 2002a; Thaler et al. 2002b).

There is also evidence for the effect of constitutive defenses against both insect herbivores and fungal pathogens. Many of the preformed secondary compounds that have been implicated in herbivore defense also affect pathogens (Harborne 1993). For instance, glucosinolates are sulfur- and nitrogen-containing secondary compounds, found in the order Capparales, including the Brassicaceae (Louda and Mole 1991). Glucosinolates and isothiocyanates – or mustard oils, the products of glucosinolate hydrolysis - mediate a number of plant interactions with insects, *e.g.*, inhibiting herbivore feeding (Mitchell et al. 1996; Shields and Mitchell 1995), stimulating specialist insect feeding (Giamoustaris and Mithen 1995) and oviposition (Huang et al. 1994; Stadler et al. 1995), and also provide resistance to fungal pathogens (Greenhalgh and Mitchell 1976; Mithen and Magrath 1992; Sarwar et al. 1998; Tierens et al. 2001). However,

much of the evidence of antimicrobial activity comes from *in vitro* studies, and direct evidence of *in vivo* effects of preformed defenses is lacking (Hammerschmidt and Schultz 1996), in part due to the difficulty of manipulating constitutive defenses (Wittstock and Gershenzon 2002).

Artificial selection experiments (Ågren and Schemske 1993; Stowe 1998), and the use of naturally occurring variants (Lambrix et al. 2001) or cultivars (Giamoustaris and Mithen 1995; Giamoustaris and Mithen 1997) provide a means of manipulating levels of constitutively expressed traits and testing for correlated effects upon multiple enemies. Modern bioengineering techniques permit the transgenic expression of compounds (Stuiver and Custers 2001). Tattersall et al. (2001) transferred the cyanogenic glucoside biosynthetic pathway, which produces dhurrin, from *Sorghum bicolor* to *Arabidopsis thaliana*. This allows the *in vivo* bioassaying of the effects of dhurrin upon crucifer specialists that would otherwise never confront this compound. Genetic engineering will offer novel tools for testing *in vivo* the effects of proteins and compounds on pathogens, and generate disease resistance crops (*e.g.*, Mora and Earle 2001).

In this paper, I describe the artificial selection of rapid cycling *Brassica rapa* for resistance to a specialist fungal pathogen, *Alternaria brassicicola* (Deuteromycetes). No particular resistance mechanism was targeted. Rather, plants expressing lower levels of disease expression were selected to establish subsequent generations. Thus, a selected population may respond via several different traits, and different populations may evolve along different avenues. In particular, a population may respond by changing the expression of a constitutive trait or the degree of induced response to infection. Selected populations were then tested for correlated responses to a number of insect herbivores:

including larvae of the specialist butterfly *Pieris rapae* (Pieridae), larvae of the generalists moth *Trichoplusia ni* (Noctuidae), adults of the flea beetle *Phyllotreta cruciferae* (Chrysomelidae), and colonies of the aphid *Brevicoryne brassicae* (Aphididae). This approach allowed me to address the following questions: (1) Rapid cycling *B. rapa* has responded rapidly to selection for traits involved in defense experiments (Ågren and Schemske 1993; Stowe 1998), and to selection for resistance to a number of fungal pathogens (Mitchell-Olds and Bradley 1996). Is there a response to selection for *A. brassicicola* resistance? (2) Costs of resistance are frequently reported, particularly of pathogen resistance (Bergelson and Purrington 1996). Is there any direct fitness cost associated with resistance? (3) Glucosinolates are the emblematic family of secondary defenses in the Brassicaceae. Does increased resistance correlate with changes in glucosinolate content or profile expression? (4) The literature points to a numbers of mechanisms that link defense mechanisms to insect herbivores and plant pathogens. Are there correlated responses of *A. brassicicola* resistance in realized levels of defense against herbivores? (5) As suggested previously, plants respond to aphid attacks via the same induced responses triggered by pathogen infection, which are distinct from the wound-response mechanisms. One might expect pathogen resistant lines to be more resistant to aphid herbivory, but not necessarily so to chewing herbivores. Is there any pattern to the response of these enemies to increased *A. brassicicola* resistance?

3.3 MATERIAL AND METHODS

Brassica rapa L. (syn *B. campestris*: Brassicaceae) is an outcrossing 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}).

Cabbage Leaf Spot (*Alternaria brassicicola* (Schwein.) Wiltshire, Deuteromycetes, Dematiaceae) is a necrotrophic pathogen causing disease in a wide range of Brassicaceae hosts. Spores landing on leaf germinate under conditions of high humidity and the hyphae penetrate host tissue. Infection manifests itself as dark, circular spots on leaves (Kucharek 1994).

Spores were collected from a diseased *Brassica oleracea*, from Riverhead, New York. The species was identified, with assistance from Dr. Margret Magrath of the Long Island Horticultural Experimental Research Station, in Riverhead, Long Island, based on host, form of leaf infection and form of spores as seen under a microscope (Changsri and Weber 1963). Spores were transferred to 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 16h-light/8h-dark photocycle (Changsri and Weber 1963). 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 a hemacytometer and adjusted to 10^6 spores mL^{-1}.

3.3.1. Selection design

The experimental design consisted of a selection and a control treatment each replicated four times. An initial population of 800 individuals was misted with a 1.5% gelatin suspension of *A. brassicicola* (10^6 sporesL^{-1}), eight days after sowing, and kept under conditions of high relative humidity (RH=90%) for the next 72 hours (Williams 1985). Relative humidity was established in a sealed room by use of a Vicks® vaporizer and a pot of warmed water. Relative humidity was checked semi-daily with the use of a psychrometer. Fourteen days after sowing, plants were submitted to a second 72h period of high humidity, after which disease severity (or disease reaction) was scored, using a categorical scale (Fox and Williams 1984; Mitchell-Olds et al. 1995; 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 total 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).

Four lines of the *Alternaria* selection treatment (A_{1-4}) were founded with twenty plants chosen among the plants exhibiting highest resistance, *i.e.*, lowest disease severity scores. Eighty plants were chosen at random from among two hundred uninoculated plants and assigned at random to 4 replicate control lines (C_{1-4}). Each subsequent generation, a population of two hundred plants from each treatment line was inoculated

and the twenty most resistant plants were mass-pollinated within-line to propagate the next generation. Control lines were grown simultaneously with treatment plants, kept under the same humidity regime and identical population size, yet misted with water.

Direct response to selection was assessed twice, after six and after seven rounds of artificial selection. In each assay, plants from all eight lines, from the control and selection treatments, were randomly distributed across three blocks of 20 x 10 individuals. Within each block, plants were immediately adjacent to one another. Each block had an independent subirrigation unit. Plants were inoculated and scored as described above. The outermost rank and file of each block was treated similarly to innermost plants, but was excluded from analysis to avoid border effects, leaving a total of 144 experimental plants per block. Thus, each assay consisted of a total of 432 experiment plants and 54 plants per line scored for disease expression. 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.

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 seven generations of selection, a random sample of forty plants per line were mass-pollinated for twelve days during peak flowering stage. The number of seeds per seed pod was counted to assess fitness costs associated with artificial selection.

3.3.2 Glucosinolate Analysis

Glucosinolates are sulfur- and nitrogen-containing compounds found in a restricted number of plant Capparales families, most notably the Brassicaceae. These preformed compounds are compartmentalized within vacuoles. When released during tissue damage, they are hydrolyzed by the enzyme myrosinase to isothiocynates (or mustard oils) (Louda and Mole 1991; Mithen 2001). They have a deterrent effect against non-specialist insect herbivores (Blau et al. 1978), but are known to stimulate feeding and oviposition by specialist insects (Bartlet and Williams 1991; Huang and Renwick 1994; Larsen et al. 1992), or have no detrimental effect on them (but see Stowe 1998). Products of glucosinolate breakdown have been found to be inhibitory to a wide range of fungi and bacteria (Olivier et al. 1999; Tierens et al. 2001). In certain cases, compounds shown to have an *in vitro* effect are ineffective defensive agents in vivo, suggesting that some pathogens have evolved means of neutralizing the hydrolysis products of glucosinolates (Sexton et al. 1999). As with insect herbivores, the effect of glucosinolates on fungi is variable. Giamoustaris and Mithen (1997) tested several lines of *Brassica napus* of varying glucosinolate leaf content. Infection level by *Leptosphaeria maculans* was uncorrelated with glucosinolate content, but positively correlated with *Alternaria* spp. infection, suggesting an affiliation for glucosinolates among fungal specialists analogous to that of insect specialists. Glucosinolate content was found to accumulate in *B. rapa* and *B. napus* after infection with *A. brassicae* (Doughty et al. 1996; Doughty et al. 1991).

I assessed whether response to seven generations of selection for increased resistance to *A. brassicicola* was correlated with changes in glucosinolate content. Plants from each treatment and control line were grown simultaneously and 14 days after

sowing five 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 following protocols are the standard procedures used in Gershenzon's laboratory. 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, 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 a random effect. Differences in glucosinolate profile, i.e. each individual glucosinolate as a separate response variable, were compared in a MANOVA with the same factorial design. Glucosinolates present in small quantities and entirely absent in some plants, were square-root transformed.

3.3.3 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, each assay consisted of 3 blocks, a total of 432 experiment plants (and 54 plants per line) submitted to a single natural enemy and scored for leaf damage or colony size.

3.3.3.1 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.

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* or of *T. ni* were placed on each plant, including border plants. 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* 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 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.

3.3.3.2 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 oil (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 (1 mm^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.

3.3.3.3 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 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.

3.3.3.4 T. ni damage under different levels of plant fertilization

In order to assess the generality of results from one of the bioassays across a gradient of environmental conditions, I compared damage by neonate *T. ni* larvae on selection and control lines grown under different fertilization regimes. Plants from all lines were grown in a total of 6 blocks of plants, as described above. Two blocks were assigned to one of three fertilization treatments. In each block, plants were supplied *ad libitium* during the course of the bioassay, via their independent subirrigation system, with solutions of Peter's 15-15-15 adjusted to provide 5 ppM N, 50 ppM N, or 100 ppM N. These levels were selected based on observations of rapid cycling *B. rapa* growth under different fertilization regimes. The regular regime under my rearing system was at 50ppM N. Under 5ppM N plant and lead size is considerably stunted, but plants do produce viable flowers. Values much larger than 100ppM N lead to leaf lesions, but at the 100ppM plants grow large healthy leaves. Although these levels are considerably higher than those used in similar experiments and of environmental levels (Throop 2002), it bears remembering that this variety was artificially selected under a high fertilization

regime, and given its particular life history requires and tolerates higher levels of fertilization.

Two neonate larvae of *T. ni* were placed on each plant, including border plants, and leaf area damaged after 48hrs of *T. ni* exposure was measured with a transparent grid (4 mm^2 grid squares). 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.

I tested whether nitrogen had an effect on leaf area eaten by *T. ni*, whether damage between selected and control lines differed across all fertilization treatment, and whether fertilization treatment affected the response of *T. ni* to selected vs. control lines. To address these questions I treated the data as a split-plot design, with fertilization level (a fixed factor) applied to an entire block (random factor), and selection treatment (a fixed factor) applied to subplots within a block. Furthermore, the random factor, line, was treated as a random factor nested within selection treatment, and I used leaf area measured before damage as a covariate.

All statistical analyses were performed on JMP (version 3.2.2, SAS Institute Inc. 1998) and JMP v.5.0.1 (SAS Institute, 2001).

3.4. RESULTS

3.4.1 Selection design

Lines artificially selected for greater resistance to *A. brassicicola* infection exhibited lower disease severity scores than control lines after six generations (disease severity scores ± SE: $\bar{x}_A = 2.28 \pm 0.133$; $\bar{x}_C = 2.80 \pm 0.135$; $F_{1,6} = 7.968$, P=0.03, Table 1) and seven generations of selection (disease severity scores ± SE: $\bar{x}_A = 2.818 \pm 0.217$;

$\bar{x}_C = 4.05 \pm 0.216$; $F_{1,6} = 20.055$, P=0.004, Table 2). Despite this consistent and significant selection treatment effect, and the lack of significant line within treatment component, pair-wise comparisons after seven generations of selection among lines, using HSD Tukey tests, reveal that certain pairs of lines from different treatments are not significantly different (Fig.1). Furthermore, selection and control treatment disease severity scores, pooled across lines, shows considerable overlap in their distributions (Fig. 2). There was no significant difference in seed number between selected and control plants, suggesting a lack of fitness cost associated with increase *A. brassicicola* resistance ($F_{1,6} = 0.0741$, P=0.7945, Fig. 3) .

3.4.2 Glucosinolate analysis

Glucosinolate analysis revealed the presence of seven different glucosinolates: 3-Butenyl (3-But), Methylpropyl (MeProp), 4-Pentenyl (4-pent), Indol-3-yl-methyl (I3M), 4-Methoxy-Indol-3-yl-methyl (4MOI3M), 2-Phenylethyl (2-Phen), and 1-Methoxy-Indol-3-yl-methyl glucosinolate (1MOI3M). 3-Butenyl was the most abundant glucosinolate in all samples (average of 97% of total glucosinolates). Other compounds were detected in varying amounts and concentrations close to detection limit (Table 3). Treatments did not differ in total glucosinolate concentration (Table 4), but multivariate analysis revealed marginally significant differences in glucosinolate profile (MANOVA: $F_{7,26} = 2.318$, P=0.056). Univariate ANOVAs comparing each glucosinolate reveal significant differences among treatments in two compounds – 4MOI3M ($F_{1,32} = 11.721$, P=0.0017) and 2-Phen ($F_{1,32} = 6.065$, P=0.0194) – both of which were expressed in lower concentrations in the selected lines (Fig. 5).

3.4.3 Bioassays

Selected lines exhibiting greater resistance to *A. brassicicola* did not differ from control lines in degree of herbivory by larval *P. rapae* ($F_{1,6}$ = 0.044, P=0.841, Fig. 6) or *T. ni* ($F_{1,6}$ = 0.871, P=0.387, Fig. 7), by *P. cruciferae* adults ($F_{1,6}$ = 4.3457, P = 0.082, Fig. 8), or in colony size of *B. brassicae* ($F_{1,6}$ = 0.1532, P = 0.708, Fig. 9). The treatment means for levels of herbivory are very similar for most enemies (Table 5). For all herbivores, except *B. brassicae*, there is a significant component of variance among lines within selection treatment (Table 6). But there is substantial overlap in line means from both treatments that explains the lack of treatment effect, and there is no line that is significantly different, using HSD Tukey pairwise tests, from all lines of the other treatment. Examination of the spatial distribution of damage and aphid colony size, using the spatial statistics software program PASSAGE (Rosenberg 2003), did not reveal any significant spatial pattern that might override a treatment effect. Previous analysis also suggests that larvae of *P. rapae* and *T. ni* do not tend to overdisperse or clump under these assay conditions (see Appendix).

An additional bioassay using neonate *T. ni* as herbivores upon plants from selection and control lines grown on different fertilization regimes revealed no significant difference between selection treatments in levels of herbivore damage (Table 7). There was no significant fertilization by selection treatment interaction. Lines selected for *A. brassicicola* resistance incurred less larval damage than control lines, across all fertilization treatments (Fig. 10), as mirrored by results, albeit nonsignificant, obtained in the previous bioassay (Table 5). Furthermore, herbivory did not differ among fertilization.

3.5 DISCUSSION

Artificial selection for decreased expression of leaf spot disease produced populations of rapid cycling *Brassica rapa* on average more resistant to infection by *Alternaria brassicicola* than control populations not subject to selection. Divergence between selected and control populations was significant after only six generations of selection and after an additional round of selection, difference between treatments increased in magnitude and statistical significance. Selected populations, although statistically more resistant to disease, exhibit a range of disease expression that spans the full spectrum of the severity scale, *i.e.*, no line was overwhelmingly resistant to disease. The presence of such continuous variation in reaction to *A. brassicicola* after seven generations of selection strongly suggests a polygenic basis for resistance.

This result is in concert with other experiments in which rapid cycling *B. rapa* responded rapidly to artificial selection using plant pathogens (Mitchell-Olds et al. 1995), or to selection of morphological and physiological components of defense (Ågren and Schemske 1992; Stowe 1998). In contrast, the same base population did not respond, after several attempts, to selection for resistance to larval lepidopteran feeding (see Appendix).

Selection did not target any particular potential defense trait and the mechanisms behind *A. brassicicola* resistance in this experiment are unknown. Conceivably, the different selection lines may even have evolved different modes of defense. I analyzed the possible role of glucosinolates in increased pathogen resistance. I found no differences between selection and control treatments in total glucosinolate concentration.

However, there were significant differences between treatments in glucosinolate profile, *i.e.*, the pattern of relative expression of particular glucosinolates.

Univariate analyses detected significant treatment effect in the expression of two glucosinolates in particular: 4-Methoxy-Indol-3-yl-methyl (4MOI3M) and 2-Phenylethyl (2-Phen). The latter is known to have negative effects upon mortality and reproduction of a number of nematodes (Potter et al. 2000; Serra et al. 2002; Zasada and Ferris 2003), and an inhibitory affect upon arbuscular mycorrhizal fungi (Vierheilig et al. 2000). In this experiment, control lines produced nine times more 2-Phen than resistant, selected lines, suggesting that if this compound does indeed play a role in the interaction with *A. brassicicola* it is a positive one. Since *A. brassicicola* is a specialized *Brassica* pathogen it is possible it has evolved to tolerate or even require specific plant signals, e.g. such as glucosinolates, for disease development. Previous work certainly suggests glucosinolates may increase susceptibility of *Brassica napus* ssp. *oleifera* to *Alternaria* (Giamoustaris and Mithen 1995; Giamoustaris and Mithen 1997).

It is worth emphasizing that change in resistance to *A. brassicicola* was accompanied by a change in glucosinolate profile, but no change in overall glucosinolate production. Total production in these populations is largely accounted for by the expression of one glucosinolate (3-Butenyl). If different enemies respond to relatively small changes in different individual glucosinolates, then natural selection might act on glucosinolate profiles without incurring the cost of profoundly changing total glucosinolate amount (Mithen et al. 1995).

Recent literature reviews have reported a substantial number of cases demonstrating a cost of resistance (Purrington 2000; Strauss et al. 2002), particularly

costs of resistance to pathogens (Bergelson and Purrington 1996). I detected no direct fitness cost of resistance to *A. brassicicola* measured as seed production, *i.e.*, selected and control lines did not differ in seed production. The absence of a cost can be due to a lack of statistical resolution (Siemens and Mitchell-Olds 1998). However, I feel the use of multiple selection lines and a reasonable replication within each line in assaying fitness averted problems of detectability. This does not exclude the possibility of direct costs expressed via other fitness components. For example, Mitchell-Olds and Bradley , compared growth rates of *B. rapa* populations resistant and susceptible to two fungal pathogens: *Leptosphaeria maculans* and *Peronospora parasitica*. While resistance to *L. maculans* had no effect on growth, resistance to *P. parasitica* was associated with a 6% decrease in growth rate.

A cost of resistance to a target species may also be expressed as an ecological cost, expressed as increased susceptibility to other enemies (Strauss et al. 2002). There is mounting evidence for conflicts between induced resistance to pathogens and herbivores (Felton and Korth 2000), but this has rarely been demonstrated *in vivo*. Resistance between pathogens and herbivores could also be positively correlated, as many of the pre-formed compounds that are antixenotic against herbivores also have negative effects upon pathogens.

I found that lines selected for resistance to *A. brassicicola* did not exhibit correlated responses to any of the four herbivores assayed. These span three insect orders (Lepidoptera, Coleoptera, and Homoptera), two modes of herbivory (leaf chewing and phloem sap sucking), and include generalist and specialist insects. This suggests a general lack of correlation between resistance to *A. brassicicola* and to insect herbivores, and that

the mechanism(s) responsible for fungal resistance in these selected lines are independent of those relevant in interactions with insects.

The lack of any correlated effect may be due to dosage-dependence of the defense traits that evolved in response to selection for fungal resistance, *i.e.*, while the magnitude of change was sufficient to alter fungal resistance, the changes are not detectable or relevant in the insect interaction. For instance, the weight of *Brevicoryne brassicae* colonies decreased when the aphids were raised on *Brassica napus* with increased concentrations of the glucosinolate 2-Phen, and *in vitro* experiments demonstrated a log-linear dose-dependent effect of *B. brassicae* survival and colony weight on 2-Phen (Cole 1996). The concentrations of 2-Phen expressed in *B. napus* were however two orders of magnitude greater than those expressed in *B. rapa* described here.

Expression of a trait correlation can be dependent upon the environmental context. An allocation trade-off may be masked under resource-rich conditions in which acquisition of the resource for both functions is unlimited, but manifest itself under resource-limited conditions (Van Noordwijk and de Jong 1986). Nitrogen is the most limiting nutrient for plants in many temperate terrestrial ecosystems (Vitousek and Howarth 1991). Nitrogen availability can have a strong effect on the interactions between plants and other organisms (Throop 2002), in part because it is a component of plant chemical defenses such as glucosinolates. Nitrogen is also an essential nutrient in insect diets (Schoonhoven et al. 1998), and plant nitrogen concentrations may affect insect consumption, development and survival rates (Henn and Schopf 2001; McCloskey and Isman 1995; Meyer 2000). I assayed for a treatment effect on feeding by *T. ni* across a fertilization gradient, and once again found no differences between selected and control

lines at any of the fertilization levels. Furthermore, there was no effect of fertilization level upon average level of *T. ni* feeding. This was the case even when comparing feeding among control lines across fertilization levels. This suggests nitrogen availability had no effect upon plant defense nor *T. ni* consumption rates.

Finally, I cannot rule out the possibility that further selection for *Alternaria* resistance, resulting in increased divergence in underlying traits, might result in correlated responses to selection. Although response to selection for fungal resistance is typically quite rapid in *B. rapa* (Edwards and Williams 1987; Miller and Williams 1986; Mitchell-Olds et al. 1995), it may take longer for trait values to become sufficiently divergent for there to be an effect on insect herbivores. In addition, there was significant variability among lines within treatments, particularly in the assays for resistance to *P. rapae* and *T. ni*. Although the overall analysis reveal no significant treatment effect, comparison of particular pairs of populations between treatments suggest the possibility of correlated effect within some lines. This also underlines the potential significance of founding or sampling effects during selection, modulating the options offered to selection, as well as the practical importance of having replicate populations.

In summary, lines of rapid cycling *B. rapa* responded rapidly to artificial selection for greater resistance to infection by a specialist fungal pathogen, *A. brassicicola*. Resistance is correlated with change in glucosinolate profile. Resistant lines did not incur a cost in terms of loss of seed production. I detected no correlation between resistance to *A. brassicicola* and a variety of insect herbivores.

Source	d.f.	SS	F
Block	2	2.62177	2.1510
Selection treatment	1	3.50282	7.9678*
Line [Treat]	6	2.63664	0.7211
Error	418	254.73663	

Table 3.1 Nested, mixed-model analysis of variance of disease severity score (square root transformed) between lines selected for *Alternaria brassicicola* resistance and control lines, after six generations of selection. Block and lines nested within treatment was considered a random effect and selection treatment a fixed factor. (* P<0.05, ** P<0.01, *** P<0.005)

Source	d.f.	SS	F
Block	2	6.81395	8.3076***
Selection treatment	1	14.6851	20.0550***
Line [Treat]	6	4.39252	1.7851
Error	420	172.24419	

Table 3.2 Nested, mixed-model analysis of variance of disease severity score (square root transformed) between lines selected for *Alternaria brassicicola* resistance and control lines, after seven generations of selection. Block and lines nested within treatment was considered a random effect and selection treatment a fixed factor. (* P<0.05, ** P<0.01, *** P<0.005)

	Alternaria selection lines	Control Lines
Total glucosinolates	26.709 ± 2.63	25.984 ± 2.259
3-But	26.030 ± 2.58	24.803 ± 2.145
MeProp	0.101 ± 0.043	0.244 ± 0.080
4-Pent	0.598 ± 0.095	0.621 ± 0.110
I3M	0.186 ± 0.019	0.250 ± 0.038
4MOI3M **	0.040 ± 0.015	0.172 ± 0.044
2-Phen *	0.021 ± 0.015	0.185 ± 0.068
1MOI3M	0.031 ± 0.012	0.019 ± 0.009

Table 3.3 Average glucosinolate content μmol glucosinolate/g dry weight ($\bar{x}$ ± 1 SE), pooled across all lines within a selection and control treatment. Two glucosinolates, 4MOI3M and 3-Phen, differed significantly between treatments using univariate ANOVAs. (* P<0.05, ** P<0.01, *** P<0.005)

Source	d.f.	SS	F
Selection Treatment	1	5.2526	0.0530 n.s.
Line [Treatment]	6	1397.3516	2.3516
Error	32	3169.1732	

Table 3.4 Analysis of variance of total foliar glucosinolate content (μmol glucosinolate/g leaf dry weight) of first pair of true leaves between lines selected for *Alternaria brassicicola* resistance and control lines, after seven generations of selection. Block and lines nested within treatment was considered a random effect and selection treatment a fixed factor. (* P<0.05, ** P<0.01, *** P<0.005)

	P. rapae	n	T. ni	n	P. cruciferae	N	B. brassicae	n
Alternaria Selection Lines	60.056 ± 2.57	215	44.009 ± 2.12	211	35.553 ± 1.74	211	46.204 ± 2.22	142
Control Lines	60.59 ± 2.37	204	49.028 ± 2.17	213	40.926 ± 1.84	211	45.542 ± 2.47	142

Table 3.5 Mean area consumed ($\bar{x}$ mm^2 ± 1 SE) by *P. rapae*, *T. ni*, and *P. cruciferae*, and mean colony size ($\bar{x}$ individuals ± 1 SE) of *B. brassicae* on lines selected for *A. brassicicola* resistance and control lines.

		P. rapae		*T.ni*			*P. cruciferae*			*B. brassicae*		
Source	d.f.	SS	F	d.f.	SS	F	d.f.	SS	F	d.f.	SS	F
Block	2	3.434	1.371	2	10.002	4.521*	2	3.199	2.1023	1	0.267	0.0546
Treatment	1	0.276	0.044	1	5.740	0.871	1	9.720	4.3457	2	0.591	0.1532
Line[Treat]	6	37.639	5.01***	6	39.575	5.963***	6	13.415	2.9393	6	22.906	0.7803
1st Leaf Area	1	22.308	17.81***	1	8.0721	7.297**	1	69.757	91.6963***	1	11.070	2.2627
Error	407	509.917		414	519.446		411	312.661		274	1340.576	

Table 3.6 Nested, mixed-model analyses of variance of leaf area damaged (square root transformed) by larvae of *P. rapae*, *T. ni*, *S. exigua*, and adults of *P. cruciferae*; and of mean colony size of *B. brassicae.* Lines nested within selection treatment and experimental block were considered random effects. The area of the first true leaf was included as a covariate to account for differences in total plant size. A significant treatment effect refers to differences between control lines and lines selected for increase *A. brassicicola* resistance. (* P<0.05; ** P<0.01; *** P<0.005)

Source	d.f.	SS	F
Nitrogen Level	2	4.50393	0.0602
Block [Nitrogen]	3	144.029	19.3071*
Selection Treatment	1	4.59183	0.9396
Nitrogen x Treatment	2	1.30895	0.2695
Treatment x Block [Nitrogen]	3	7.47133	2.3430
Line [Treatment]	6	20.3149	3.5688*
Nitrogen x Line [Nitrogen]	12	11.383	0.8924
1st Leaf Area	1	13.3879	12.5953***
Error	820	871.5975	

Table 3.7 Split plot analysis of leaf area damaged (square root transformed) by larvae of *T.ni* on lines of selected and control plants, across 6 blocks of plants supplied with 3 different fertilization regimes (5ppm N, 50 ppM N, and 100 ppM N). Nitrogen level and selection treatment were considered fixed factors, while block and line nested within selection treatment were considered random effects. The area of the first true leaf was included as a covariate to account for differences in total plant size. Levels of damage did not differ across nitrogen treatments, or between selected and control lines. A non-significant nitrogen level-selection treatment interaction indicates that level of damage on selected vs. control plants is uniform across the levels of fertilization assayed. (* $P<0.05$; ** $P<0.01$; *** $P<0.005$)

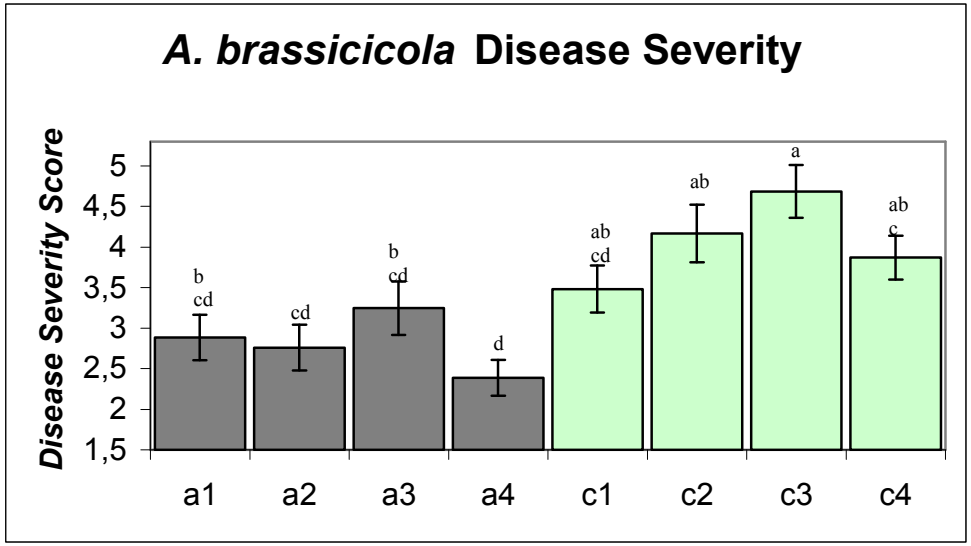

Fig. 3.1 Average disease severity score caused by *Alternaria brassicicola* ($\bar{x}$ ± 1 SE), between control lines and lines selected for seven generation for resistance to *A. brassicicola*. Selected lines were significantly more resistant to *A. brassicicola* (Table 2). Letters indicate which lines were significantly different from one another according to pair-wise comparisons using Tukey HSD tests.

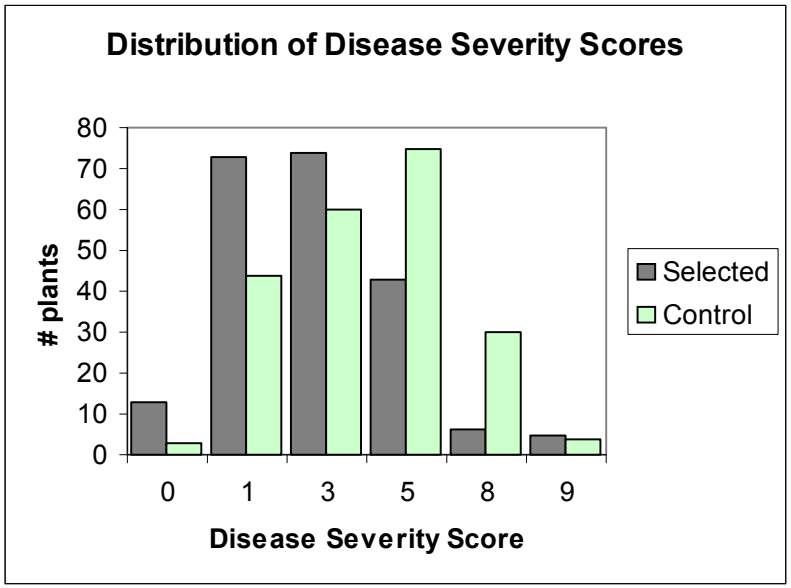

Fig. 3.2 Distribution of disease severity scores caused by *Alternaria brassicicola* (0=disease absent; 1=very low expression, a low number of small foci of disease; 3=low expression, affecting <20% of leaf area; 5=intermediate conspicuous expression, affecting 20-75% of leaf tissue; 8= high expression, covering 75-95% of tissue; 9= very high expression, affecting nearly 100% of plant). Disease data are pooled from replicate populations from each treatment, in an assay done after seven generations of artificial selection for resistance to *A. brassicicola*.

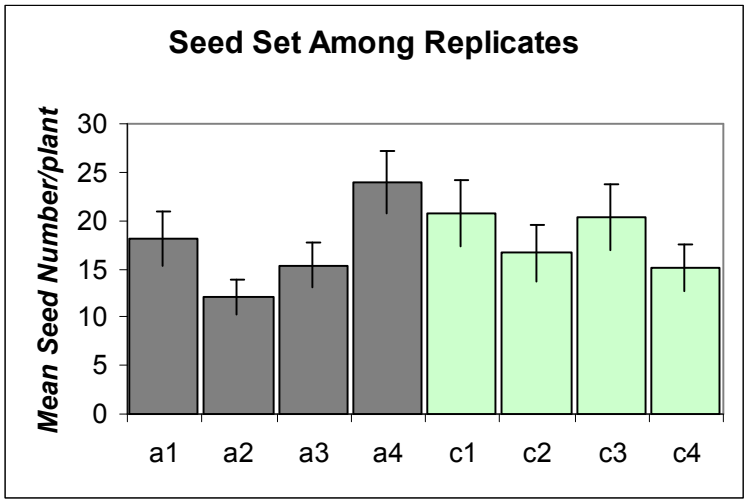

Fig. 3.3 Seed production of plants from all selected and control lines ($\bar{x}$ seed number/plant ± 1 SE), measured in the absence of the fungal pathogen. Plants were crossed with plants from same line, each plant acting both in its female and male function. There were no significant differences in seed production between treatments ($F_{1,6} = 0.0741$, P=0.7945).

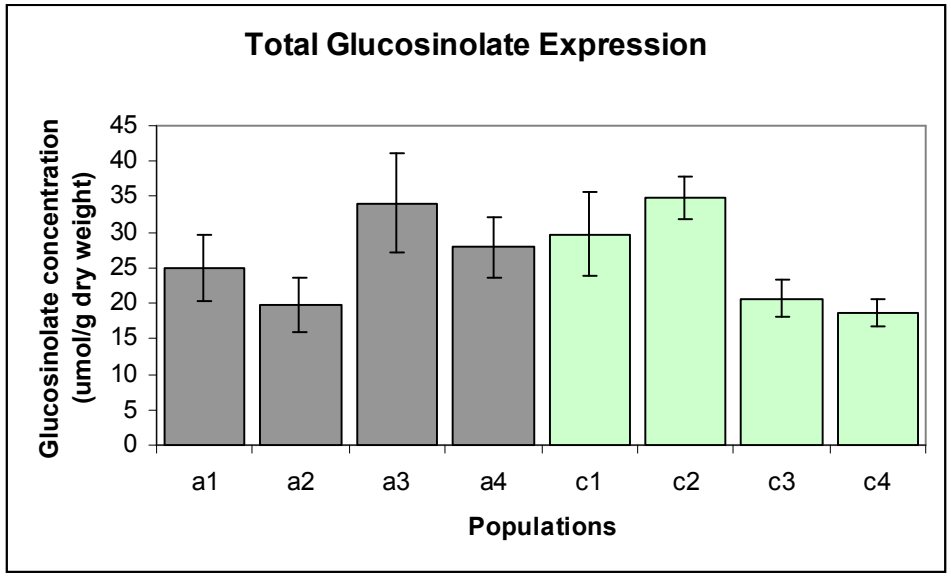

Fig. 3.4 Total glucosinolate content of each selection line ($\bar{x}$ μmol/g leaf dry weight ± 1 SE). Analysis of variance revealed no significant differences among selection treatments (see Table 4).

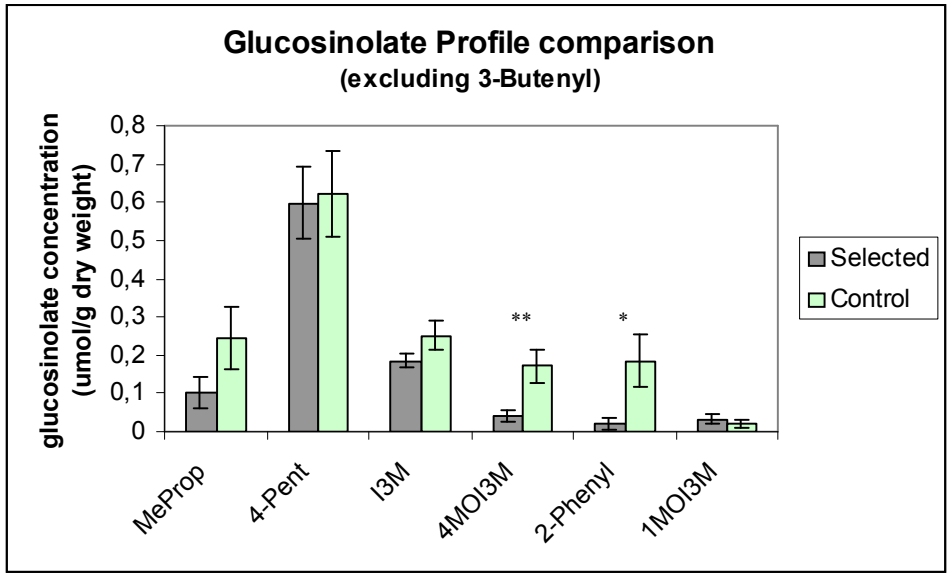

Fig. 3.5 Concentration of Methylpropyl (MeProp), 4-Pentenyl (4-pent), Indol-3-yl-methyl (I3M), 4-Methoxy-Indol-3-yl-methyl (4MOI3M), 2-Phenylethyl (2-Phen), and 1-Methoxy-Indol-3-yl-methyl glucosinolate (1MOI3M) pooled over lines within selection treatment. A sixth identified glucosinolate, 3-Butenyl (3-But), corresponded to 97% of total glucosinolate content, and its pattern of expression is closely reflected in Fig. 4. A MANOVA revealed difference between selection treatments in glucosinolate profile, and subsequent univariate analyses revealed significant differences in 4MOI3M ($F_{1,32} = 11.721$, P=0.0017) and 2-Phen content ($F_{1,32} = 6.065$, P=0.0194).

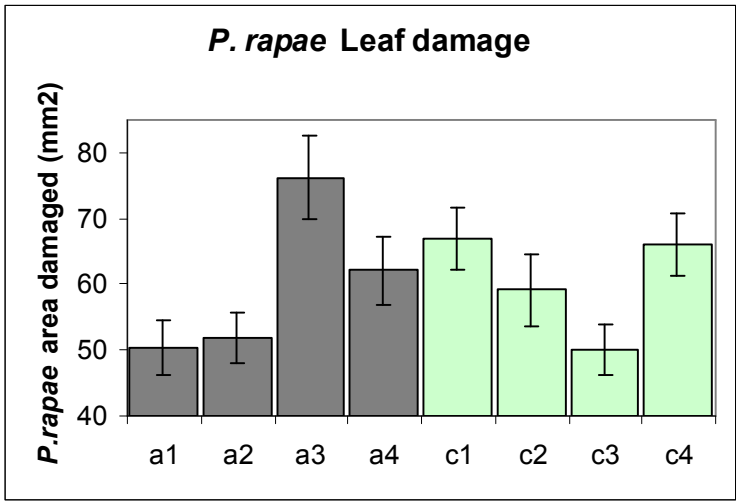

Fig. 3.6 Average leaf area damaged by first instar larvae of *Pieris rapae* ($\bar{x}$ $mm^2 \pm 1$ SE). There were no significant differences between selection and control lines ($F_{1,6} = 0.044$, $P = 0.8409$) and significant variation among lines within treatments ($F_{6,407} = 5.01$, $P < 0.005$).

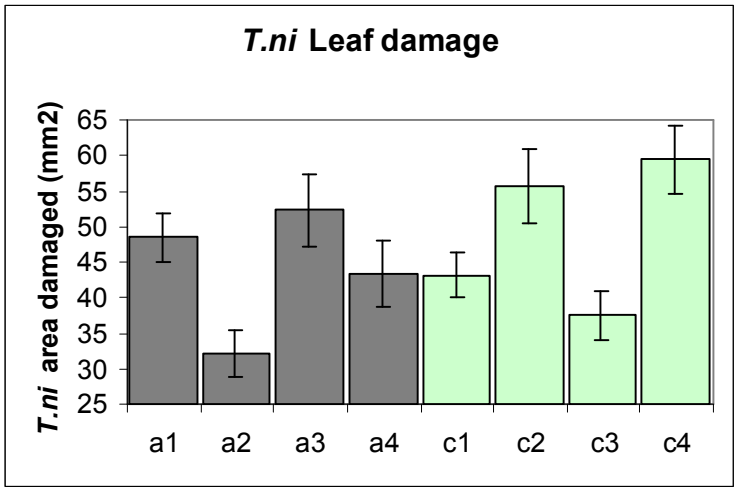

Fig. 3.7 Average leaf area damaged by first instar larvae of *Trichoplusia ni* ($\bar{x}$ $mm^2 \pm 1$ SE). There were no significant differences between selection and control lines (ANOVA, $F_{1,6} = 0.871$, $P = 3868$) and significant variation among lines within treatments ($F_{6,414} = 5.96$, $P < 0.005$).

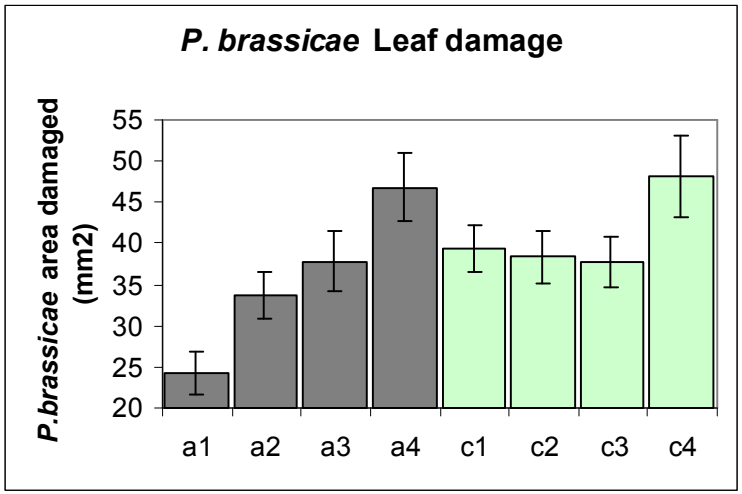

Fig. 3.8 Average leaf area damaged by adults of *Phyllotreta cruciferae* ($\bar{x}$ $mm^2 \pm 1$ SE). There were no significant differences between selection and control lines (ANOVA, $F_{1,6} = 4.3457$, $P = 0.0822$).

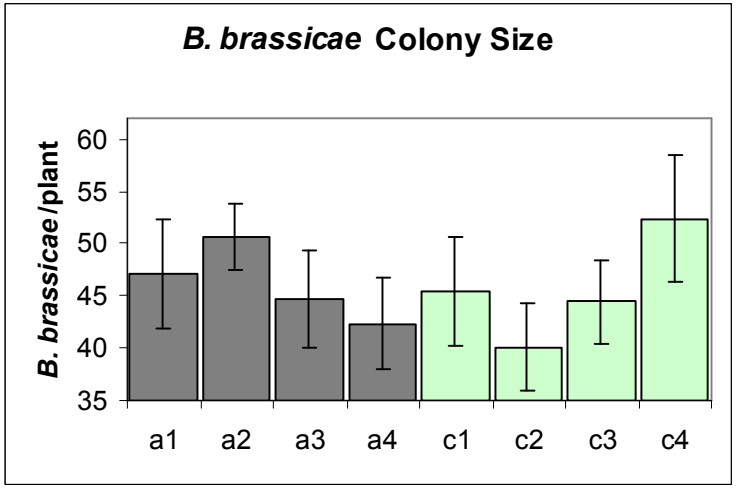

Fig. 3.9 Average colony size of B*revicoryne brassicae* ($\bar{x}$ # individuals ± 1 SE). There were no significant differences between selection and control lines (ANOVA, $F_{1,6} = 0.1532$, $P = 0.7078$).

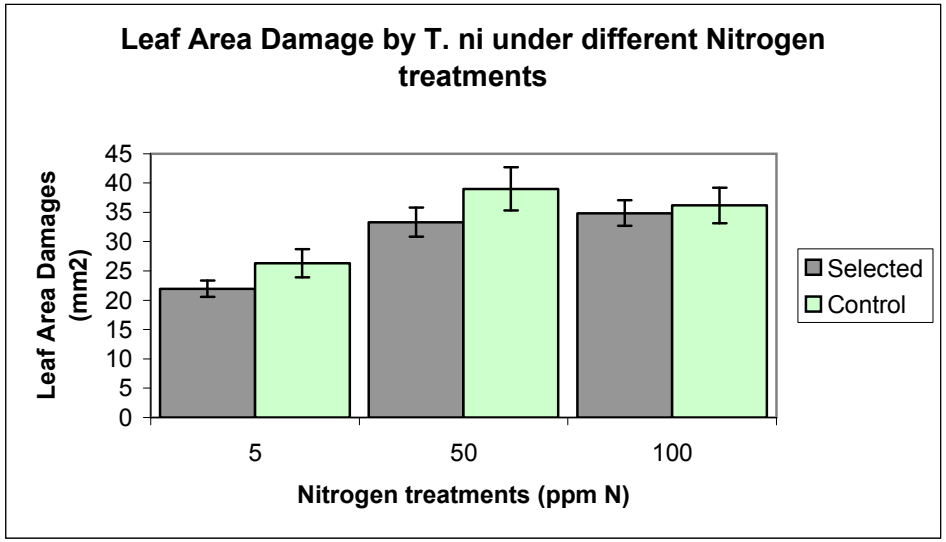

Fig. 3.10 Leaf area damaged ($mm^2 \pm SE$) by *T. ni*, during a period of 48h, of selection and control lines grown under three fertilization treatments (5, 50, and 100 ppm N).

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