Chapter 4

Comparison of induced physiological responses in Chinese cabbage towards herbivory and fungal infection

Abstract

Fungal infection of Chinese cabbage leaves by *Alternaria brassicae* has earlier been shown to have detrimental effects on larval development of the chrysomelid beetle *Phaedon cochleariae*. Furthermore, adults of this leaf beetle avoid fungus-infected cabbage leaves for oviposition and feeding. Vice versa, herbivory had no impact on fungal growth. In this study we investigated physiological responses of the host plant to herbivore attack and fungal infection in order to elucidate the mechanisms of the described ecological interactions between the fungus and the herbivore. Changes in primary (water, C/N ratio, protein, sucrose) and defence-related plant compounds (glucosinolates, anthocyanins, peroxidase) were measured. Herbivory and fungal infection reduced the sucrose concentration of leaves and increased amounts of indole glucosinolates as well as of total anthocyanins. In addition, water content was slightly lower in insect-damaged but not in infected leaves. Higher levels of peroxidase activity resulted exclusively from fungal infection. C/N ratio and total protein content remained unaffected by either treatment. The implications of the induced plant changes on the herbivore are discussed.

Key words - tripartite interactions, phytopathogenic fungus infection, herbivory, induced resistance, cross effects, nutrients, glucosinolates, anthocyanins, peroxidase, Chinese cabbage, *Phaedon cochleariae, Alternaria brassicae.*

Introduction

Plants have to cope with a multitude of attackers such as pathogenic fungi and herbivores. Damage to plant tissue caused by these antagonists may have a profound impact on host physiology (Hatcher and Ayres, 1997). The alterations of the plant's physiology may be the direct result of fungal growth (involving e.g. the release of phytotoxins and phytohormons by the parasite) or of wounding by the herbivore. But very often pathogens and herbivores also evoke a variety of active plant responses, including the induction of defensive secondary compounds and shifts in primary metabolism, so-called 'civilian responses', which may enable the plant to fend off or to tolerate attackers (Baldwin and Preston, 1999; Hammerschmidt, 1999). Certain plant responses may be induced specifically by either pathogens or herbivores while other responses lack specificity with regard to the inducing agent. Likewise, the effects of plant responses on the challenge organisms can be also specific or general (Karban and Kuc 1999; Stout and Bostock, 1999). However, little is known about the integration of plant reactions to multiple enemies in spite of a wealth of studies concerning inducible responses to either pathogens or herbivorous arthropods (Paul et al. 2000). In the field, herbivorous insects and plant pathogenic fungi frequently co-occur on the same host plant. Therefore, fungi and herbivores may interact indirectly, mediated by the induced physiological changes of the host plant. The relationship between the antagonists may be beneficial or detrimental to one or both sides (Hatcher 1995).

The ecological effects of such tripartite interactions have previously been studied in a system including the crop plant *Brassica rapa* ssp. *pekinensis* (Chinese cabbage), the leaf beetle *Phaedon cochleariae,* and the perthotrophic fungus *Alternaria brassicae* (Rostás and Hilker, submitted, a-c): Fungal growth was not affected by feeding damage caused by larvae of *P. cochleariae*. In contrast, larval performance was impaired when larvae were fed with the non-symptomatic parts of fungally infected Chinese cabbage leaves. Larvae feeding on infected Chinese cabbage leaves suffer reduced growth rates. The consumption of this type of suboptimal food drastically enhances larval susceptibility towards the entomopathogenic fungus *Metarhizium anisopliae.* The adverse effect of fungal infection on larval development, although plant-mediated, is locally restricted to leaves where *A. brassicae* is also present. However, larval performance is not affected when larvae consume Chinese cabbage leaves that were previously fed upon by conspecific larvae.

Not only the chrysomelid larvae, but also the adults were shown to respond to fungal disease of their host plant. Female beetles avoided diseased leaves for feeding and oviposition. They were also deterred by leaves that had been damaged by conspecific larvae.

The present study investigated several traits of plant primary and secondary metabolism of herbivore-damaged and fungus-infected leaves of Chinese cabbage that might be relevant for these observed ecological effects of fungus-infected leaves on larval development and adult behaviour in *P. cochleariae*. Only few have focused on a direct comparison of responses caused by both pathogens and herbivores on the same host plant (e.g. Stout et al., 1999).

In particular, we assessed: a) leaf water content, b) C/N ratio, c) total proteins, d) sucrose concentration, e) concentrations of individual glucosinolates, f) total anthocyanin concentrations, and g) peroxidase activity in herbivore-damaged and fungally infected Chinese cabbage leaves.

a) Among the various factors that can change the suitability of a host plant for insect consumers is the water content of leaves (Slansky & Scriber, 1985). Pathogenic fungi often enhance the dry matter to water ratio of the infected plant by an increase of fungal tissue, by inducing plant cell wall enforcements or by destroying the leaf epidermis (Duniway and Durban, 1971; Hatcher, 1995; Götz, 1996).

b) The carbon to nitrogen (C/N) ratio is a general indicator of food quality as it may inform about the availability of nutritionally valuable nitrogen in relation to undigestible structural carbohydrates. Growth efficiency of many insect species is correlated with plant nitrogen content (Schoonhoven et al., 1998). A high C/N ratio can therefore be detrimental to herbivores (Clancy, 1992). Shifts in carbon and nitrogen concentrations may be caused by the whole spectrum of primary and secondary plant responses following fungal infection and herbivory. These changes may be due to increased photosynthesis, re-allocation of storage compounds, the synthesis of proteins or nitrogen-containing allelochemicals as well as many other responses of the plant (Ayres 1992; Baldwin & Preston, 1999).

c) Additionally, we analysed total protein content as the most important nutrient for herbivorous insects.

d) The concentration of sucrose in herbivore-damaged and fungus-infected leaves was measured, because sucrose is known as a phagostimulant for several leaf beetles and other insects (Dadd, 1985; Matsuda, 1988).

e) In addition to sucrose, glucosinolates may stimulate feeding in crucifer specialists like the leaf beetle *P. cochleariae* (Nielsen, 1978; Wallsgrove et al. 1999; Fahey et al. 2001). However, the stimulatory effect of glucosinolates may be dose-dependent: specialists have been shown to feed less on individuals of *Brassica rapa* that contained higher glucosinolate levels than on plants of the same population with low glucosinolate concentrations (Siemens and Mitchell-Olds, 1996). Glucosinolates are known to be inducible by plant pathogenic fungi and herbivorous insects (Hopkins et al., 1998; Ludwig-Müller et al., 1998).

f) Anthocyanins belong to the large group of flavonoids and are ubiquitous in petals, stems, and leaves of angiospermes. Primarily they are known as the compounds that give flowers their red or blue colour or protect the plant against ultraviolet radiation (Harborne and Williams, 2000). However, cyanidin-3-*ß*-glucoside (chrysanthemin) has also been reported as a major factor of resistance in cotton towards *Heliothis virescens* (Hedin et al., 1983). The defensive role of proanthocyanidins (precursors of anthocyanins) against herbivores has been established (Harborne and Williams, 1995).

g) Finally, we measured the activity of soluble leaf peroxidases (POX). These are widespread plant enzymes, which use hydrogen peroxide to oxidize phenolics and other substrates. They are considered to contribute to plant defence against herbivores and fungi in several ways: products from phenolic oxidation may bind to dietary proteins and inactivate fungal proteins, damage membranes and contribute to cell wall reinforcement. Increased activity of peroxidases may be induced by herbivore damage and by pathogenic infection. However, the biological functions of peroxidases *in planta* have been difficult to verify and are not yet resolved (Constabel, 1999; Østergaard et al., 2000).

In this study, the above mentioned physiological parameters will be compared between fungus-infected and healthy plants as well as between herbivore-damaged and unattacked plants. Differences will be discussed with respect to the described ecological effects of the tripartite interactions between Chinese cabbage, the fungus *A. brassicae* and the leaf beetle *P. cochleariae.*

Materials and Methods

General

Chinese cabbage plants (*Brassica rapa* L. ssp. *pekinensis* cv. 'Kantonner') were grown from seed (obtained from Saatzucht Quedlinburg GmbH) in 7 x 8 x 7 cm plastic pots in a greenhouse at ca. 20°C. Standard potting soil (Einheitserde Typ P, Kausek GmbH and Co. KG, Mittenwalde, Germany) was used and an additional dose of fertilizer was applied once three weeks after germination. Light from 400 W sodium vapour lamps supplemented natural daylight, providing an illumination of > 10,000 lx for 16 h a day. Plants were assigned to herbivore or fungus treatment when the 5th leaf (1st leaf = oldest leaf) had reached maximum size (4 - 5 weeks). Only the 5th leaf was used for analyses.

Strain BBA 64878 of *Alternaria brassicae* (Berk.) Sacc., the causal organism of blackspot disease, was obtained from the Biologische Bundesanstalt (Berlin-Dahlem, Germany). The fungus was cultured on potato-dextrose-agar (PDA) at 20°C without induction of sporulation. Cultures used for inoculation were five days old.

A laboratory colony of *Phaedon cochleariae* (F.) was maintained in a climate chamber at 20°C, 70% r.h. and a photocycle of L18:D6. Larvae and adults were fed on Chinese cabbage leaves.

Herbivore treatment and fungal inoculation of plants

Experimental plants were selected for equal size and arranged in a completely randomised design. A clip cage (∅ 21 mm, h: 18 mm) containing five second-instar beetle larvae was attached to the 5th leaf of an experimental plant. After a period of 24 h and 48 h, respectively, the clip cage with the larvae was shifted to an undamaged part of the leaf. After a feeding period of 72 h in total and removal of about 9 % of the leaf area, the leaf was excised and used for analyses (see below). Control plants received identical treatment with an empty clip cage.

For the inoculation of Chinese cabbage with the fungus *A. brassicae* a method described by Gröntoft and O'Connor (1990) was adopted. The 5th leaves of Chinese cabbage plants were inoculated with small mycelium-containing agar discs (Ø 5 mm) punched out from behind the growth front of Petri dish cultures using a cork borer. Two discs were laid with their mycelium-containing side onto the adaxial surface of a leaf half. Upon each disc, and

underneath it on the abaxial side of the leaf, a piece of firm foam material (10 x 10 x 7 mm) was placed. An insect needle (No. 00) was pierced through inoculum, leaf, and foam material, fixing the 'sandwich' construction. Control plants were treated equally but agar discs contained no mycelium. Following inoculation, treated plants and controls were placed under a tent made of thin transparent plastic foil in a completely randomised design for 5 d. The tent ensured an atmosphere of 100% relative humidity thus enhancing fungal growth. Harvested leaves were cut along their midveins and the leaf halves bearing no symptoms of fungal infection were used for chemical analyses.

Chemical analyses

The 5th leaves of herbivore-damaged and fungally infected plants and their corresponding controls were detached and immediately submerged in liquid nitrogen. Samples were then stored at -70°C until needed for analyses. Freeze-dried leaf tissue powder was used in all analyses except for measurements of water content and POX activity. For further preparation samples were lyophilised for 3 d and ground in a planetary micromill. All spectrophotometric analyses were carried out using an UV-Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Freiburg, Germany). Samples from five plants were assessed in triplicate if not stated otherwise.

Analyses of primary compounds

a) The water content of herbivore-damaged and pathogen-infected leaves was determined from the 5th leaves of twenty plants from each treatment group. Leaves were excised and the succulent petioles and midveins were removed. The remaining laminas were weighed and then left to dry at 80°C for 2 d. Dried leaves were weighed again and percentage water content was calculated.

b) Total carbon and nitrogen content was analysed using a CHN-analyser (LECO CHN-932, Typ 600-800-532). Two milligrams of tissue powder were weighed into 50-mg tin capsules. Carbon and nitrogen concentrations were determined in duplicate.

c) Total protein content was determined after Bradford (1976) and Jones et al. (1989). Thirty milligrams of lyophilised tissue powder was added to 2 ml 0.1 N NaOH. The suspension was agitated on a vortex mixer for 3 s, left to extract for 30 min, agitated again for 3 s, and then centrifuged for 5 min at 12,000*g*. The supernatant was decanted and vortex mixed for 3 s. Aliquots of 100 µl of the supernatant or 100 µl of 0.1 N NaOH (blank) were

each mixed with 1 ml Bradford dye reagent to which 3 mg/ml soluble polyvinylpyrollidone (PVP; MW: 40,000) had been added. The absorbance of the leaf extract was read at 595 nm against the dye reagent/NaOH blank. Bovine serum albumin was used as a standard.

d) Sucrose concentrations of treated and control Chinese cabbage leaves were determined enzymatically. A commercial test kit was used (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, no. 716 260). Thirty milligrams of lyophilised tissue powder were extracted for 1 h in 2 ml aq. bidest. The homogenate was then centrifuged for 15 min at 12,000*g* and the supernatant transferred into 1.5-ml vials. Enzymatic hydrolysis of sucrose was done according to the test kit manual except that only a quarter of the suggested quantities of all buffers and enzyme suspensions was used. Extinction was read at 340 nm.

Analysis of secondary compounds

e) Glucosinolates were extracted and measured as described by Kiddle et al. (2001). Essentially samples (3 x 40 mg for each sample) of tissue powder were extracted with v/v MeOH. The extract was bound to pre-activated Sephadex A-25, and the glucosinolates were desulfated with purified *Helix* sulfatase for at least 16 h. The desulfo-glucosinolates were eluted with water and stored at –20°C until analysed by reverse-phase HPLC (Kiddle et al., 2001). Sinigrin was used as the internal standard.

f) Relative concentrations of total anthocyanins were analysed colorimetrically (Manicelli et al. 1984). Tissue powder (3 x 10 mg for each sample) were vortex mixed with 1 ml of 1% v/v HCL in 100% MeOH in a screw-top microtube. Samples were centrifuged at 4°C for 10 min at 17,000*g*. The supernatant was then carefully removed and absorbance of anthocyanins was measured at 530 nm. A second reading at 657 nm assessed contaminating chlorophylls that absorb in the 530 nm region. Anthocyanin specific absorption unit was $AU = A_{530}-A_{657}$.

g) To assay peroxidase (POX) activity, 1 g of fresh foliage was homogenised in 2 ml of 50 mM ice-cold sodium phosphate buffer (pH 7.0, containing 0.5 mM EDTA, 5 mM sodium-metabisulfite, and 40 ml/l protease inhibitor cocktail, Sigma, Deisenhofen, Germany, P 2714). Acid-washed sand (100-200 mg/g tissue) in the mortar improved homogenisation. The homogenate was added to 1.25% w/v polyvinylpolypyrrolidone (PVPP) and 1.25% w/v amberlite XAD-4 in a beaker (25 ml) and left on ice for 5 min. The homogenate was then

filtered through a muslin cloth into a pre-chilled centrifuge tube and centrifuged at 4°C for 20 min at 14,000*g*. The supernatant was removed and immediately used as the enzyme source. A 50-µl aliquot of foliar homogenate was assayed with 10 mM 10 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) and 10 mM H_2O_2 (30% v/v) in 50 mM NaH_2PO_4 (pH 6.0). POX activity was estimated at 414 nm and 25°C.

Statistics

Student's *t*-tests were used to analyse differences between herbivore-damaged or fungus-infected leaves and their corresponding controls. Due to differing treatments (see above) statistical testing was not performed between herbivore-damaged and fungus-infected leaves.

Table 1 *Primary compounds measured in herbivore-damaged and fungally infected Chinese cabbage leaves and their respective controls. 'Herbivory' = leaves damaged by second-instar larvae of* Phaedon cochleariae *for 72 h. 'Fungus' = leaves 5 days after infection with* Alternaria brassicae. *n = 5 (water content: n = 20). Values represent means and standard deviations (in parenthesis). Asterisks denote significant differences (***P < 0.001, *P < 0.05; Student's t-test for independent samples).*

	Treatment			
	Herbivory	Control	Fungus	Control
Water content [%]	89.8 (1.59)***	91.4 (0.68)	91.6 (0.81)	91.7 (0.63)
C/N	6.6 (0.54)	7.8 (1.29)	6.1 (0.82)	6.0 (0.24)
Total protein [mg/g foliar dry weight]	61 (4.0)	59 (2.3)	57 (6.7)	57 (7.7)
Sucrose [g/100g foliar dry weight]	1.5 (0.34)*	2.2 (0.61)	1.2 (0.52)*	2.2 (0.92)

Results

Analyses of primary compounds (Table 1)

a) Water content of Chinese cabbage leaves significantly decreased after three days of herbivory by second-instar larvae of *P. cochleariae*. However, the reduction did not exceed 2%. In contrast, leaves infected by *A. brassicae* did not differ ($P = 0.847$) in their mean water content from healthy control leaves.

b) The C/N ratio did not significantly differ in leaves receiving either herbivore ($P = 0.096$) or fungus ($P = 0.835$) treatment when compared with their respective controls.

c) Total protein contents of Chinese cabbage leaves did not significantly change as a consequence of herbivore or fungal activity (herbivore: $P = 0.376$; fungus: $P = 0.773$).

d) Herbivory and plant pathogenic infection reduced sucrose content as indicated by a 31% loss of foliar sucrose in case of herbivore-damaged leaves and a 47% decrease in pathogen-infected leaves. In both treatments the reduction was significant (herbivore: $P = 0.018$; fungus: $P = 0.035$).

Table 2 *Secondary compounds measured in herbivore-damaged and fungally infected Chinese cabbage leaves and their respective controls. 'Herbivory' = leaves damaged by second-instar larvae of* Phaedon cochleariae *for 72 h. 'Fungus' = leaves 5 days after infection with* Alternaria brassicae. *n = 5. Values represent means and standard deviations (in parenthesis). Asterisks denote significant differences (***P < 0.001; Student's t-test for independent samples).*

	Treatment			
	Herbivory	Control	Fungus	Control
Rel. anthocyanin conc. [AU x 10^3]	271 (31.4)***	36 (6.1)	173 (35.3)***	34 (4.9)
POX [dA_{414} x 10^2/min/g foliage]	326 (106.6)	356 (106.2)	1482 (418.0)***	544 (148.8)

Analyses of secondary compounds (Table 2, Fig. 1)

e) Production of glucosinolates was induced in herbivore-damaged and in fungus-infected leaves (Fig. 1). Chinese cabbage leaves on which larvae of *P. cochleariae* had fed upon showed a 2.6-fold increase in total glucosinolate content compared with untreated control leaves ($P < 0.001$). Glucosinolate content of leaves infected with the fungus *A. brassicae* was

5.9 times higher than of healthy tissue ($P < 0.001$). This effect was entirely due to an increase in the concentrations of indole glucosinolates (Fig. 1). Glucobrassicin (3-indole methylglucosinolate) was 12.3 times higher in herbivore-damaged leaves while the increase in fungus-infected leaves was 11.6-fold (herbivore: $P = 0.004$; fungus: $P = 0.008$). An accumulation of 4-MeO-glucobrassicin (4-MeO-indole methylglucosinolate) was observed in

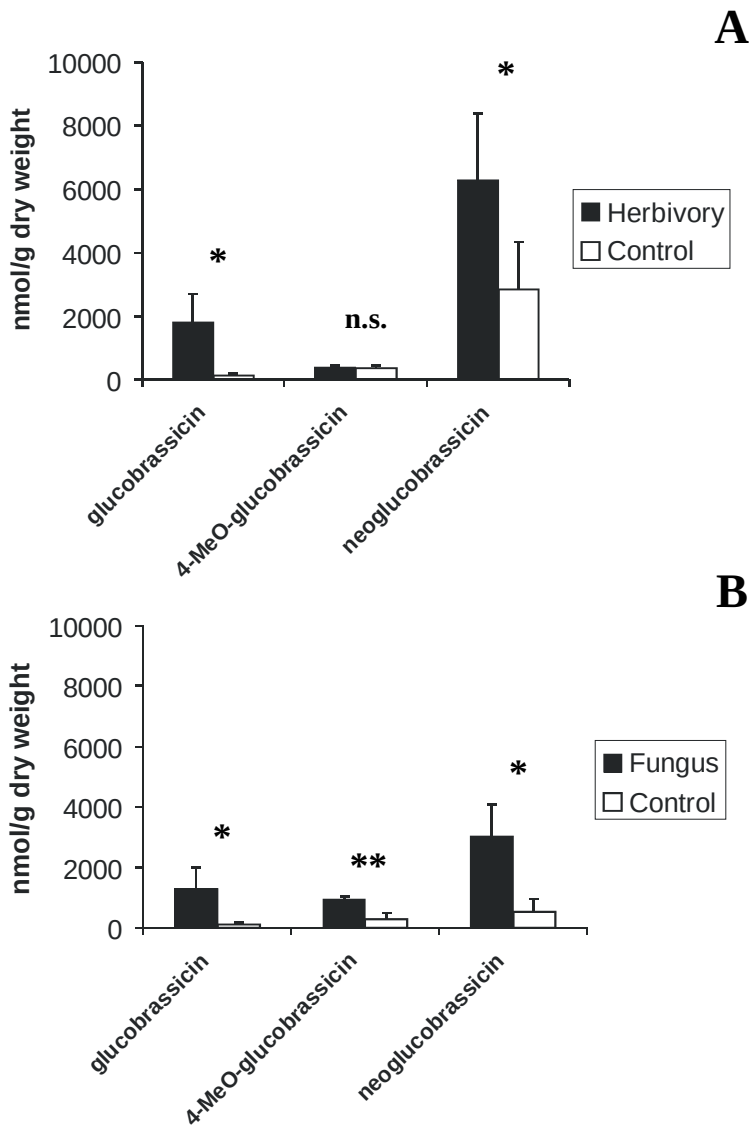

Figure 1 *Concentrations of indole glucosinolates in herbivore-damaged* (**A**) *and fungally infected* (**B**) *Chinese cabbage leaves. 'Herbivory' = leaves damaged by second-instar larvae of* Phaedon cochleariae *for 72 h. 'Fungus' = leaves 5 days after infection with* Alternaria brassicae. *n= 5. Values represent means and standard deviations. Asterisks denote significant differences (***P < 0.001, **P < 0.01, *P < 0.05, n.s. = not significant; Student's t-test for independent samples).*

leaves treated with the fungus (3.5-fold, $P = < 0.001$). In herbivore-damaged leaves a slight increase of about 10% was measured, however, this was no significant difference from the control ($P = 0.693$). Elevated concentrations of neoglucobrassicin (N-MeO-3-indole

methylglucosinolate) were found in both treatment groups (herbivore: 2.2-fold, $P = 0.027$; fungus: 5.9-fold, $P = 0.002$). Aliphatic glucosinolates were measured only in trace amounts and these were not affected by either treatment. Concentrations of aromatic glucosinolates were below detectability.

f) Total anthocyanin content was 7.5 times higher in leaves affected by herbivory when compared with undamaged controls ($P < 0.001$). An increase in the anthocyanin concentration by the factor 5.1 was observed in fungus-infected leaves ($P < 0.001$) (Table 2).

g) Activity of POX assayed with ABTS as the hydrogen donor differed in herbivore and fungus treated plants (Table 2). Previous feeding by the herbivore did not result in increased POX activity ($P = 0.767$) in Chinese cabbage leaves. However, POX activity was significantly enhanced in leaves infected by the fungus compared to the control treatment (2.7-fold, $P < 0.001$).

Discussion

In this study we could show that herbivory and fungal infection partly induced similar but also differential responses in leaves of Chinese cabbage.

The water content of leaves on which larvae of *P. cochleariae* had fed for 3 d was significantly reduced. It seems obvious that wounding of leaf tissue leads to an increased loss of water. However, this reduction (-1.6%) might be negligible as the total water content of healthy Chinese cabbage leaves was high (> 90%, Tab. 1). Consequently, no signs of wilting were observed. Fungally infected leaves, on the other hand, did not differ in their water content from healthy leaves. This is consistent with studies that measured the leaf water content of fungally infected plants in conjunction with herbivore consumption (Kingsley et al., 1983; Hatcher et al., 1994, 1995).

No shifts in the C/N ratio of Chinese cabbage leaves were observed following herbivory or fungal infection. The carbon and nitrogen contents were comparable to results obtained from analyses of commercially grown Chinese cabbage plants (Müller, 1999). Likewise, neither of both treatments altered the total content of proteins in the leaves. Contrasting this, levels of sucrose concentration were lower in both wounded and infected leaves. It is conceivable that in case of herbivore-damaged leaves the decrease in sucrose concentration might be a genuine

plant response. Exporting sucrose may be a mechanism for re-allocation of valuable nutrients to other unaffected plant parts for compensatory growth. This has also been hypothesised for the observed reduction in the concentrations of total free sugars in herbivore-damaged oak leaves (Valentine et al., 1983). The lower sucrose content of the uninfected half of a fungus-infected leaf is most likely due to an altered sink-source relationship caused by the pathogen. Köhle (1989) showed that radiolabelled ^{14}C-sucrose accumulated only in those halves of oilseed rape (*Brassica napus*) leaves that were infected with *Alternaria brassicicola*. Changes in leaf nutrients caused by the activity of herbivores and fungi may vary greatly depending on several factors as e.g. the timing and intensity of feeding damage or infection and the system that has been studied (Hatcher, 1995; Karban and Baldwin, 1997). An increase in carbohydrates other than sucrose, in wounded and infected leaves, may have contributed to the stabile C/N ratio after pathogen or herbivore attack.

Following herbivory and fungal infection, a distinct rise of the concentrations of the indole-glucosinolates glucobrassicin, 4-MeO-glucobrassicin, and neoglucobrassicin was observed. Constitutive levels of glucosinolates were higher in control plants of the 'herbivore' treatment group than in the 'fungus' treatment group. This was probably due to different treatment conditions. Plants inoculated with *A. brassicae* were kept in a tent with higher humidity and higher temperatures. However, a more pronounced induction of glucosinolates was observed in the infected plants. Differing responses were found with regards to 4-MeO-glucosinolate: this compound was induced in leaves infected with the fungus, but not in plants damaged by herbivory. Koritsas et al. (1991) demonstrated very similar effects in the stem tissue of *B. napus* cv. Ariana that had been damaged by the chrysomelid beetle *Psylliodes chrysocephala*. Aerial infestation stimulated the production of glucobrassicin (6-fold), 4-methoxy-glucobrassicin (2-fold), and neoglucobrassicin (23-fold), while levels of aliphatic and aromatic glucosinolates remained too low to be detected. Accumulation of glucosinolates was also observed in leaves of *B. napus* (cv. Cobra, cv. Bienvenue) following *A. brassicae*-infection (Doughty et al., 1991). The increase was mainly the result of higher levels of indole and aromatic glucosinolates, while concentrations of aliphatic glucosinolates were variable.

Chinese cabbage leaves responded to feeding damage and fungal infection, respectively, by raising the levels of total anthocyanins. Accumulation of anthocyanins is induced by a range of stimuli including UV light, low temperature, phytohormones, and pathogens (Lo and

Nicholson, 1998). Interestingly, six-carbon (C_6-) volatiles and methyl jasmonate, which are released by insect-damaged plant tissues, have also been demonstrated to induce the production of anthocyanins (Franceschi and Grimes, 1991; Bates and Rothstein, 1998). These volatiles play a role in the plant's defence response against herbivores and may activate the expression of phenylpropanoid-related genes and the production of proteinase inhibitors (Karban and Baldwin, 1997; Bates and Rothstein, 1998).

A significant increase in the activity of POX in the uninfected half of *A. brassicae*-infected leaves could be observed. This contrasted with results obtained from Chinese cabbage leaves damaged by *P. cochleariae,* where no significant difference between treated and control leaves was apparent. In one of the few studies investigating the specificity of host plant responses to herbivores and pathogens the same pattern was evident (Stout et al., 1999). Tomato plants inoculated with the pathogenic fungus *Phytophthora infestans* had increased POX activities. Wounding by *Helicoverpa zea,* on the other hand, showed no such effects. However, the induction of POX by insect feeding is not uncommon and has been demonstrated in tomato by other insects and also in cotton (Bi et al., 1997; Stout et al., 1998). Enhanced POX activity due to infection by *A. brassicae* or *A. brassicicola* was measured in *B. juncea* with higher activities found in susceptible cultivars (Gupta et al., 1990).

Considering the observed phytochemical responses, what can be inferred from our results that might explain the adverse effects of plant pathogenic fungus infection on the larval performance of *P. cochleariae*? Firstly, there were no changes in the nutrient status of leaves except for a decrease in sucrose concentration as a result of fungal infection. Secondly, fungal infection invoked higher levels of indole glucosinolates, anthocyanins and POX activity. When interpreting these data it is important to be aware that the larval performance of *P. cochleariae* is not affected when larvae consume leaves that were previously fed upon by conspecific larvae (Rostás & Hilker, submitted, b). Consequently, one might suggest that all plant responses equally induced by the beetle *and* the fungus may be excluded as factors responsible for reduced host plant quality, i.e. changes of the concentrations of sucrose, anthocyanins, glucobrassicin, and neo-glucobrassicin (Fig. 2) In this study, the compounds exclusively induced by *A. brassicae* are 4-MeO-glucobrassicin and POX. However, it seems unlikely that 4-MeO-glucobrassicin can be considered as a resistance factor, since *P. cochleariae* is a crucifer specialist and therefore is expected to be able to detoxify

glucosinolates (Louda & Mole 1991). Enhanced POX activity, on the other hand, is more likely to have contributed to an increased resistance of fungally infected leaves against the herbivore. In cotton, POX, among other oxidative components, was discussed as a contributing factor in resistance against herbivores (Bi et al., 1997). Likewise, over-expression of POX in transgenic tobacco leaves conferred resistance against *Helicoverpa zea* (Dowd and Lagrimini, 1997).

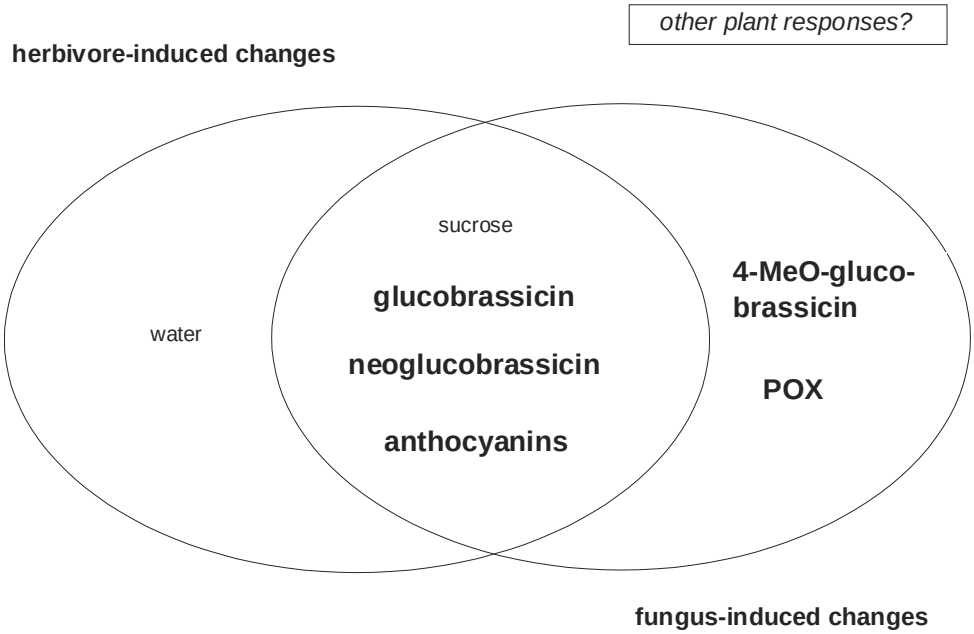

Figure 2 *Overlapping and differential responses of Chinese cabbage leaves due to feeding damage caused by* Phaedon cochleariae *and fungal infection with* Alternaria brassicae. *Compounds written in large bold (small non-bold) letters showed significant increases (decreases) in their concentrations due to herbivory or fungal infection compared with corresponding controls.*

Which of the measured physiological factors could have affected the behavioural response of adult *P. cochleariae* towards fungus-infected cabbage leaves and may be relevant for the deterrent effect of diseased leaves? When trying to answer this question, it must be considered that adult *P. cochleariae* are not only deterred by fungus-infected leaves, but also by leaves that had been damaged by conspecific larvae (Rostás and Hilker, submitted, c). This implies that, although there is some overlap of the plant responses to herbivory and fungal infection (Fig. 2), none of the observed physiological changes can be excluded as putative resistance factors. Two different resistance mechanisms, maybe with a certain degree of overlap, may have elicited the same reaction in the beetles when confronted with fungus-infected or herbivore-damaged leaves.

Even though the potential role of POX as a factor reducing host plant suitability to *P. cochleariae* is advocated here (see above), it is important to emphasize that plant defences against herbivores and pathogens are complex phenomena. Certainly, an array of yet unmeasured factors, as e.g. fungal toxins, proteinase inhibitors or phytoalexins, may also have contributed to the resistance effects of the fungus-infected Chinese cabbage plants on the leaf beetle (Bains &Tewari, 1987; Baur et al., 1998; Lim et al., 1996). Future research involving feeding experiments with isolated inducible phytochemicals or transgenic plants will have to further elucidate the underlying mechanisms of plant-mediated cross-effects between *A. brassicae* and *P. cochleariae*.

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References

Ayres, P. G. 1992. Pests and Pathogens - Plant Responses to Foliar Attack. Bios Scientific Publishers, Oxford.

Bains, P. S. and Tewari, J. P. 1987. Purification, chemical characterization and host-specifity of the toxin produced by *Alternaria brassicae*. *Physiol. Mol. Plant Pathol.* 30:259-271.

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

Baldwin, I. T. and Preston, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137-145.

Bates, N. J. and Rothstein, S. J. 1998. C_6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. *The Plant J.* 16:561-569.

Bi, J. L., Murphy, J. B., and Felton, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. *J. Chem. Ecol.* 23:97-117.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Clancy, K. M. 1992. The role of sugars in western spruce budworm nutritional ecology. *Ecol. Entomol.* 17:189-197.

Constabel, C. P. 1999. A survey of herbivore-inducible defensive proteins and phytochemicals, pp. 137-166 *in* A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture. APS Press, St. Paul.

Dadd, R. H. 1985. Nutrition: Organisms, pp. 313-390 *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 4, Regulation: Digestion, Nutrition, Excretion. Pergamon, Oxford.

Doughty, K. J., Porter, A. J. R., Morton, A. M., Kiddle, G., Bock, C. H., and Wallsgrove, R. 1991. Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.) leaves. II. Response to infection by *Alternaria brassicae* (Berk.) Sacc. *Ann. Appl. Biol.* 118:469-477.

Dowd, P.F. and Lagrimini, L.M. 1997. Examination of different tobacco (*Nicotiana* spp.) types under- and overproducing tobacco anionic peroxidase for their leaf resistance against *Helicoverpa zea*. *J. Chem. Ecol.* 23:2357-2370.

Duniway, J. M. and Durbin, R. D. 1971. Some effects of *Uromyces phaseoli* on the transpiration rate and stomatal response of bean leaves. *Phytopathology* 61:409-411.

Fahey, J. W., Zalcman, A. T., and Talalay, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5-51

Franceschi, V. R. and Grimes, H. D. 1991. Induction of soybean vegetative storage proteins and anthocyanins by low-level atmospheric methyl jasmonate. *Proc. Nat. Acad. Sci. USA* 88:6745-6749.

Götz, M. 1996. Zur vegetativen und generativen Entwicklung der obligat biotrophen Parasiten in den Pathosystemen *Triticum aestivum/Blumeria graminis* und *Phaseolus vulgaris/Uromyces appendiculatus*. Ph.D. Thesis, Technische Universität Braunschweig.

Gupta, S. K., Gupta, P. P., Yadava, T. P., and Kaushik, C. D. 1990. Metabolic changes in mustard due to alternaria leaf blight. *Indian Phytopathol.* 43:64-69.

Gröntoft, M. and O'Connor, D. 1990. Greenhouse method for testing of resistance of young *Brassica* plants to *Alternaria brassicae*. *Plant Breed.* 105:160-164.

Hammerschmidt, R. 1999. Induced disease resistance: how do induced plants stop pathogens? *Physiol. Mol. Plant Pathol.* 55:77-85.

Harborne, J.B. and Williams, C.A. 1995. Anthocyanins and other flavonoids. *Natur. Prod. Rep.* 12:639-657.

Harborne, J. B. and Williams, C. A. 2000. Advances in flavonoid research since 1992. *Phytochemistry* 55:481-504.

Hatcher, P. E. 1995. Three-way interactions between plant pathogenic fungi, herbivorous insects and their host plants. *Biol. Rev.* 70:639-694.

Hatcher, P. E. and Ayres, P. G. 1997. Indirect interactions between insect herbivores and pathogenic fungi on leaves, pp. 133-149 *in* A. C. Gange and V. K. Brown (eds.). Multitrophic Interactions in Terrestrial Systems. Blackwell Science, Oxford.

Hatcher, P. E., Ayres, P. G., and Paul, N. D. 1995. The effect of natural and simulated insect herbivory, and leaf age, on the process of infection of *Rumex crispus* L. and *R. obtusifolius* L. by *Uromyces rumicis* (Schum.) Wint. *New Phytol.* 130:239-249.

Hatcher, P.E., Paul, N.D., Ayres, P.G., and Whittaker, J.B. 1994. The effect of a foliar disease (rust) on the development of *Gastrophysa viridula* (Coleoptera: Chrysomelidae). *Ecol. Entomol.* 19:349-360.

Hedin, P. A., Jenkins, J. N., Collum, D. H., White, W. H., Parrott, W. L., and MacGown, M. W. 1983. Cyanidin-3-β-glucoside, a newly recognized basis for resistance in cotton to the tobacco budworm *Heliothis virescens* (Fab.) (Lepidoptera: Noctuidae). *Experientia* 39:799-801.

Hopkins, R. J., Griffiths, D. W., Birch, A. N. E., and McKinley, R. G. 1998. Influence of increasing herbivore pressure on modification of glucosinolate content of swedes (*Brassica napus* spp. *rapifera*). *J. Chem. Ecol.* 24:2003-2019.

Jones, C. G., Hare, J. D., and Compton, S. J. 1989. Measuring plant protein with the Bradford assay. *J. Chem. Ecol.* 15:979-992.

Karban, R. and Baldwin, I.T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

Karban, R. and Kuc, J. 1999. Induced resistance against pathogens and herbivores: an overview, pp. 1-15 *in* A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses against Pathogens and Herbivores. APS Press, St. Paul.

Kiddle, G. A., Bennett, R. N., Botting, N. P., Davidson, N. E., Robertson, A. A. B., and Wallsgrove, R. M. 2001. High performance liquid-chromatography separation of natural and synthetic desulfoglucosinolates and their chemical validation by spectroscopic, NMR, and CI-MS methods. *Phytochem. Methods* 12:226-242.

Kingsley, P., Scriber, J. M., Grau, C. R., and Delwiche, P. A. 1983. Feeding and growth performance of *Spodoptera eridania* (Noctuidae: Lepidoptera) on 'vernal' alfalfa as influenced by *Verticillium* wilt. *Prot. Ecol.* 5:127-134.

Koritsas, V. M., Lewis, J. A., and Fenwick, G. R. 1991. Glucosinolate response of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (*Psylliodes chrysocephala*). *Ann. Appl. Biol.* 118:209-221.

Köhle, H. 1989. Untersuchungen zur Physiologie des *Alternaria*-Befalls von Raps. *Z. Pflanzenkrankh. Pflanzensch.* 96:225-238.

Lim, C. O., Lee, S. I., Chung, W. S., Park, S. H., Hwang, I., and Cho, M. J. 1996. Characterization of a cDNA encoding cystein proteinase inhibitor from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) flower buds. *Plant Mol. Biol.* 30:373-379.

Lo, S. C. and Nicholson, R. L. 1998. Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. *Plant Physiol.* 116:979-989.

Louda, S. and Mole, S. 1991. Glucosinolates: chemistry and ecology, pp. 123-164 *in* G.A. Rosenthal and M.R. Beerenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, San Diego.

Ludwig-Müller, J., Schubert, B., Pieper, K., Ihmig, S., and Hilgenberg, W. 1997. Glucosinolate content in susceptible and resistant Chinese cabbage varieties during development of clubroot disease. *Phytochemistry* 44:407-414.

Manicelli, A. L. 1984. Photoregulation of anthocyanin synthesis. *Plant Physiol.* 75:447-453.

Matsuda, K. 1988. Feeding stimulants of leaf beetles, pp. 41-56 *in* P. Jolivet, E. Petitpierre, T.H. Hsiao (eds.). Biology of Chrysomelidae. Kluwer Academic Publishers, Dordrecht.

Müller, C. 1999. Chemische Ökologie des Phytophagenkomplexes an *Tanacetum vulgare* L. (Asteraceae). Ph.D Thesis, Freie Universität Berlin.

Nielsen, J. K. 1978. Host plant discrimination within Cruciferae: feeding responses of four leaf beetles (Coleoptera: Chrysomelidae) to glucosinolates, cucurbitacins and cardenolides. *Entomol. Exp. Appl.* 24:41-54.

Østergaard, L., Teilum, K., Mirza, O., Mattsson, O., Petersen, M., Welinder, K. G., Mundy, J., Gajhede, M., and Henriksen, A. 2000. *Arabidopsis* ATP A2 peroxidase. Expression and high resolution structure of a plant peroxidase with implications for lignification. *Plant Mol. Biol.* 44:231-243.

Paul, N. D., Hatcher, P. E., and Taylor, J. E. 2000. Coping with multiple enemies: an integration of molecular and ecological perspectives. *Trends Plant Sci.* 5:220-225.

Rostás, M. and Hilker, M. in press. Asymmetric plant-mediated cross-effects between a herbivorous insect and a phytopathogenic fungus. *Agri. Forest Entomol.*

Rostás, M. and Hilker, M. submitted, a. Indirect interactions between a phytopathogenic and an entomopathogenic fungus in support of the slow-growth, high-mortality hypothesis.

Rostás, M. and Hilker, M. submitted, b. Feeding damage by larvae of the mustard leaf beetle deters conspecific females from oviposition and feeding.

Schoonhoven, L. M., Jermy, T., and van Loon, J. J. A. 1998. Insect-Plant Biology. Chapman & Hall, London.

Siemens, D. H. and Mitchell Olds, T. 1996. Glucosinolates and herbivory by specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): Consequences of concentration and induced resistance. *Environ. Entom.* 25:1344-1353.

Slansky, F. and Scriber, J. M. 1985. Food consumption and utilization, pp. 88-163 *in* G.A. Kerkut and L.I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 4, Regulation: Digestion, Nutrition, Excretion. Pergamon, Oxford.

Stout, M. J. and Bostock, R. M. 1999. Specificity of induced responses to arthropods and pathogens, pp. 183-209 *in* A. A. Agrawal, S. Tuzun, E. Bent (eds.). Induced Plant Defenses Against Pathogens and Herbivores. APS Press, St.Paul.

Stout, M. J, Fidantsef, A. L., Duffey, S. S., and Bostock R. M. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiol. Mol. Plant Pathol.* 54:115-130.

Stout, M. J., Workman, K. V., Bostock, R. M., and Duffey, S. S. 1998. Stimulation and attenuation of induced resistance by elicitors and inhibitors of chemical induction in tomato (*Lycopersicon esculentum*) foliage. *Entomol. Exp. Appl.* 86:267-279.

Valentine, H. T., Wallner, E., and Wargo, P. M. 1983. Nutritional changes in host foliage during and after defoliation, and their relation to the weight of gypsy moth pupae. *Oecologia* 57:298-302.

Wallsgrove, R. M., Doughty, K., and Bennett, R. N. 1999. Glucosinolates, pp. 523-562 *in* B. K. Singh (ed.). Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker Inc., New York.