The role of the glucosinolate-myrosinase system for the interaction of Brassicaceae with the turnip sawfly *Athalia rosae* (L.)

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Chapter 1

Introduction

Vascular plants are in general immobile and tied to their immediate environment. In contrast to mobile animals, changes within the habitat need to be coped with directly at the site. Plants therefore require plastic mechanisms of defence against abiotic and biotic stresses (Rosenthal and Berenbaum, 1991). Unpalatability towards insects, one major group of herbivores, is achieved *via* morphologic characters, e.g. trichomes or leaf toughness, or *via* defensive secondary metabolites. The chemical nature of these bioactive compounds varies greatly: from alkaloids, over terpenoids and cyanogenic glycosides to glucosinolates (Rosenthal and Berenbaum, 1992). Furthermore, plants can apply phenological or environmental escape mechanisms, e.g. high regrowth capacity, simultaneous leaf expansion, or occurrence in habitats with low densities of herbivores.

In this study the interaction of Brassicaceae with the turnip sawfly *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) is examined with emphasis on the glucosinolate-myrosinase system. The larvae of this herbivorous sawfly are specialised to feed on glucosinolate-containing crucifers. Short-term impacts of larval feeding on the physiology of white mustard (*Sinapis alba* L.), the responses' specificity and their potential defence effects are investigated. Furthermore, the defensive properties of seven plant species are evaluated with regard to nutrition, mechanical and chemical defence and their individual and combined importance for the long-term performance and preference of *A. rosae*.

1.1 The glucosinolate-myrosinase system

The binary glucosinolate-myrosinase system is a very prominent plant defence mechanism (Louda and Mole, 1991). Glucosinolates, once known as mustard oil glucosides, are amino

acid derived compounds which are hydrolysed upon tissue damage by the enzymes myrosinases (Figure 1.1). The resulting breakdown products, called mustard oils, are mostly toxic (Wittstock *et al.*, 2003). The system is primarily found in the plant order Brassicales and especially within the family Brassicaceae (Rodman, 1991). Within this clade a monophyletic origin is assumed including 15 plant families, e.g. Capparaceae, Caricaceae, and Tropaeolaceaee. However, the genus *Drypetes* (Euphorbiaceae) contains species which produce glucosinolates and thus this particular defence mechanism was at least developed twice during the evolution of terrestrial plants (Rodman *et al.*, 1998).

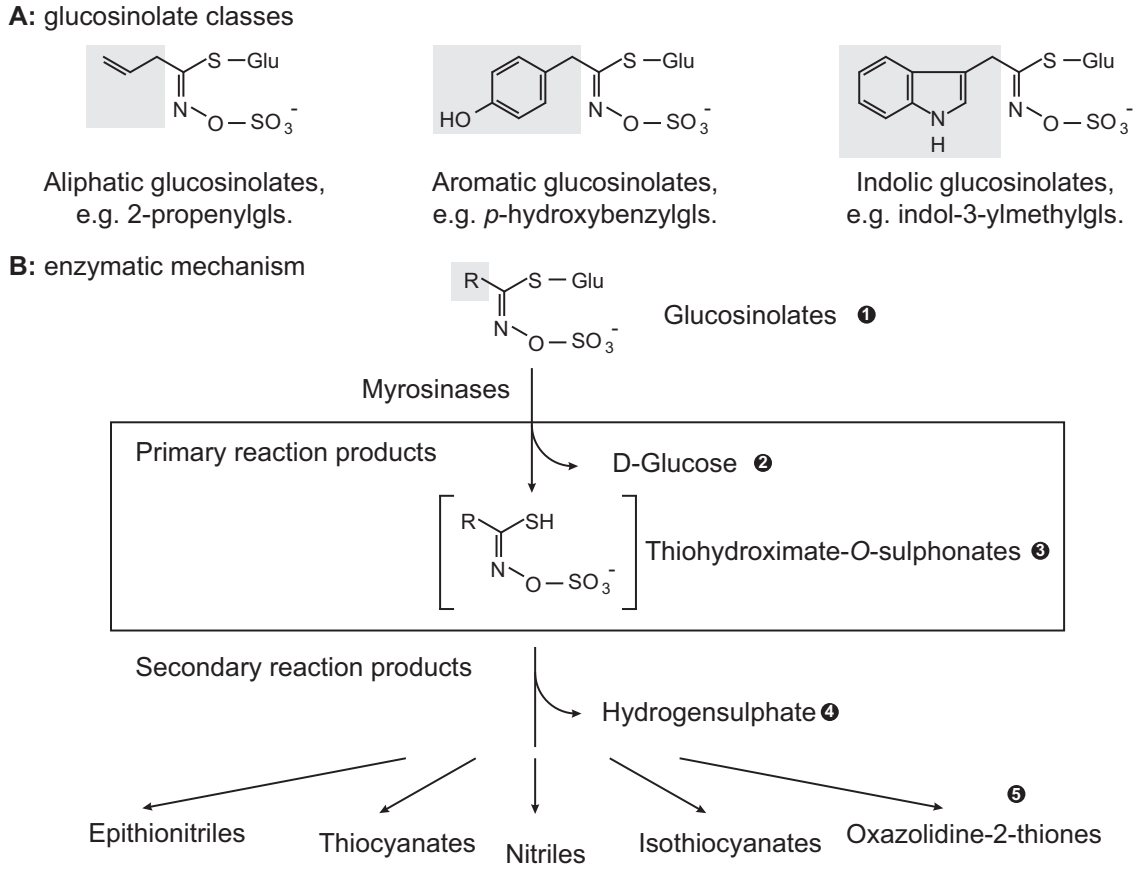

Figure 1.1: The glucosinolate-myrosinase system (modified from Wittstock and Halkier, 2002). A - Examples of aliphatic, aromatic and indolic glucosinolate structures; B - enzymatic mechanism: upon tissue damage glucosinolates and myrosinases interact to form the primary reaction products D-glucose and thiohydroximate-*O*-sulphonates. The aglucone is unstable and rearranges to yield hydrogensulphate and one or more compounds of a variety of further decomposition products called mustard oils. Numbers in black circles refer to metabolites for possible detection of activity (Chapter 2). Abbreviations - gls: glucosinolate, R: modified side chain, Glu: D-glucose.

Glucosinolates were first isolated by Robiquet and Boutron-Charlard in 1831: sinalbin (*p*-hydroxybenzylglucosinolate) from white mustard (*S. alba*) and sinigrin (2-propenyl-glucosinolate) from black mustard (*Brassica nigra* (L.) W.D.J.KOCH) (Figure 1.1A). The structure in which an amino acid derived core is sulphur-linked to a β-D-glucopyranose moiety was first proposed by Ettlinger and Lundeen in 1956. Chemically, glucosinolates are β-thioglucoside-*N*-hydroxysulphates (Fahey *et al.*, 2001). The processes of glucosinolate biosynthesis in crucifers can be differentiated into three major independent steps: first putative side chain elongation of amino acids, secondly glucone formation and thirdly side chain modifications (Wittstock and Halkier, 2002). The initial formation of the glucosinolate core structure is mediated by cytochrome P 450-monooxygenases which converse amino acids by *N*-hydroxylation and subsequent decarboxylation into their corresponding aldoximes. These enzymes were found in all plant tissues of Chinese cabbage and oilseed rape (Bennett *et al.*, 1995). Subsequently, sulphur from cystein, glucose *via* uridine diphosphate glucose (UDPG) and sulphate from 3'-phosphoadenosine-5'-phosphosulphate (PAPS) are added. Side chain elongation occurs for aliphatic and aromatic amino acids, during which up to 9 additional methylene groups can be incorporated. Glucosinolates with an aliphatic side chain are derived from alanine, methionine, valine, leucine or isoleucine. Aromatic glucosinolates originate from the precursors phenylalanine or tyrosine, whereas the amino acid tryptophan yields indole glucosinolates. These distinctions and the side chain modifications are of particular importance, because the side chain's structure largely implies the nature of the degradation products.

Glucosinolate degrading enzymes are thioglucoside glucohydrolases denoted myrosinases (since 2001 EC 3.2.1.147, formerly EC 3.2.3.1; reviewed in Rask *et al.*, 2000). They have been found in all glucosinolate-containing plants investigated so far. Additionally, myrosinase activity has been found in some fungi, bacteria and insects. Myrosinases promote the cleavage of D-glucose resulting in an unstable aglucone which releases sulphate and different final degradation products (Bones and Rossiter, 2006). Factors influencing the nature of the breakdown products are side chain structure, reaction conditions like pH or the presence of ferrous ions, as well as the presence of specific proteins, e.g. epithiospecifier proteins (ESPs). Genetic data obtained from *S. alba* and *Brassica napus* L. showed that the enzymes are encoded by a gene family which comprises of at least three subfamilies called MA, MB and MC (Rask *et al.*, 2000). These subfamilies are distinguished by their relative DNA sequence similarity and their glycosylation extent (Xue *et al.*, 1995). Functional MA myrosinase enzymes are dimeric glycoproteins with molecular masses of about 130 to 150 kDa. For MB and MC myrosinases a complexion state with myrosinase binding proteins and myrosinase associated proteins is proposed resulting in molecular masses of up to 800 kDa (Eriksson *et al.*, 2001, 2002). Purified myrosinases were obtained from different plant species (Chapter 2, Table 2.1). For soluble MA enzymes, temperature and

pH optima were found in the ranges of 37 to 70 °C and at pH values of 4 to 9, respectively. Furthermore, ascorbic acid has marked effects on myrosinase activity displaying a dual nature. At concentrations of 0.3 to 0.7 mM, ascorbic acid increases the reaction velocity, whereas concentrations higher than that are effectively reducing the hydrolysis rates. The role of ascorbate in the molecular mechanism is proposed to be that of a catalytic base (Burmeister *et al.*, 2000). For some purified enzymes strong substrate specificity was reported, whereas others seem to degrade an array of different glucosinolates. Up to now only soluble MA, but not complexed MB or MC isoforms were tested in pure form for their enzyme characteristics.

In intact plant tissues, enzyme and substrate have to be inactive in order to prevent self intoxification of the plant. Spatial separation renders the system dormant until the tissue is destroyed, a circumstance which caused its description as the "mustard oil bomb" (Lüthy and Matile, 1984). Evidence collected recently suggests that the necessary compartimentalization is found at the level of distinct cells (Kelly *et al.*, 1998; Koroleva *et al.*, 2000; Andréasson *et al.*, 2001a). Myrosinases are located in vacuoles of specialized idioblasts called myrosin cells and glucosinolates can be found in vacuoles of most other cell types. Almost all tissue types investigated so far, e.g. leaves, stems, or roots, contained myrosin cells. The distribution of myrosin cells is species-specific. In *Arabidopsis thaliana* (L.) Heynh. they are located among phloem parenchyma cells, whereas in *B. napus* they occur in phloem as well as ground tissue of leaves (Andréasson *et al.*, 2001a). Destruction of plant tissue containing myrosin cells and glucosinolates results in the triggering of the "bomb". As glucosinolates are degraded, various end products of different toxicity arise (Wittstock *et al.*, 2003). Isothiocyanates are toxic to non-adapted insects by fumigation, upon contact and after ingestion. Nitriles and thiocyanates seem to be less toxic. The defensive properties of epithionitriles and oxazolidine-2-thiones are still unknown for insects. However, the latter interfere with hormone production in the thyroid gland causing goitres in mammals (Halkier and Gershenzon, 2006). Isothiocyanates react with amino groups of proteins cleaving disulphide bonds *in vitro* (Kawakishi and Kaneko, 1987). Thiocynanates interact with the insect's detoxification metabolism, i.e. gluthathione-*S*-transferases, and as a result hydrogencyanide is released (Kawakishi and Kaneko, 1985).

1.2 Herbivores on glucosinolate-containing plants

In evolutionary terms, the so-called arms race between plants and herbivores should lead to adaptations or avoidance mechanisms on the insects' side, if plant natural products display detrimental effects (Louda and Mole, 1991). Non-adapted herbivores ("food generalists")

should consequently restrict their host range *via* feeding or oviposition. The restriction can take place on two different levels: the preingestive phase of food selection and the postingestive phase of potential toxicity. Adapted herbivores ("food specialists") overcome the physiological constraints leading to toxicity and hence render the usually toxic food harmless.

Generalist herbivores found on glucosinolate-containing plants are mostly arthropods, molluscs, nematodes and birds (Louda and Mole, 1991; Frenzel and Brandl, 2003). Against generalist insects, glucosinolates and mustard oils function as oviposition and feeding inhibitors (Bodnaryk, 1991; El-Sayed *et al.*, 1996; Halkier and Gershenzon, 2006). Most generalists use Brassicaceae as host plants only to a certain percentage in their diet because of adverse effects on survival and performance (Bodnaryk, 1991; Newman *et al.*, 1996). The dilution thus obtained may aid in avoidance of the toxic effects. However, even these insects need broadband detoxification or tolerance mechanisms. Blau *et al.* already showed in 1978 that an unspecialized Apiaceae-specialist died when fed a diet containing allylglucosinolate. In contrast, the oligophagous feeder was only moderately affected and the Brassicaceae-specialist did not show any effects even at the highest concentration tested. However, caterpillars of another crucifer-specialist, *Plutella xylostella* L. (Lepidoptera: Plutellidae), although insensitive to glucosinolates, were even more susceptible to isothiocyanates than generalist caterpillars (Siemens and Mitchell-Olds, 1996). The volatile hydrolysis products formed by the reaction of glucosinolates with myrosinases are responsible for antibiotic or antifungal effects, as well as insect repellence and toxification in many cases (Chew, 1988; Newman *et al.*, 1992; Lazzeri *et al.*, 1993; Li *et al.*, 2000; Wittstock *et al.*, 2003). In general, glucosinolates are now thought to be "bitter-tasting" deterrents, whereas the breakdown products are the biologically active toxic principle.

There are now several examples known which illustrate how insects, mainly specialists, can overcome the "mustard oil bomb" (Ratzka *et al.*, 2002; Müller and Wittstock, 2005). The detoxification of myrosinase-catalyzed hydrolysis of glucosinolates is outcompeted in *P. xylostella* by a sulphatase secreted into the gut lumen. The resulting desulphoglucosinolates cannot be hydrolysed by myrosinases and thus no mustard oils can arise. Recently, sulphatase activity was accordingly determined in a generalist locust (*Schistocerca gregaria*) which temporarily restricts feeding to glucosinolate-containing plants (Falk and Gershenzon, 2007). In *Pieris rapae* L. (Lepidoptera: Pieridae) a different protein-factor is found in the gut (Wittstock *et al.*, 2004). The nitrile-specifier protein (NSP) interferes with the glucosinolate degradation process. Instead of toxic isothiocyanates, predominantly less toxic nitriles are produced which are furthermore excreted with the faeces. Highly adapted insects concentrate glucosinolates in their body tissues, a phenomenon called sequestration (Duffey, 1980). Within the haemolymph high concentrations of glucosinolates can

be reached. This spatial separation of substrate and enzyme is realized by several insects including aphids, bugs, and sawflies (Müller *et al.*, 2001; Aliabadi *et al.*, 2002; Bridges *et al.*, 2002). By leaf-chewing *A. rosae* sawflies, glucosinolates are taken up into the haemolymph, but myrosinases remain in the gut. By sap-feeding bugs of *Murgantia histrionica* (Hahn) (Heteroptera: Pentatomidae) and aphids of *Brevicoryne brassicae* (L.) and *Lipaphis erysimi* (Kaltenbach) (Homoptera: Aphididae), only glucosinolate containing solutions are ingested. The stored glucosinolates were shown to protect its insect owners against predators of the next trophic level (Francis *et al.*, 2001; Müller *et al.*, 2002; Müller and Arand, 2007). After ingestion by predators, prey-myrosinases found in muscle cells of the aphids or those produced by microflora commonly found in animal guts may trigger the insect "mustard oil bomb" (Bridges *et al.*, 2002; Halkier and Gershenzon, 2006).

Specialist herbivores not only circumvent but also exploit the plants' chemical defences. Irrespective of physiological adaptation, a range of specialists were shown to use non-volatile glucosinolates themselves as short-range contact cues in host recognition, feeding, and oviposition (Städler *et al.*, 1995; Renwick, 2002; Reifenrath *et al.*, 2005). The volatile mustard oils can in addition to these functions also aid in long-range host location (Courtney, 1983). Furthermore, sequestration by insects actually confers benefits to the herbivore *via* general defensive metabolites. From the plants' perspective, this is a dilemma called the "lethal plant defence paradox" (Price *et al.*, 1980; Fordyce, 2001). It was postulated that a possible solution for plants could be the massive induction of metabolites within plant tissues in order to kill early, susceptible instars of sequesterers (Malcolm and Zalucki, 1996). In contrast, the plant should respond to feeding of later, resistant instars with a reduction of this particular defence to prevent awarding benefits to the insect. However evidence of an actual realization in Brassicaceae-specialist interactions is very sparse (Müller and Sieling, 2006).

1.3 Variation in plant defence

As described above the single components of the glucosinolate-myrosinase system are considerably variable. Hitherto more than 120 different structures were described for glucosinolates and also many different myrosinase isoforms were isolated (Rask *et al.*, 2000; Fahey *et al.*, 2001). Next to this qualitative variation, quantitative variation of both components are widely described. Phylogenetic origin (e.g. species, genus, and tribe association) and ontogenetic state, i.e. age of single tissues and whole plants are internal causes (Clossais-Besnard and Larher, 1991; Porter *et al.*, 1991; Rodman *et al.*, 1998; Charron *et al.*, 2005a,b), whereas environmental conditions (e.g. light, soil nutrients and moisture, and herbivore or pathogen pressure) are external causes (Bones *et al.*, 1994; Doughty *et al.*,

1995; Rostás *et al.*, 2002; Reifenrath and Müller, 2007; Schonhof *et al.*, 2007). This picture is not static for any given set of conditions, but is subject to short-term variation. Sudden, un-predictable changes of the abiotic or biotic environment can be answered by changes of plant physiology or morphology, a phenomenon known as induction (Karban and Baldwin, 1997; Agrawal, 1999a; Underwood *et al.*, 2005). All of these processes shaping the defence status of a plant or tissue are interwoven; one determining the other and *vice versa*.

The urge to understand a picture this complex led to the development of an array of hypotheses for predicting plant-insect interactions and their ultimate causes (Stamp, 2003). But a general theory of plant defence "has the characteristics of immature theory" (Stamp, 2003, p. 49, ll. 5-6) and is still under development (Agrawal, 2007). Coevolution of plants and herbivores in an arms race is clearly very important. However, the complex insect-plant relationships are also influenced by abiotic factors, host plant attributes and predators. One of the first hypotheses to be formulated was the "optimal defence hypothesis" stating that "The plant's limited supply of defensive compounds should be concentrated in those regions in which their presence would most increase the fitness of the plant" (1974, p. 309, ll. 14-16) and "It follows (*from metabolic costs*) that there is selection for allocation of the plant's limited supply of defense compounds in ways that most increase fitness" (1979, p. 66, ll. 9-11) (McKey, 1979, 1974). Two complexes of sub-hypotheses can be distinguished within this framework, which are more testable than the original ones (Stamp, 2003). First, defences are allocated within an individual in proportion to risk of herbivory and value of the plant tissue, and in inverse proportion to the cost of defence (genotypic expression). Second, defence is increased in the presence of enemies and a trade-off between growth and reproduction on one side and defence on the other side exists (phenotypic expression). Not all of the results obtained so far support this view, but the hypothesis has never been firmly rejected (Stamp, 2003). In terms of induction responses within individual plants, it is still a very intriguing framework for plant-insect researches (Chapters 3 and 4). Most of the earlier hypotheses on plant defence theory were developed in view of one defence against a prominent herbivore only. It was thought for a long time that any sufficiently effective defence should trade-off against a second redundant one by selection (Agrawal, 2007). Reinforcement of this trade-off under nutrient limitation is therefore likely because of allocation costs associated with the production of one particular defence. However, plants contain multiple defences against a variety of opponents and abiotic stresses. Next to the glucosinolate-myrosinase system, other defence strategies are commonly found in Brassicaceae. Trichomes and alkaloids are present in many species (Beilstein *et al.*, 2006; Brock *et al.*, 2006). Proteinase inhibitors are widely found which reduce the nutritive value of an insect's meal by blocking the digestion of proteins (Winterer and Bergelson, 2001; Fan and Wu, 2005). Phenylpropanoids and in particular flavonoids are widespread groups of secondary metabolites (Harborne and Williams, 2000)

and one function is the protection against ultraviolet light (Reifenrath and Müller, 2007). Phytoalexins, e.g. the widely known camalexin from *A. thaliana* are effective in reducing pathogen infections (Pedras *et al.*, 2002; Bednarek *et al.*, 2005). The univariate trade-off model cannot satisfactorily include all abiotic and biotic selection pressures. Multivariate approaches for plant defence theory have increased over the last few years (Kursar and Coley, 2003; Koricheva *et al.*, 2004; Leimu and Koricheva, 2006). These included various defences over a wide phylogenetic range of plants. The resulting hypothesis rejects the idea of obligate trade-offs between two defences (Agrawal, 2007). However, trade-offs should occur at higher levels of organisation, e.g. the suite of tolerance traits versus resistance traits. Recently, these reports cumulated in the description of plant defence syndromes (Agrawal and Fishbein, 2006). Agrawal and Fishbein concluded that combinations of the plant traits "edibility" and "feeding barrier" can be distinguished into three syndromes. The first was called "low nutritional quality" syndrome, where low edibility should render a plant non-attractive for herbivores and thus defences would not be needed. In the "nutrition and defence" syndrome, plants with higher edibility should require equally higher defences. The highest edibility could then only be obtained by phenological escape or regrowth strategies in plants of the "tolerance/escape" syndrome, because the biosynthesis of defence is costly (Chapter 5). Deliberate experimental testing of this hypothesis and the implied associations of single defence traits has not been conducted until now.

1.4 Study system and scope of work

The turnip sawfly *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) is distributed in the temperate zone of the Eurasian continent with two distinct subspecies: the European *A. rosae* ssp. *rosae* (L.) and the Asian *A. rosae* ssp. *ruficornis* Jakovlev (Abe, 1988). The larvae of this species are oligophagous feeders on Brassicaceae (Oishi *et al.*, 1993) and can reach pest status on some crop species in Europe (Riggert, 1939; Sáringer, 1976). The larvae are able to concentrate certain glucosinolates of their host plants within their haemolymph (Müller *et al.*, 2001). These stored glucosinolates are employed by the larvae as a protection mechanism against the next trophic level (Müller *et al.*, 2002; Müller and Arand, 2007). When these "easy bleeders" are attacked by invertebrate predators with pointed mouthparts, the integument disrupts easily and a droplet of haemolymph is released (Boevé and Schaffner, 2003). No myrosinase activity was detected in body tissues of *A. rosae* (Müller and Wittstock, 2005). Therefore, the presented glucosinolates can serve directly as deterrents. After ingestion, they could also be degraded to toxic mustard oils by myrosinase-producing micro-flora found in most animal gut systems (Halkier and Gershenzon, 2006). The adults of this species are nectar-feeders found predominantly on

Apiaceae. They were shown to respond to several plant chemicals, e.g. clerodendrins, glucosinolates and isothiocyanates (Nishida *et al.*, 2004; Barker *et al.*, 2006). The particular host plant traits, which are important for larval development, are not yet known. When investigating the performance of *A. rosae* on three plant species of Brassicaceae, developmental times and adult weights differed significantly between groups fed on either of the three Brassicaceae species, *Barbarea stricta* ANDRZ., *B. nigra*, or *S. alba* (Müller and Arand, 2007). In contrast, the performance was only slightly influenced by glucosinolate and myrosinase levels using inbred homozygous lines of *Brassica juncea* CZERN. which displayed three combinations of substrate and enzyme levels (high/high, low/low, and high/low, Müller and Sieling, 2006).

As a leaf-chewing sequesterer, the larvae are normally faced with both beneficial glucosinolates AND detrimental myrosinases. Until now, several methods were developed to measure purified, soluble enzymes. As described above, myrosinases in plant tissues can occur in a soluble (dimers) or in an insoluble form (complexes). The applicability of existing methods to turbid pellet suspensions is therefore very important. Furthermore, using crude plant extracts instead of purified enzymes can lead to erroneous measurements. One common source of error is the presence of endogenous substrate (Segel, 1975), i.e. in this case glucosinolates in plant extracts used for myrosinase activity measurements. Another source of error is the interference of several plant metabolites with detection methods (Kleinwächter and Selmar, 2004). Therefore, a central point of this work was the development of a reliable myrosinase assay for both soluble and insoluble myrosinases which can characterize potential processes occurring in damaged plant tissues (Chapter 2).

To determine the role of the glucosinolate-myrosinase system for the interaction with the turnip sawfly, I want to focus on two fields of interest in particular: variation within plants and between plant species (Figure 1.2). Induction responses of Brassicaceae were widely described for many herbivores including specialists (Chapter 3). But only one sequestering insect species (a sap-sucking aphid, *B. brassicae*) was used as induction agent (Mewis *et al.*, 2005). As described above, sequestering specialists pose a problem for plants ("lethal plant defence paradox") because these insects can benefit through the plant's chemical defence. Furthermore, most studies described only glucosinolate levels but not myrosinase activities. In the following, short-term induction responses of glucosinolates and myrosinases after herbivory of sequestering sawfly larvae on white mustard (*S. alba*) are presented. *S. alba* is used by the turnip sawfly as a common host in Germany and larval infestation can reach high densities in agricultural fields (personal observation; Besenhofer, 2003). Furthermore, only impacts of artificial damage (Koritsas *et al.*, 1991; Bodnaryk, 1992) or plant hormone application (Bodnaryk, 1994; Bennett *et al.*, 1997) on plant physiology were studied in this plant species. The patterns of induction evoked by feeding of herbivorous

A. rosae larvae within a plant individual are followed time- and space-dependent. Next to its specificity according to larval instar (young *versus* old), the patterns of responses to this specialist are compared to those evoked by feeding of a generalist herbivore and by mechanical wounding. Furthermore, the subsequent feeding and oviposition behaviours of larvae and adults on these induced plants need to be considered to evaluate the resistance or defence properties of plant induction responses. These short-term interactions focus on variation within plant individuals. In contrast, a long-term approach was used to also evaluate the importance of variation between plant species for the performance and preference of the turnip sawfly. Within this context, multiple defences were studied for possible correlations. These included next to the glucosinolate-myrosinase system, trichomes, proteinase inhibitors and nutritional components. The defensive properties of seven plant species and two leaf-ages are investigated to evaluate the "plant defence syndromes hypothesis". Larvae of the turnip sawfly were reared to adulthood on these 14 groups of host tissues. Preference of adult females was assessed in oviposition bioassays. Several performance and preference parameters were recorded to estimate which determine insect fitness most. Cluster analyses on plant and insect data were used to find associations between the 14 groups. Regression analyses were afterwards applied to see which single plant traits were most important for insect parameters.

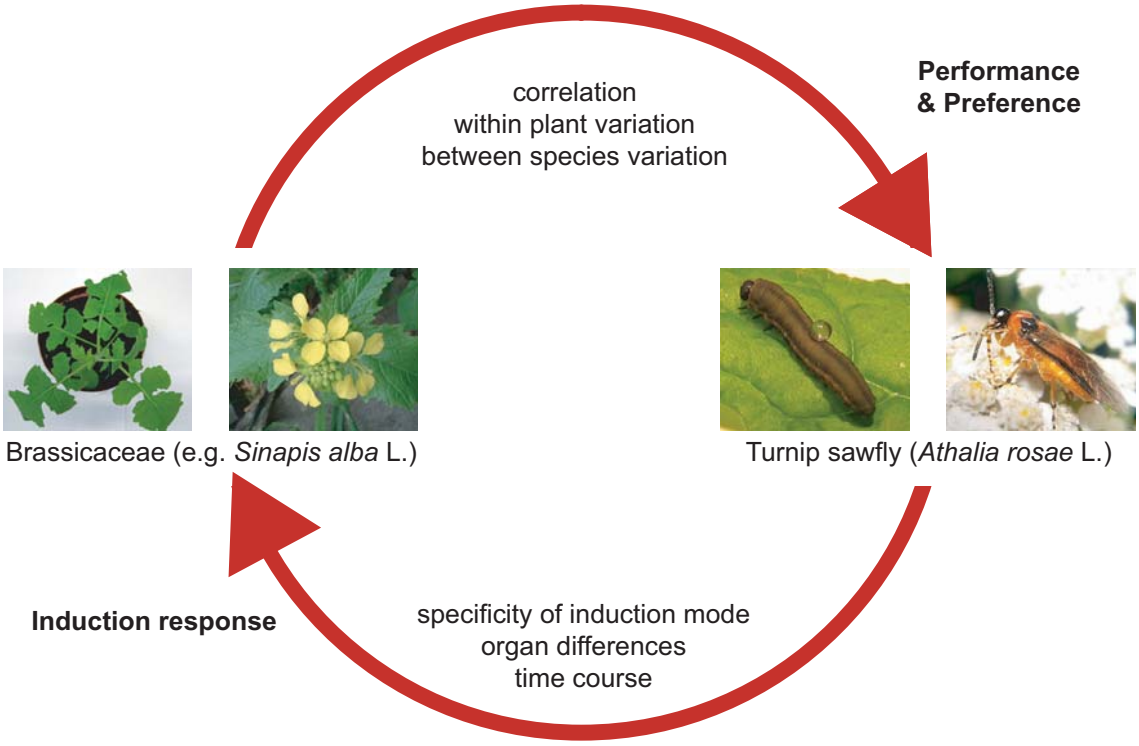

Figure 1.2: Scheme of presented plant-insect interactions.

1.5 Hypotheses and questions

Considering published results on Brassicaceae-herbivore interactions and plant defence theory, I hypothesise for variation within plant individuals that from the plants' perspective a sequestering insect should not induce increases in glucosinolates, but rather in myrosinase activity. These reactions should accordingly be distinct from those evoked by a generalist or by mechanical wounding which should include increases in glucosinolate, as well as myrosinase activity levels. Feeding by early instars should have a higher impact on plant defence compared to later instars. Behavioural responses should be mediated by these plant induction responses especially for a specialist insect known to respond to glucosinolates and mustard oils.

For variation between plant species I hypothesise that multiple defences in several species of Brassicaceae can be grouped into syndromes. The development of highly adapted *A. rosae* larvae should be impaired by mechanical, but not by chemical plant defences. If mechanical and chemical defences represent two independent strategies of the "nutrition and defence" syndrome, performance and preference should reflect plant defence syndromes.

In particular I want to answer the following questions:

Variation within plant individuals and short-term effects

- Which responses are triggered by feeding of sequestering *A. rosae* larvae in tissues and organs around the damage site of two different cultivars of *S. alba* and how long do they last?
- How are induction responses in *S. alba* influenced by insect specialism, insect instar, and damage mode?
- Why is the elicited response of the plant different between young and old instars, generalists and specialists?
- What are the consequences of plant induction responses on the short-term behaviour of larvae and adults of *A. rosae*?

Variation between plant species and long-term effects

- How is the glucosinolate-myrosinase system correlated to other plant defences and nutrition?
- How are long-term developmental parameters of *A. rosae* correlated?
- Which are the key components of Brassicaceae that influence the performance and preference of *A. rosae*?

Chapter 2

Revised determination of soluble and insoluble myrosinase activities in plant extracts

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Abstract - The enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147, formerly EC 3.2.3.1) catalyses the hydrolysis of glucosinolates after tissue damage in plants of the order Brassicales. The various enzyme isoforms occur either as free soluble dimers or as insoluble complexes. We propose a reliable method for determination of both soluble and insoluble myrosinase activity concentrations in partially purified plant extracts. The procedure requires the removal of endogenous glucosinolates through ion-exchange columns previous to enzyme measurements. Myrosinase activity was assayed in continuous mode by photometric quantification of the released glucose using glucose-oxidase with peroxidase and colourimetric indicators. The measurement of the colored product at 492 nm had a favourable signal to noise ratio both in clear extract solutions (free dimers) and in turbid pellet suspensions (insoluble complexes). No interferences by ascorbic acid were found in continuous analyses. With the recommended sample preparation methods and assay conditions potential activities in damaged plant tissues can be characterized which function as a plant defence mechanism.

Key words: Continuous assay, glucosinolates, GOD-PAP-method, myrosinase binding proteins.

2.1 Introduction

The association of the enzyme myrosinase with glucosinolates termed the "mustard oil bomb" is mainly found in the plant order Brassicales (formerly named Capparales; Bremer *et al.*, 2003). This plant defence system is important both from a commercial and a scientific point of view, because it was shown to play a key role in plant resistance (Halkier and Gershenzon, 2006). Furthermore, many crop species, e.g. canola, cabbage and mustards, belong to the Brassicaceae family.

Glucosinolates are β-thioglucoside-N-hydroxysulphates that share a common structure, in which a D-glucose moiety and a sulphate group are tied to an aglucone synthesized from amino acids and their chain elongated analogues (Fahey *et al.*, 2001). The amino acids predominantly determine the side-chain structure (Figure 1.1A; Wittstock and Halkier, 2002). Upon tissue damage, the separately stored myrosinases hydrolyse glucosinolates to yield glucose and the aglucones, thiohydroximate-O-sulphonates as primary reaction products (Figure 1.1B; Iori *et al.*, 1996; Andréasson and Jørgensen, 2003). The aglucones are unstable and release hydrogensulphate, as well as a variety of further decomposition products called mustard oils, e.g. isothiocyanates, nitriles, thiocyanates, epithionitriles or oxazolidine-2-thiones (Bones and Rossiter, 2006). Various of these hydrolysis products are toxic to an array of organisms, e.g. herbivorous animals like insects or grazing mammals and plant pathogens like fungi or bacteria (Halkier and Gershenzon, 2006). In addition to these ecological roles, some degradation products also promote anticarcinogenic activity in humans (Verhoeven *et al.*, 1997). Not only concentrations of the substrate should be evaluated in the plant tissue, but also activities of myrosinases to characterize the potential defence of Brassicales (Li *et al.*, 2000; Müller and Martens, 2005; Müller and Sieling, 2006; Martin and Müller, 2007) or the production of health-beneficial mustard oils (van Eylen *et al.*, 2006, 2007).

Myrosinase proteins are encoded by a family of genes which is divided in at least three subfamilies (MA, MB and MC; reviewed in Rask *et al.*, 2000). These subfamilies are separated by sequence similarity and by size of the proteins due to different extents of glycosylation and are expressed differently depending on plant organ and developmental stage (Falk *et al.*, 1992; Xue *et al.*, 1992). MA myrosinases occur as free soluble dimers, whereas MB and MC isoforms are bound in complexes of high molecular weight of up to 800 kDa which can be solubilized only with denaturing agents (Eriksson *et al.*, 2001, 2002). The complexed forms were found in most tissues of oilseed rape (*B. napus*) and white mustard (*S. alba*; Taipalensuu *et al.*, 1997a,b; Eriksson *et al.*, 2001). In the following, we will refer to these two native states of myrosinase proteins as soluble and insoluble isoforms whose different solubility properties need to be considered when determining myrosinase activities.

Methods for quantification of the activity of purified soluble isoforms are well established. Apart from the unstable thiohydroximate-*O*-sulphonates, all possible compounds have by now been used to determine myrosinase activity (Kleinwächter and Selmar, 2004). The decrease of glucosinolates in a direct spectrophotometric assay or the formation of hydrogensulphate using a pH-stat were monitored directly (Palmieri *et al.*, 1982, 1987). Mustard oils were also determined by their spectrophotometric absorbance (Bellostas *et al.*, 2006). Indirect enzyme coupled methods were used to quantify the amount of released glucose (Wilkinson *et al.*, 1984; Palmieri *et al.*, 1987; Kleinwächter and Selmar, 2004). However, there are much fewer studies published which are concerned with the activity determination of insoluble myrosinases (Finiguerra *et al.*, 2001; Eriksson *et al.*, 2002; Pontoppidan *et al.*, 2005).

The objective of the present work was to establish a method for the determination of both soluble and insoluble myrosinase activity concentrations in partially purified extracts of plant tissue which closely describes the *in situ* situation. The assay is based on a protocol described previously (Siemens and Mitchell-Olds, 1998; Li *et al.*, 2000; Wallace and Eigenbrode, 2002) for measurements of soluble myrosinases, but was highly modified to fulfill our needs. Specifically, we focused on assay conditions, sample preparation, and detection methods. The conditions of the reaction mixture of plant tissue samples can be alike those found in the enzyme's typical environment or optimal for the enzyme. These two parameters are not necessarily identical, as in the case of myrosinases: the pH of crushed leaf material is about 6 to 6.5 (Wilkinson *et al.*, 1984), but myrosinase optima can vary from pH 4 to 9 (for a compilation of literature data see Table 2.1). The temperatures of living plant tissues in natural environments range from 0 to over 40 °C but enzyme optima are also found at up to 75 °C (Table 2.1; Bones and Slupphaug, 1989) where most plant tissues are not able to survive.

To test activities reliably with well defined substrate concentrations, a preceding efficient removal of internal substrates from these extracts is crucial (Segel, 1975). We elucidate mechanisms and solutions for problems of myrosinase activity measurements in crude or partially purified plant extracts. The role of endogenous glucosinolates and the influence of ascorbic acid on glucose determination are evaluated. We propose a method that is quick, reliable and in many aspects close to the processes taking place in damaged plant tissues.

Table 2.1: Enzyme properties of purified soluble myrosinases.

Plant species	Enzyme source	pH optimum	Temperature optimum	AsA optimum	AsA inhibition	K_m (-/+ AsA)	v_{max}	Reference(s)
Armoracia rusticana Gaertn., Mey & Scherb.	Mature roots	5.7	45 °C	0.5	n.d.	0.128 / n.d.	0.62 ‡	Li and Kushad (2005)
Brassica napus L. cv. Niklas	Seeds	5.2 - 5.5	70 – 75 °C	0.3 - 0.5	2	0.07 / 0.5	n.d.	Bones and Slupphaug (1989)
cv. Panter	Seeds	4.5 - 5	60 °C	0.7	5 - 10	0.03 / 0.5	n.d.	Björkman and Lönnerdal (1973)
Brassica rapa L. em. Metzg.	Leaves	5.5	n.d.	n.d.	n.d.	0.045 / n.d.	2.5	El-Sayed and Jwanny (1994)
ssp. *rapa*	Leaves						19.7	Jwanny and El-Sayed (1994)
Crambe abyssinica Hochst. cv. Cebeco 9402	Seeds	7 - 9	37 °C	n.d.	n.d.	0.2 / 1.1	54 ‡ - 200 †	Bernardi *et al.* (2003)
Lepidium sativum L.	5-day-old seedlings (incl. roots)	5.5	n.d.	n.d.	n.d.	0.3 / n.d.	2 ‡ - 3.2 ‡	Durham and Poulton (1990)
Raphanus sativus L.	Mature roots	6 - 6.5	37 °C	n.d.	1	0.47 / n.d.	n.d.	Jwanny *et al.* (1995)
	7-8-day-old seedlings (excl. roots)	n.d.	n.d.	n.d.	n.d.	0.02 / 0.25	2 ‡ - 280 †	Shikita *et al.* (1999)
Sinapis alba L.	Seeds	4.2 - 6	60 °C	0.7	5 - 10	0.06 / 0.4	n.d.	Björkman and Lönnerdal (1973)
						0.2 / 1.0	60 ‡ - 500 †	Bernardi *et al.* (2003)
Tropaeolum majus L.	Mature leaves of flowering plants	n.d.	n.d.	2	$\gg$8	0 / 0.34	0.84 †	Kleinwächter and Selmar (2004)

Notes: K_m [mM]; v_{max} [μmol min^{-1} mg^{-1} protein]; AsA [mM]; † measured in the presence of ascorbic acid, ‡ measured in the absence of ascorbic acid; Abbreviations: AsA - ascorbic acid, n.d. - property not determined.

2.2 Materials and methods

2.2.1 Plant material and processing of samples

Seeds of *Sinapis alba* L. cultivar Silenda (Kiepenkerl, Norken, Germany) were germinated in unfertilized soil (pH 6) at 25 °C, 16:8 hours L:D, and 70 % r.h. Cotyledons of 5-day-old seedlings were harvested to test for extraction procedure, column properties and measurements of specific myrosinase activities. To test for substrate specificity of soluble and insoluble myrosinases, second youngest leaves of 3-week-old plants (5 to 6 true leaves) were used. Harvested cotyledons or true leaves were weighed individually in Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. Four consecutive leaves (age categories 1 and 2 according to Brown *et al.*, 2003) of 6-weeks-old plants of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 were harvested per plant to analyse effects of freeze-drying on myrosinase activities. The leaves were cut longitudinally and two right and two left halves were combined per sample. 20 replicate pairs were prepared. One sample of each pair was stored at −80 °C, the other was freeze-dried.

2.2.2 Myrosinase assay

Myrosinase activity was determined by photometric quantification of released glucose from standardized amounts of externally added substrate. Frozen or freeze-dried leaf material [50 - 200 mg fresh weight (f.wt.)] was finely pulverized in 2 mL Eppendorf tubes in a pre-cooled mixing mill (Retsch, MM301, Haan, Germany) using stainless steel beads (diameter 7 mm) and 60 seconds shaking at an oscillation frequency of 25 Hz. All following extraction steps were carried out on ice. The pulverized plant material was extracted three times in 500 μL Tris-EDTA extraction buffer (200 mM Tris, 10 mM EDTA, pH 5.5), and centrifuged at 16,060 × g for 10 min at 4 °C. Supernatants were subsequently applied to diethylaminoethyl (= DEAE) Sephadex A-25-columns [400 μL void volume, diameter 9 mm, bed height 10 mm, 0.1 g DEAE Sephadex A-25 (Sigma-Aldrich, St. Louis, Missouri, USA) swollen in 2 mL of 0.5 M acetic acid buffer, pH 5] to remove internal glucosinolates (see 2.2.3). The pellets were kept for assays of insoluble myrosinases (see later). The ion exchange procedure was carried out in a refrigerator at 4 °C. Samples were eluted from columns with further 500 μL Tris-EDTA buffer which yielded a total volume of 2 mL of these purified extracts. Protein concentration was determined following (Bradford, 1976) and the concentration was adjusted to $200 \pm 25\,\mu g\,mL^{-1}$ for true leaf extracts of *S. alba* and to $50 \pm 10\,\mu g\,mL^{-1}$ for extracts of cotyledons of *S. alba* and true leaves of *A. thaliana*. These extracts were used to determine activity concentrations of soluble myrosinases. The remaining pellets were re-suspended in 500 μL Tris-EDTA buffer and taken for two further

tests. To assess the activity of insoluble myrosinases exclusively, 250 μL pellet suspensions were diluted with 750 μL Tris-EDTA buffer to yield a total volume of 1 mL. Samples to measure mixtures of soluble and insoluble myrosinases and thus total myrosinase activities were prepared from 250 μL of pellet suspensions which were centrifuged at 16,060 $\times$ g for 10 min at 4 °C. The supernatants were discarded and the remaining pellets were re-suspended in 1 mL of the purified protein extract. Thereby, a suspension was obtained with a reconstituted ratio of soluble and insoluble myrosinases alike the original extract but free from interfering internal glucosinolates. Obtained suspensions were diluted according to adjustments to specific protein concentrations of the corresponding supernatants.

Of each sample, 4×100 μL of the purified extract, of the pellet suspension alone and of the combination of both were applied each to individual cells of a 96-cell microplate. Two cells served as background controls (specific blank) to correct for internal glucose levels and light scattering, and 25 μL phosphate buffer (0.2 M, pH 6.5) were added. In the other two cells, 25 μL of 2 mM glucosinolate (*S. alba*: *p*-hydroxybenzylglucosinolate, glucosinolates.com, Copenhagen, Denmark; *A. thaliana*: indol-3-ylmethylglucosinolate, Phytoplan, Heidelberg, Germany) in phosphate buffer were added as substrate. All tests were carried out with the adjusted protein concentration of extracts and suspensions and additionally with diluted ones at half the protein concentration. A glucose dilution series was included on each microplate.

After addition of 50 μL freshly mixed colour reagent, including glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, Fluka, Buchs, Switzerland) (57 U mL^{-1}), peroxidase (EC 1.11.1.7, from horseradish, Fluka, Buchs, Switzerland) (5.6 U mL^{-1}), 2.8 mM 4-aminoantipyrine (Fluka, Buchs, Switzerland), 30.7 mM phenol and 0.136 M imidazole (GOD-PAP-method; Trinder, 1969; Kunst *et al.*, 1984) the reaction was started at room temperature (23 $\pm$ 0.5 °C). The release of glucose was determined by measuring the absorbance of the colored product *N*-(4-antipyryl)-*p*-benzoquinone imine at 492 nm on a microplate photometer (Multiskan EX, Thermo Labsystems, Vantaa, Finland) for 45 minutes. Absorbance was read every minute and the plate was shaken between measurements to prevent pellet suspensions from sedimentation.

Means of the two replicate measurements were calculated after subtraction of the means of the background controls. Glucose concentrations were calculated from a linear standard curve of the dilution series for each time point separately. A linear part of at least 10 time points of the reaction progress curve was selected (usually between 10 and 30 min) to determine myrosinase activities. The capacity of a tissue sample to hydrolyse glucosinolates is given as myrosinase activity concentration in nanomoles of glucose released per minute of one milligram of fresh weight according to the following equation:

$$\text{Activity [nmol min}^{-1}\text{ mg}^{-1}\text{ f.wt.]} = \frac{\Delta\,\text{glucose (test-blank) [nmol]} \times \text{(total volume / test volume)}}{\Delta\,\text{time [min]} \times \text{sample fresh weight [mg]}}$$

2.2.3 Glucosinolate-retarding properties of Sephadex A-25

To test conditions in which endogenous glucosinolates are sufficiently removed from crude plant extracts, column properties of DEAE Sephadex A-25 (Sigma-Aldrich, St. Louis, Missouri, USA) were studied with glucosinolate standards and extracts of 5-day-old cotyledons. The amounts of standard glucosinolates were chosen equivalent to leaf or cotyledon material with high glucosinolate levels of 4 % of dry weight at a sample size of 100 mg fresh weight with 90 % water content (Fahey *et al.*, 2001). Extracts of 5-day-old cotyledons, 2-propenylglucosinolate (Sigma-Aldrich, Steinheim, Germany, Figure 1.1A) solution in nanopure water (4.9 mM, 20 μL) and *p*-hydroxybenzylglucosinolate (Figure 1.1A) solution in phosphate buffer (2 mM, 50 μL) were applied separately to pre-cooled Sephadex columns. Previous to sample applications, column material (400 μL void volume, diameter: 9 mm, bed height: 10 mm, 0.1 g Sephadex swollen in 2 mL of 0.5 M acetic acid buffer, pH 5) was washed three times with acetic acid buffer to saturate DEAE-binding sites. Two subsequent washes with Tris-EDTA buffer replaced acetic acid buffer in the void volume. Cotyledon tissue was treated as described above for myrosinase sample preparation with three consecutive extraction steps. Columns with standards were eluted four times with 500 μL extraction buffer (200 mM Tris, 10 mM EDTA, pH 5.5) to mimic the obligate multiple step extraction procedure and kept in the refrigerator. Glucosinolate concentrations present in flow-through solutions were determined by the standard glucosinolate extraction procedure involving binding of glucosinolates onto the ion exchange groups (DEAE) of the Sephadex A-25 matrix of further columns, addition of benzylglucosinolate (Phytoplan, Heidelberg, Germany) as internal standard, incubation with purified sulphatase overnight (EC 3.1.6.1, type H-1, from *Helix pomatia*, 14.000 units (gram solid)$^{-1}$; Sigma, Taufkirchen, Germany; 19.6 U sample^{-1} dissolved in 0.02 M acetic acid buffer, pH 5; for purification see Graser *et al.*, 2001) and HPLC of the eluted desulphoglucosinolates (Heaney and Fenwick, 1981; Martin and Müller, 2007). To analyse remaining glucosinolates on the original columns, these were treated directly with sulphatase after addition of internal standard, incubated overnight, and eluates were analysed for desulphoglucosinolates by HPLC following Martin and Müller (2007).

2.2.4 Substrate specificity of soluble and insoluble myrosinases

To test the substrate specificity of both soluble and insoluble myrosinases of *S. alba*, activities were tested against five glucosinolates using extracts of fully-expanded leaves

(~ 300 mg). Purified extracts and suspensions of three leaf samples from different plants were each split equally in five portions, to test activities under identical conditions. As substrates, four glucosinolates naturally occurring in *S. alba* leaves were used, namely benzylglucosinolate, *p*-hydroxybenzylglucosinolate, phenylethylglucosinolate (Phytoplan, Heidelberg, Germany) and indol-3-ylmethylglucosinolate. 2-Propenylglucosinolate (Sigma-Aldrich, Steinheim, Germany) was also tested because it can be easily obtained and has therefore been used in a number of studies (Palmieri *et al.*, 1982; Wilkinson *et al.*, 1984; Eriksson *et al.*, 2001). All glucosinolate solutions (2 mM) were prepared in phosphate buffer (see 2.2.2). Measurements of soluble and insoluble myrosinase activity concentrations were elaborated individually and in combination as described above (2.2.2).

2.2.5 Effects of ascorbic acid on glucose determination

Ascorbic acid can interfere with glucose determinations using the colour reagents mentioned above in the absence of myrosinase (Kleinwächter and Selmar, 2004). To establish whether ascorbic acid amounts originally present in the plant tissue interfere with myrosinase activity determinations by the GOD-PAP-method, glucose and ascorbic acid were mixed and tested in various concentrations. The tests were performed on microplates in a total volume of 225 μL which consisted of 150 μL glucose solution, 25 μL ascorbic acid solution and 50 μL of colour reagent. Glucose solution (stock: 1 mM in nanopure water) was diluted so that the reaction mixtures contained amounts of 20, 30, 40, 50, 60, 80 and 100 nanomoles. Ascorbic acid solution (stock: 28.4 mM in nanopure water) was diluted resulting in concentrations of 0.01, 0.05, 0.1, 0.2 and 0.3 mM in the reaction. All glucose amounts were treated with all ascorbic acid concentrations in quadruplicate. One set of glucose amounts served as a control and was treated only with 25 μL of nanopure water. After addition of colour reagent (see 2.2.2), the development of colour production was recorded at 492 nm on a microplate photometer for 2 hours. Intervals between measurements lasted 15 seconds for the first 5 minutes and 60 seconds afterwards and the microplate was shaken during these periods.

2.3 Results

2.3.1 Freeze-drying effects on myrosinase activity concentrations

Freeze-drying of *A. thaliana* leaf tissue resulted in reduced activity concentrations of soluble myrosinases compared to frozen material (T-test for matched pairs: $t = $-12.99, $df = 19$, $P<0.001$; Table 2.2). A previous experiment with cotyledons of *S. alba* had revealed that

there were no differences in activity measurements between fresh and frozen leaf material (data not shown). No insoluble myrosinase activities could be detected in pellet suspensions alone or in combination with purified extracts in *A. thaliana* leaves in accordance with Barth and Jander (2006). The correlation between the activity concentrations of frozen and freeze-dried samples was only weak (Pearson's correlation coefficient: $R^2 = 0.16$, $F_{1,18} = 4.68$, $P < 0.05$). As the ratio of frozen to freeze-dried samples varied highly, a general factor could not be determined.

Table 2.2: Properties of purified extracts of plant tissues containing only soluble isoforms, mean ± SE. As substrates *p*-hydroxybenzylglucosinolate for *S. alba* and indol-3-ylmethylglucosinolate for *A. thaliana* were used.

Sample source	N	Myrosinase activity concentration †	Protein concentration ‡	Specific myrosinase activity §
		Sinapis alba L. cv. Silenda:		
Cotyledons	5	0.17 ± 0.02	4.45 ± 0.15	37.5 ± 4.6
Young, true leaves	3	0.04 ± 0.01	3.56 ± 0.64	9.5 ± 2.5
		Arabidopsis thaliana (L.) Heynh., ecotype Columbia:		
Frozen leaves	20	0.12 ± 0.01	3.63 ± 0.43	32.5 ± 4.6
Freeze-dried leaves	20	0.07 ± 0.01	3.09 ± 0.44	23.8 ± 4.1

Notes: N - number of replicates; † [nmol min^{-1} mg^{-1} f.wt.], ‡ [μg mg^{-1} f.wt.], § [nmol min^{-1} ng^{-1} protein].

2.3.2 Glucosinolate-retarding properties of Sephadex A-25

About 90 % of glucosinolate standards were retarded at the matrix using DEAE Sephadex A-25 (2-Propenylglucosinolate: 83 ± 0.4 %; *p*-hydroxybenzylglucosinolate: 92 ± 2.9 %; mean ± SE, n = 3). Using 5-day-old cotyledons, the main glucosinolate *p*-hydroxybenzylglucosinolate was mainly retained on DEAE Sephadex A-25 columns (recovered total amount 36.7 ± 7.8 nmol $sample^{-1}$, 99 ± 0.1 % on column; mean ± SE, n = 5). Accordingly, the flow-through which is used for myrosinase activity measurements contained only small amounts of glucosinolates when using the Sephadex DEAE A-25 columns. Suspensions

of column material of DEAE Sephadex A-25 were tested for residual myrosinase activity after sample application according to the procedure for pellet suspensions. No activity was detected within 24 hours with these suspensions (data not shown).

2.3.3 Activity and substrate specificity of soluble and insoluble myrosinases

Myrosinase activity concentrations of soluble isoforms were about four times higher in cotyledons of *S. alba* than in young true leaves (Table 2.2). The activity concentrations of insoluble myrosinases were about two times higher in cotyledons than in true leaves (0.34 ± 0.01 nmol min^{-1} mg^{-1} f.wt., mean $\pm$ SE, n = 5, compared to 0.14 ± 0.02 nmol min^{-1} mg^{-1} f.wt., mean $\pm$ SE, n = 3). Soluble and insoluble myrosinases of *S. alba* true leaf extracts displayed high activities against the aromatic *p*-hydroxybenzylglucosinolate, benzylglucosinolate and phenylethylglucosinolate (Figure 2.1). In contrast, indol-3-ylmethylglucosinolate and the aliphatic 2-propenylglucosinolate were degraded on average at lower rates.

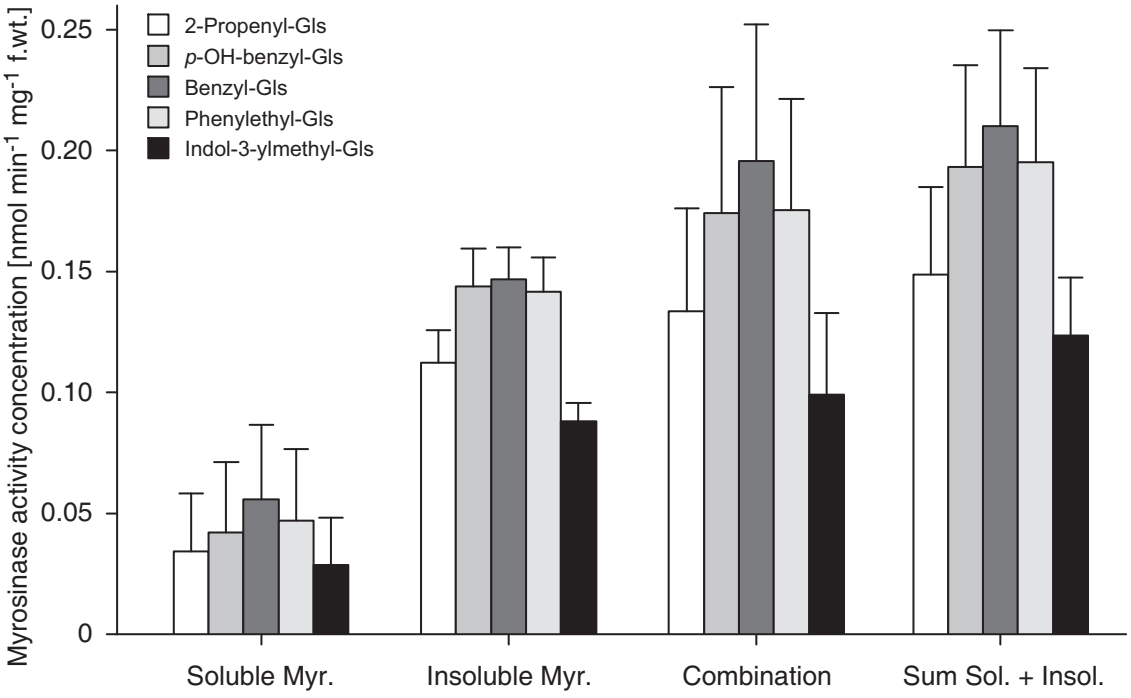

Figure 2.1: Substrate specificity in three independently prepared plant extracts (mean $\pm$ SE, n = 3) of soluble and insoluble myrosinase isoforms (myr.), a mixture of both and the arithmetic sum of soluble (sol.) and insoluble (insol.) activities. Young leaves of 3-week-old *S. alba* plants were analysed. Extracts were purified on DEAE Sephadex A-25 columns. *p*-OH-benzyl-Gls = *p*-hydroxybenzylglucosinolate; Gls = glucosinolate.

2.3.4 Effects of ascorbic acid on glucose determination

In plant tissues, ascorbic acid levels range from around 1 to 10 μmol g^{-1} fresh weight (for a compilation of literature data see Table 2.3). In our assays, these would relate to concentrations of 0.01 to 0.4 mM (for estimation details see Table 3). Concentrations of 0.01 to 0.3 mM of ascorbic acid reduced absorption values for glucose detection (Figure 2.2A) whereas those higher than that could prevent colouration (data not shown; Kleinwächter and Selmar, 2004). Regarding our glucose dilution series, the slope of the resulting curve depending on ascorbic acid concentrations remained constant, whereas the y-intercept was reduced proportionally to the amount of ascorbic acid present in the assay. Thus, the amount of glucose determined the maximum ascorbic acid concentration that could be present for color development (x-intercept, Figure 2.2B; $y = 0.005x \pm 0.003$; Pearson's correlation coefficient: $R^2 = 0.99$, $F_{1,26} = 6190.7$, $P<0.001$). In contrast to absolute absorption values, colour production rates were not affected by ascorbic acid concentrations (Figure 2.2C).

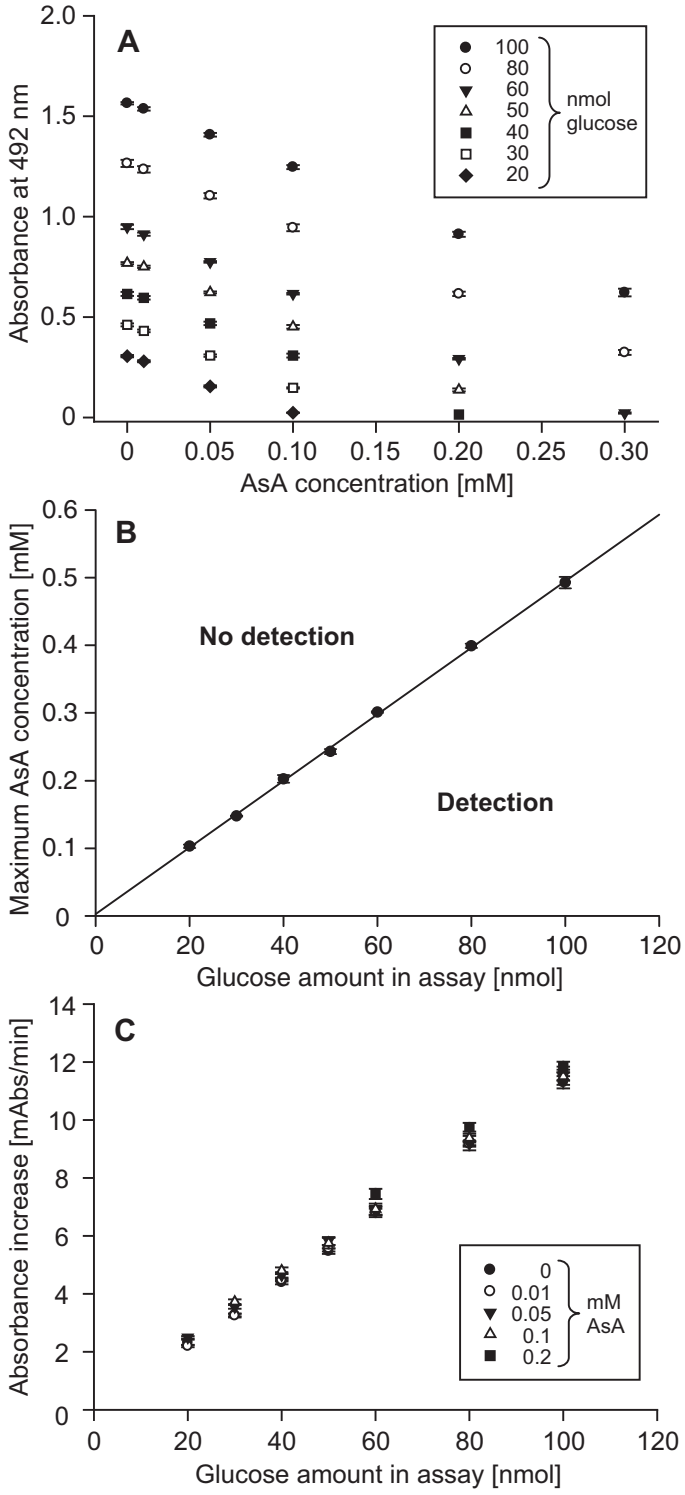

Figure 2.2: Influence of different ascorbic acid concentrations on glucose colour production (mean ± SE, n = 4). A - Absolute values of maximum absorbance at 492 nm between 75 and 85 min after start of reaction with glucose oxidase, peroxidase, 4-aminoantipyrine, phenol and imidazole in a volume of 225 μL; B - maximum ascorbic acid concentrations for glucose detection (x-intercept of panel A). Colour production only occurs below the regression line; C - colouration rates of glucose amounts depending on ascorbic acid (= AsA) concentrations between 10 and 30 min after start of reaction.

2.4 Discussion

2.4.1 Sample preparation

Plant tissue samples for myrosinase activity determinations should be processed as little as possible to prevent unwanted decomposition processes. A previous experiment revealed that there were no differences in activity measurements between fresh and frozen leaf material (data not shown). Freeze-drying significantly reduced myrosinase activity concentrations of samples (Table 2.2). To yield complete cell extracts, cryogenic grinding with steel beads in a vibration mill is most appropriate. These cell extracts can be subsequently processed without material losses (Markert, 1995). The separation of mortar ground seed tissue required at least three subsequent extraction steps and centrifugation in between at a minimum of 10,000 × g. Eriksson and co-workers extracted already 90 % of soluble myrosinase activity with two subsequent extractions and could demonstrate complete separation of soluble and insoluble isoforms by Western blot analysis after repeated extraction (Eriksson *et al.*, 2001). We obtained about 95 % of soluble myrosinase activity (data not shown) in *S. alba* cotyledons with two extraction steps of cryogenic ground tissue. Three extraction steps are therefore obligate for a sufficient separation of myrosinase fractions. Consequently, the supernatant contains next to the soluble myrosinase isoforms all other soluble proteins and metabolites, whereas the cell debris and insoluble proteins and molecules are present in the pellet which is, however, free from endogenous glucose and glucosinolates.

In assay mixtures containing sub-saturating concentrations of externally added substrate, varying endogenous substrate levels would lead to erroneous measurements of enzyme activities (Segel, 1975) and should thus be removed from the extracts previous to enzyme measurements. The selective binding of glucosinolates using purified standards or plant samples was efficient on DEAE Sephadex A-25 ion exchange columns which are extensively used in the normal extraction procedure for glucosinolates from plant tissues (Heaney and Fenwick, 1981; Agerbirk *et al.*, 2001). After application of 100 nmol 2-propenylglucosinolate or *p*-hydroxybenzylglucosinolate, the corresponding flow-through would contain about 100-fold less endogenous glucosinolate in the assay (~ 0.5 nmol in 100 μL per well) compared to the externally added 50 nmol of glucosinolate (in 25 μL of a 2 mM solution). In comparison, a gel filtration medium like Sephadex G-25, which has been previously used in myrosinase preparations of plant extracts (Siemens and Mitchell-Olds, 1998; Li *et al.*, 2000; Wallace and Eigenbrode, 2002), is non-interactive and its separation characteristics require the application of a volume smaller than the void volume of the column. Repeated loading of separate extractions onto a gel filtration column will not retain glucosinolates from the plant extracts and is therefore not applicable (Amer-

sham Handbook, 2002, 2004). The complete preparation procedure should be carried out on ice using cool buffers and columns. However, even at 4 °C myrosinase activity is only slowed down but not entirely inhibited (Björkman and Lönnerdal, 1973). Thus, products of myrosinase hydrolysis can be formed which in turn could lead to inhibition of enzyme activity in the assay to some degree (Shikita *et al.*, 1999). By usage of DEAE Sephadex A-25 however, substrate and enzymes of each extraction step are separated rapidly (flow rate: 500 µL in ~60 sec), so that uncontrolled product formation during preparation for enzyme measurements can be prevented as far as possible.

2.4.2 Assay conditions

The composition of the reaction mixture and the temperature conditions for myrosinase measurements were chosen in relation to the *in situ* situation of myrosinases in plants after tissue damage. Assays were conducted at the weakly acid pH value of crushed leaf material (value of 6) and at room temperature (23 ± 0.5 °C). Neither ascorbic acid nor protease inhibitors or other effectors were added, as they could modify enzyme activities in an unknown manner (see also Palmieri *et al.*, 1988). Almost all purified myrosinases studied are activated by ascorbic acid at low concentrations (0.3 to 2 mM) whereas they are inhibited at higher concentrations (>2 mM), however, with highly species-specific ranges (Table 2.1; Björkman and Lönnerdal, 1973; Bones and Slupphaug, 1989; Kleinwächter and Selmar, 2004). The multiple extractions of plant tissue samples resulted in a pellet that was deprived of all soluble cofactors. By mixing pellet and glucosinolate-free supernatant a combination could be obtained which supplied an identical milieu for both isoforms and allowed for realistic measurements of total myrosinase activity concentrations. In young leaves of pre-flowering mature plants of *S. alba* (Figure 2.1) as well as in *Brassica juncea* (L.) CZERN. (Müller and Sieling, 2006), the sum of the activity concentrations of soluble and insoluble isoforms tested separately had the same values as the activity concentrations of both fractions tested in combination. Thus, in these tissues the milieu of the supernatant is not crucial for the activity of pellet myrosinases. Nevertheless, at other developmental stages, or in different tissues or plant species, mixing of soluble and insoluble myrosinases could lead to divergent results.

In plants, qualitative and quantitative glucosinolate composition is species- and organ-specific and in many cases one compound is predominant (Fahey *et al.*, 2001; Brown *et al.*, 2003). In *S. alba* leaf and stem tissue *p*-hydroxybenzylglucosinolate makes up about 90 % of all glucosinolates present (Martin and Müller, 2007). We found that in young leaves of *S. alba* soluble and insoluble myrosinases hydrolysed all five glucosinolates tested with slightly different activities against the various glucosinolates (Figure 2.1). Similarly,

rather unspecific substrate activities of myrosinases were found in seeds of *B. napus* and *S. alba* (Björkman and Lönnerdal, 1973). In contrast, soluble and total myrosinases, i.e. a mixture of soluble and insoluble forms, of *Crambe abyssinica* HOCHST. were reported to be highly specific for 2(*S*)-2-hydroxy-3-butenylglucosinolate (epi-progoitrin; Bernardi *et al.*, 2003; Finiguerra *et al.*, 2001). For the determination of a plant tissue's capacity to produce hydrolysis products it is important to use a glucosinolate that naturally co-occurs with the myrosinases in question (Finiguerra *et al.*, 2001), preferably the most dominant glucosinolate. Otherwise, the enzyme's degradation capacity with respect to a different substrate might be under- or overestimated in comparison to *in situ* conditions.

The natural contents of glucosinolates in plants are about 1 % of the dry weight in *Brassica* vegetables, but can approach 10 % in the seeds of some plants (Fahey *et al.*, 2001). For a plant sample used for enzyme activity measurements that contains glucosinolates of about 1 % of dry weight this equals to a hypothetical homogeneous concentration of $\sim 3\,\mathrm{mM}$ in the tissue water (for 90 % water content of fresh weight, and a molar weight of glucosinolate on average of $400\,\mathrm{g\,mol^{-1}}$). This concentration is higher than most K_m-values reported to date for myrosinases (Table 2.1) and near or in the range of substrate saturation for soluble *S. alba* seed myrosinase (Hochkoeppler and Palmieri, 1992; Wilkinson *et al.*, 1984). Reaction mixtures imitating the milieu of plant tissues should contain substrate concentrations in a similar range. However, individual glucosinolates are partly difficult to obtain, expensive or need to be isolated from plant material. We therefore applied around 10-times less glucosinolate than would be optimal.

2.4.3 Detection

Soluble protein content varies according to age and type of plant tissues and plant species (Table 2.2; Martin and Müller, 2007; Reifenrath and Müller, 2007). The relationship of protein concentration and myrosinase activity is expressed as "specific activity" (Units $\mathrm{mg^{-1}}$ protein) which is used to characterize an enzyme's activation status (Moss, 1984; Stranger and Mitchell-Olds, 2005). In contrast, the concentration of myrosinase activity in relation to tissue fresh weight is a measure of the plant tissue's capacity to produce potentially toxic end products for defence against attacks by pathogens or herbivores, which is of interest especially for ecological and plant breeding studies. Nevertheless, a determination of soluble protein in the extract is necessary because the velocity of the enzyme needs to be in a range of time resolution and in the range of absorbance that is measurable. For tissue of true leaves of *S. alba* the optimum range of protein concentration in our tests was $200\,\mu\mathrm{g\,mL^{-1}}$, but for mature *A. thaliana* leaves and cotyledons of *S. alba* it was four-times lower. These figures result mainly from differences in specific activity of

myrosinases in the different tissues and plant species, but less from differences in protein concentrations (Table 2.2). The observed differences in specific activity could be due to varying numbers of myrosinase isoforms or due to different isoforms with altered turn-over numbers, e.g. because of conformation changes or differences in concentrations of cofactors in the sample. Furthermore, extraction procedures add volume to the original sample resulting in an inevitable dilution of all components, i.e. enzymes and cofactors. The concentrations of these components in plant material are higher than in the assays which might affect myrosinase activities. In our assay, we incorporated the analysis of myrosinase activities at two different protein concentrations of each extract and suspension. The two values obtained for myrosinase activity concentrations should be identical when they are corrected for the dilution. This way, dilution effects caused by effectors present in the sample or by the erroneous presence of endogenous substrate are easily detected.

Enzyme activities can be determined as the rate of disappearance of a substrate or the rate of appearance of a product (Segel, 1975). In case of myrosinase measurements, decrease of glucosinolates or formation of primary reaction products, such as glucose or thiohydroximate-*O*-sulphonates, or secondary reaction products, such as hydrogensulphate or further decomposition products ("mustard oils", e.g. isothiocyanates or nitriles), could be analysed (Figure 1.1B ❶-❺). Apart from the unstable thiohydroximate-*O*-sulphonates, all possible compounds have by now been used to determine myrosinase activity, mainly of soluble isoforms (Wilkinson et al., 1984; Palmieri et al., 1987; Kleinwächter and Selmar, 2004; Bellostas et al., 2006; and references therein). Some widely applied methods for the detection of myrosinase activities cannot be applied to turbid samples. The absorbance of a typical clear plant extract in Tris-EDTA extraction buffer was about 18-times higher in the UV range (200-350 nm) compared to the visible range of the light spectrum (400-700 nm) due to absorption by proteins, DNA or other metabolites (data not shown; see also Kleinwächter and Selmar, 2004). Additional light scattering in turbid samples, i.e. in pellet suspensions, yields an unfavourable signal to noise ratio. Thus, the direct measurement of the decrease of glucosinolates at a wavelength of 229 nm, preferable for activity measurements of purified enzymes, and the indirect measurements of developing glucose detected by glucose-hexokinase combined with glucose-6-phosphate dehydrogenase (GDH-method) at 340 nm are not suitable for an application in turbid samples (Figure 1.1B ❶ - ❸).

The analyses of the dynamic development of the enzyme reaction are essential and furthermore limit the choice of detection methods. Most of the mustard oils (Figure 1.1B ❺) generated by the decay of thiohydroximate-*O*-sulphonates cannot be easily analysed taking also the dynamic development of the reaction into account (Kleinwächter and Selmar, 2004). Additionally, condensation products with metabolites of the plant extracts

can occur (Bones and Rossiter, 2006) whose detection can be very laborious. Recently, a method using micellar electrokinetic capillary chromatography was successfully used to determine substrate and product of myrosinase catalysed reactions (Bellostas et al., 2006). This method requires only very low amounts of soluble enzyme preparation, but it is not stated whether insoluble myrosinases can be measured accordingly. The pH-stat assay was used successfully to determine kinetics of soluble and insoluble myrosinases *via* hydrogensulphate (Finiguerra et al., 2001; Figure 1.1B ❹). However, dialysis of all solutions is not only time-consuming, but also removes all low molecular substances, e.g. ascorbic acid, that affect myrosinase activity (Palmieri et al., 1987).

The advantages of determination of D-glucose release for myrosinase measurements are plenty: glucose is a common metabolite, for which a variety of commercial detection kits is available, endogenous glucose in the plant extract can be corrected for by background controls without glucosinolate, and standard curves are easily incorporated in the assays. Most of the glucose detection kits are enzyme coupled kits which generate a coloured product that can be measured photometrically, enabling users to conduct high-throughput assays on microplate photometers. In the GOD-PAP-method, glucose is oxidized by the enzyme glucose oxidase to form gluconic acid and hydrogenperoxide (Trinder, 1969; Kunst et al., 1984). In the presence of peroxidase, hydrogenperoxide reacts with 4-aminoantipyrine to form *N*-(4-antipyryl)-*p*-benzoquinone imine, which can be detected photometrically at 500 nm where turbidity and background absorption are negligible compared to the UV range. Glucose oxidase and peroxidase have been widely used for glucose determinations with various dyes (Björkman and Janson, 1972; Blake and McLean, 1989; Raba and Mottola, 1995; Wielanek and Urbanek, 1999; Pontoppidan et al., 2005) or coupled to an oxygen Clark electrode (Palmieri et al., 1987). Kleinwächter and Selmar (2004) reported that ascorbic acid interfered with the GOD-PAP-method by competing with the peroxidase for hydrogenperoxide. This resulted in reduced absorbance. We found the same reduction (Figure 2.2A) which was dependent on the glucose amount present in the reaction mixture (Figure 2.2B). Furthermore, colour production rates in the absence of myrosinase were not affected (Figure 2.2C). The dynamic determination of myrosinase activities is therefore free from ascorbic acid interferences compared to end-point measurements where absolute values could vary drastically.

2.4.4 Conclusions

Our study demonstrates that an existing enzyme assay for myrosinase activity measurements could be modified to measure both soluble and insoluble myrosinase isoforms of partially purified plant extracts and to exclude sources of inaccuracies. Free dimers and

insoluble complexes are only separated by repeated extraction steps of effectively disrupted cells of plant tissue. Ion-exchange columns should be used to remove endogenous glucosinolates from crude extracts. Myrosinase activity can then be measured as glucose release from standardized concentrations of externally added substrate for every sample. A dynamic measurement of glucose *via* the GOD-PAP-method has a favourable signal to noise ratio and is free from ascorbic acid interferences. It allows synchronous measurements of the reaction by which the linear part of the reaction progress curve can be determined. The adaptation for any plant species with the appropriate glucosinolate is easy. Thereby, it is possible to reliably determine myrosinase activities taking place in damaged plant tissues and thus characterize potential ecological or physiological processes of the plant and its environment.

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Table 2.3: Ascorbic acid concentrations in tissues of several plant species of Brassicaceae.

Plant species	Plant age	Tested organ(s)	AsA concentration [a]	Assay concentration [b]	Reference(s)
Arabidopsis thaliana (L.) Heynh., ecotype Columbia	6-week-old plants	Leaves	∼2.5	0.11	Lorence *et al.* (2004)
		Stems	∼2.3	0.10	
		Flowers	∼8	0.36	
		Roots	<1	0.04	
		Siliques	∼7	0.31	
	10-day-old plants	Leaves	∼1	0.04	Sasaki-Sekimoto *et al.* (2005)
		Induction by JA	after induction ∼2	0.09	
Brassica juncea (L.) Czern.	14-day-old plants	Roots	7 - 9	0.31 - 0.40	Wang *et al.* (2004)
	10-day-old plants	Whole seedlings	∼0.3	0.01	Qadir *et al.* (2004)
Brassica oleracea L. var. *italica* Plenck [c]	85-days and older	Edible portions of inflorescences	2.3 - 6.8	0.1 - 0.3	Vallejo *et al.* (2003)
Brassica oleracea L. var. *capitata* [d]	At edible maturity	Uniform sized inflorescences	0.3 - 1.3	0.01 - 0.06	Singh *et al.* (2006)
Sinapis alba L.	3-day-old seedlings	Roots	1.7 - 3.4	0.08 - 0.15	Pihakaski and Pihakaski (1978)
		Hypocotyls	1.1 - 3.4	0.05 - 0.15	
		Cotyledons	0.6 - 3.4	0.03 - 0.15	
Sinapis incana L. [e]	Field collection of mature plants	"edible portions of plants"	0.69	0.03	Salvatore *et al.* (2005)
Sinapis nigra L. [f]			0.63	0.03	
Diplotaxis erucoides (L.) DC.		=presumably leaves	0.82	0.04	

Notes: [a] Ascorbic acid (AsA) concentration [μmol g^{-1} f.wt.]; [b] Assay ascorbic acid concentration [mM]: sample weight 200 mg, normal extraction procedure, 2 mL total extract volume, 100 μL of extract in 225 μL reaction mixture; [c] - [f] Official names according to SysTax-Database (`http://www.biologie.uni-ulm.de/systax/`): [c] *Brassica oleracea* L. ssp. *oleracea* convar. *botrytis* (L.) Alef. var. *italica* Plenck, [d] *Brassica oleracea* L. ssp. *oleracea* convar. *capitata* (L.) Alef. var. *capitata* L., [e] *Hirschfeldia incana* (L.) Lagr.-Foss.; [f] *Brassica nigra* (L.) W.D.J.Koch; JA - Jasmonic acid.

Chapter 3

Induction of plant responses by a sequestering insect: Relationship of glucosinolate concentration and myrosinase activity

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Summary - Induction of plant allelochemicals is of particular ecological importance for interactions with herbivores that can make use of induced metabolites by incorporating them for their own defence. Induction patterns in white mustard, *Sinapis alba*, were investigated following herbivory of the turnip sawfly, *Athalia rosae*, which sequesters plant glucosinolates. Larvae of different age were allowed to feed for 24 hours on young leaves of pre-mature, non-flowering plants. Changes in primary and secondary metabolites were recorded in the damaged leaves (local) and in the adjacent leaves and stems (systemic) for several days. Organ and time specific patterns were evident. Local responses included increases in glucosinolate concentrations, soluble and insoluble myrosinase activity and glucose levels, while systemic responses in leaves were restricted to increases in myrosinase activities and glucose. All effects were strongest immediately after feeding and declined mostly within a day. Stems had overall lower constitutive levels of glucosinolates and myrosinase activities than leaves. Feeding by one large larva had a greater impact on the plant's physiology than feeding by three small ones, even though both treatments resulted in quantitatively similar leaf destruction. Local increase in glucosinolate concentration could be beneficial for larvae, while conspecifics feeding on induced adjacent leaves might be negatively affected due to higher myrosinase activity levels. The results are discussed in the context of the "optimal defence hypothesis" and the "lethal plant defence paradox".

Zusammenfassung - Die Induktion pflanzlicher Allelochemikalien ist vor allem für Interaktionen mit Herbivoren von ökologischer Bedeutung, die diese induzierten Metabolite zu ihrer eigenen Verteidigung in ihr Körpergewebe einbauen. Reaktionen des Weißen Senfs *Sinapis alba* auf den Fraß von Larven der Blattwespe *Athalia rosae* wurden ort- und zeitabhängig untersucht. Die Larven zeichnen sich durch die Fähigkeit aus, Glucosinolate zu sequestrieren. Junge Blätter von nicht blühenden Pflanzen wurden für 24 Stunden von Larven unterschiedlichen Alters befressen. Anschließend wurden Veränderungen von Primär- und Sekundärmetaboliten in lokalen befressenen, sowie in benachbarten systemischen Blättern und Sprossachsenabschnitten an den vier folgenden Tagen untersucht. Die gefundenen Reaktionen waren zeitabhängig und spezifisch für das jeweils betrachtete Organ. Lokale Reaktionen umfassten Anstiege im Gehalt von Glucosinolaten, löslichen und unlöslichen Myrosinaseaktivitäten und Glucose, während sich systemische Antworten in Blättern auf Anstiege in Myrosinaseaktivität und Glucosegehalt beschränkten. Alle gefundenen Effekte waren direkt nach der Fraßperiode am stärksten und nahmen in den meisten Fällen bis zum Folgetag ab. Sprossachsenabschnitte hatten niedrigere konstitutiv vorhandene Konzentrationen an Glucosinolaten und Myrosinaseaktivitäten als Blattgewebe. Der Fraß einzelner größerer Larven zeigte stärkeren Einfluss auf die Pflanzenphysiologie als der von Gruppen kleiner Larven, obwohl beide Behandlungen zu einer quantitativ ähnlichen Blattbeschädigung führten. Der lokale Anstieg von Glucosinolaten könnte für die Larven vorteilhaft sein, während Artgenossen, die an induzierten benachbarten Blättern

fressen, möglicherweise durch erhöhte Myrosinaseaktivitäten beeinträchtigt werden. Die Ergebnisse werden vor dem Hintergrund der „optimal defence hypothesis“ und dem „lethal plant defence paradox“ diskutiert.

Key words: *Sinapis alba* (white mustard), Brassicaceae, *Athalia rosae* (turnip sawfly), primary metabolites, “lethal plant defence paradox”, “optimal defence hypothesis”.

3.1 Introduction

The distribution, concentration, and composition of various toxic or bitter tasting plant secondary metabolites such as glucosinolates are known to depend on many factors, e.g. nutritional state, age, tissue, genotype, population, or species (Clossais-Besnard and Larher, 1991; Visvalingam *et al.*, 1998; Fahey *et al.*, 2001; Brown *et al.*, 2003; Charron *et al.*, 2005a,b). In many cases, these compounds confer efficient defence against pathogens or herbivores (Blau *et al.*, 1978; Bennett and Wallsgrove, 1994; Siemens and Mitchell-Olds, 1996; Ludwig-Müller *et al.*, 1997; Li *et al.*, 2000; Wittstock and Gershenzon, 2002). McKey (1974, 1979) proposed an adaptive hypothesis to explain variability of defence levels within a plant individual. According to this so-called "optimal defence hypothesis", tissues that are most valuable to the plant are expected to have the highest constitutive defence levels, and their secondary metabolites to be least inducible by damage. In plants with one single shoot, we expect the stem's tissue to have a high constitutive level of defence, because damage would result in reduced water and nutrient supply for all tissues above, and thus reproduction success of the whole plant would be affected dramatically. In contrast, damage to assimilating leaves, except to cotyledons (Wallace and Eigenbrode, 2002), should be less severe for reproduction, as has been shown, for example, for *Raphanus sativus* L. (Mauricio and Bowers, 1990). Thus, in leaves inducible responses rather than constitutive defences are likely to be observed. On the other hand, vulnerability of tissue might be higher for leaves than for stems since the latter are often well protected by physical features. Thus, value and vulnerability need to be ranked when evaluating optimal defence (McKey, 1974). Induced defences (reviewed in Dicke and Hilker, 2003) are thought to have evolved as a cost saving strategy for plants growing under nutrient limited conditions (Agrawal, 1999a, 2005; Cipollini *et al.*, 2003b). Given an energetic cost of plant allocation from growth to defence, plant fitness could be enhanced by rising defence levels only in the presence of herbivory (Agrawal, 1999a, 2005). In Brassicaceae, induced responses have been widely studied using a large diversity of organisms, chemical signals or mechanical wounding (Shattuck, 1993; Menard *et al.*, 1999; Rostás *et al.*, 2002; Agrawal and Kurashige, 2003). Brassicaceae contain, amongst other mechanisms, a binary defence system consisting of preformed substrates and hydrolytic enzymes, namely glucosinolates and myrosinases (Halkier, 1999; Rask *et al.*, 2000; Andréasson and Jørgensen, 2003; Kliebenstein *et al.*, 2005). Glucosinolates consist of a thioglucoside moiety linked to a variety of amino acid-derived side chains (Andréasson and Jørgensen, 2003). Myrosinases (β-thioglucoside glucohydrolases) occur in various isoforms (reviewed in Rask *et al.*, 2000). Some of these are soluble under non-denaturing conditions. Others have been shown to form a complex with two groups of proteins, myrosinase-associated proteins (MyAPs) and myrosinase-binding proteins (MBPs, Eriksson *et al.*, 2001, 2002). These complexes are

insoluble in non-denaturing buffers but the myrosinase subunits are still active. Upon tissue disruption the separately stored glucosinolates and myrosinases interact to form various mostly toxic products (Halkier, 1999; Wittstock *et al.*, 2003).

So far, in induction studies on Brassicaceae both parts of the putative defence system have rarely been investigated simultaneously (Siemens and Mitchell-Olds, 1998; Andréasson *et al.*, 2001b). In most induction studies, either glucosinolates or myrosinases have been addressed, whereby important relationships might have been missed. Using microorganisms or herbivores as eliciting agents, it has been shown that especially glucosinolates with an indolic side chain increase in concentration (Koritsas *et al.*, 1991; Ludwig-Müller *et al.*, 1997; Bartlet *et al.*, 1999; Agrawal, 1999a; Rostás *et al.*, 2002). Increases of glucosinolates caused a higher resistance of plants against specialist herbivores in several cases (Agrawal *et al.*, 2002; Mewis *et al.*, 2005). Also, activity of soluble myrosinases can increase after damage (Siemens and Mitchell-Olds, 1998; Pontoppidan *et al.*, 2003, 2005). Additionally, transcript and protein levels of MyAPs and MBPs were shown to be inducible by damage or treatment with the plant hormone jasmonate (Taipalensuu *et al.*, 1996, 1997a; Pontoppidan *et al.*, 2003, 2005). Thus, studies monitoring the activity of either soluble or bound myrosinases only are likely to underestimate total activity of a plant's tissue. Therefore, we established a method to determine myrosinase activity of soluble and insoluble fractions of the enzyme, and correlated these with glucosinolate levels of the same tissues.

In previous studies, periods of herbivory as well as the time between treatment and sampling of tissues varied from less than an hour to a few weeks (Cipollini *et al.*, 2003a; Strauss *et al.*, 2004; Pontoppidan *et al.*, 2005). Systemic responses were examined after treatment of leaves with plant hormones, i.e. jasmonates or salicylates, in younger parts (only leaves) and older parts (only roots) of plants (Bodnaryk, 1994; Ludwig-Müller *et al.*, 1997, 2002; van Dam *et al.*, 2003). In contrast, responses to herbivore feeding were analysed only in younger tissue and mostly at a distance of two or more leaves from the site of damage (Bartlet *et al.*, 1999; Traw and Dawson, 2002). Direct neighbouring tissues have rarely been monitored (Koritsas *et al.*, 1991; Pontoppidan *et al.*, 2005). Thus, little is known about the short-term pattern of metabolite induction in the older and younger stems and leaves directly surrounding the site of damage.

Herbivores used as inducing agents have so far included few generalists and a variety of crucifer specialists (Bodnaryk, 1992; Siemens and Mitchell-Olds, 1998; Traw and Dawson, 2002; Pontoppidan *et al.*, 2003, 2005), in particular caterpillars of *Pieris* spp. (Traw and Dawson, 2002; Agrawal and Kurashige, 2003; Strauss *et al.*, 2004), which do not sequester glucosinolates (Müller *et al.*, 2003), or aphids (Pontoppidan *et al.*, 2003; Mewis *et al.*, 2005). The turnip sawfly, *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae), is oligophagous on species of Brassicaceae and can reach pest status on some crop species

(Riggert, 1939; Sáringer, 1976, 1989; Šedivý and Vašák, 2002). The larvae sequester certain glucosinolates of their host plants within their haemolymph (Müller *et al.*, 2001; Müller and Wittstock, 2005) and use it in part for their own defence (Müller *et al.*, 2001, 2002; Müller and Brakefield, 2003). For plants, sequestering insects represent a special problem, termed "lethal plant defence paradox" (Price *et al.*, 1980), as these herbivores benefit from their hosts' putative defence compounds. For insects, sequestration of secondary compounds from host plants may be costly (Camara, 1997; Kelley *et al.*, 2002), and for *A. rosae* additional costs might result from the necessity to efficiently circumvent hydrolysis of glucosinolates. Since *A. rosae* is not only able to overcome the glucosinolate-myrosinase system but also to use it for its own protection (Müller *et al.*, 2001, 2002; Müller and Brakefield, 2003), we were particularly interested in the response of the plant to such a herbivore. We specifically addressed the question of whether feeding of approximately the same amount of leaf tissue by three young *A. rosae* larvae or by one old larva might result in different induction patterns. In many insect species, first-instar larvae are more susceptible to secondary plant metabolites than older ones (Zalucki *et al.*, 2002). Consequently as a possible solution to the lethal plant defence paradox, a vigorous increase of the (putative) plant defence levels in response to feeding by younger larvae was expected, while a rather weak increase should result from induction by older ones, as proposed earlier for the interaction of monarch butterfly larvae (*Danaus plexippus* L.) with milkweed (*Asclepias syriaca* L., Malcolm and Zalucki, 1996).

In this study, we investigated induction patterns in the herbivore wounded leaf and upwards and downwards from the damaged site in the adjacent stem parts and leaves over several days. We determined glucosinolate concentrations as well as activities of soluble and insoluble myrosinases and also analysed these tissues for water, protein and sugar content to examine nutritional changes. This setup enabled us to reveal relevant induction responses with a high resolution in the host plant upon damage of a sequestering insect.

3.2 Materials and methods

3.2.1 Planting and induction experiment

Seeds of *Sinapis alba* L. (cultivar: Salvo, obtained from Advanta Seeds B.V., Kapelle, Netherlands) were sown in mid May 2004 and kept in a climate chamber at 25 °C, 16:8 hours light-dark-cycle, and 70 % relative humidity (light source: Osram L 58 W/25 Universal White, 4150 lumens, Osram, Munich, Germany). Seven days later, the seedlings were transferred to individual pots (diameter: 9 cm; height: 7 cm) with unfertilised soil (peat, pH 6). After 21 days, when five to six true leaves had developed, plants received

one of the following four treatments on the second youngest leaf: (a) three small male larvae (two days after egg hatch, first larval instar) of *A. rosae* enclosed with the leaf in a muslin bag (8 x 10 cm, 1 mm mesh width); (b) one large male larva (six days after egg hatch, third larval instar) in a bag; (c) a bag only; (d) no treatment. Seven replicates per treatment and per day of harvest were prepared. Plants of all treatments were placed in random order in the climate chamber. Sawfly larvae were taken from a laboratory culture established from a field collection in Germany in the previous autumn.

Larvae and muslin bags were removed after 24 hours. At that time, an estimated amount based on leaf weights of about 37 and 43 % had been consumed by three small larvae and one large larva, respectively. Harvests were conducted immediately after feeding (day one) and on days two, three, and five. Seven plants of each treatment were harvested per day. Samples were taken from damaged leaves, as well as the flanking upper and lower leaves and stems. Leaves and stems were cut longitudinally, weighed, frozen in liquid nitrogen, and stored at $-80\,^{\circ}$C for later analysis. This allowed us to analyse both glucosinolate and myrosinase levels from the same tissue parts.

3.2.2 Analysis of glucosinolates

Frozen samples were freeze-dried, weighed, and pulverised in a mill (Retsch, MM301, Haan, Germany). Glucosinolates were extracted in 80 % methanol after addition of 20 μL of a 5 mM solution of allylglucosinolate (Merck, Darmstadt, Germany) as internal standard. Glucosinolate analysis was performed by conversion to desulphoglucosinolates as described elsewhere (Agerbirk *et al.*, 2001). For analysis of desulphoglucosinolates, samples were subjected to HPLC analysis on a 1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Elution was accomplished on a Supelco C-18 column (Supelcosil LC-18, 250 x 4.6 mM, diameter 5 μm, Supelco, Bellefonte, Pennsylvania, USA) with a gradient (solvent A: water, solvent B: methanol) of 0-5 % B (10 min), 5-38 % B (24 min), followed by a cleaning cycle (38-100 % B in 4 min, 6 min hold, 100 to 0 % B in 5 min, 7 min hold). Peaks were quantified by the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, applying the response factors as used by (Müller *et al.*, 2001; Brown *et al.*, 2003). Desulphoglucosinolates were identified by comparison of retention times and UV spectra to those of purified standards. The identities of the desulpho derivatives of *p*-hydroxy(*p*-OH)-benzylglucosinolate (sinalbin), benzylglucosinolate (glucotropaeolin), indol-3-ylmethylglucosinolate (glucobrassicin), and 4-methoxyindol-3-ylmethylglucosinolate (4-methoxy-glucobrassicin) had been confirmed in earlier studies (Müller *et al.*, 2001; Müller and Martens, 2005). Three minor compounds were considered as

aromatic glucosinolates due to their characteristic UV spectra but could not be further identified. For these, an estimate response factor of 0.5 was used according to the factor for the aromatic sinalbin.

3.2.3 Analysis of myrosinase activity

Myrosinase activity was determined by photometric quantification of released glucose from the externally added substrate *p*-OH-benzylglucosinolate (obtained from glucosinolates.com, Denmark), which is the most dominant glucosinolate in *S. alba*. Myrosinase extraction followed a slightly modified protocol described previously (Siemens and Mitchell-Olds, 1998; Li *et al.*, 2000; Müller and Martens, 2005; Chapter 2). Frozen leaf material was pulverised in a mill (Retsch, MM301, Haan, Germany), extracted three times in 500 μL extraction buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice, and centrifuged at 13,000 rpm (16,060 $\times$ g) for 10 min at 4 °C. A control experiment had revealed that fresh and frozen samples resulted in similar measurements and that three subsequent extraction steps were sufficient to extract virtually all soluble myrosinases from the tissue (data not shown; see also Eriksson *et al.*, 2001). Supernatants were subsequently subjected to ion-exchange in 400 μL Sephadex A25-columns (0.1 g DEAE Sephadex A25 welled in 2 mL of 0.5 M acetic acid buffer, pH 5; Sigma-Aldrich, St. Louis, Missouri, USA) to eliminate internal glucosinolates, and samples were eluted from columns with further 500 μL buffer. Protein concentrations were determined (see below), and extracts were adjusted to 200 μg mL^{-1} soluble protein per leaf sample and to 20 μg mL^{-1} per stem sample where possible. For determination of activity of soluble myrosinases, 100 μL of each extract were added to four individual cells on a 96-cell microplate with 25 μL of 1.9 mM *p*-OH-benzylglucosinolate/phosphate buffer in two cells as substrate. The other two cells served as background controls and 25 μL phosphate buffer were added instead of glucosinolate. Released glucose was measured by addition of 50 μL freshly mixed colour reagent, including glucose oxidase, peroxidase, 4-aminoantipyrine and phenol (Trinder, 1969; Siemens and Mitchell-Olds, 1998). The glucose release was determined by measuring the absorbance at 490 nm on a Multiskan EX (Thermo Labsystems, Vantaa, Finland) for 45 min (with 1 measurement min^{-1} and shaking mode between measurements) at room temperature (22 $-$ 24 °C). Means of the two replicate measurements were calculated after subtraction of the means of the background controls. A glucose standard curve was included in each assay. The resulting enzyme kinetics were analysed for a linear range of enzyme activity (usually between 10 and 30 min) and a time-frame of at least 10 time points was used to determine enzyme activity. It has been shown that ascorbate in high concentrations can interfere with the glucose colour reaction by decreasing absorption values (Kleinwächter and Selmar, 2004). However, under our assay conditions rates of colouration were not

affected by ascorbate (Chapter 2). The remaining pellets were dissolved in 500 μL buffer and two further assays performed. To assess the myrosinase activity of insoluble myrosinases exclusively, 250 μL pellet suspension were diluted with 750 μL buffer to yield a total volume of 1 mL. To assess the interaction of soluble and insoluble myrosinases and measure total myrosinase activity, 250 μL of pellet suspension were centrifuged at 13,000 rpm (16,060 $\times$ g) for 10 min at 4 °C. The supernatants were discarded and the dry pellets were dissolved in 1 mL of corresponding filtered protein extract solution. Thereby, a a suspension was obtained with a reconstituted ratio of soluble and insoluble myrosinases alike the original extract but free from interfering internal glucosinolates. Measurements of glucose release were conducted in the same way as for the supernatants.

3.2.4 Analyses of primary metabolites and water content

Protein concentration was determined with Bradford reagent (Sigma) by using bovine serum albumin (1.4 mg mL^{-1} in extraction buffer) as a standard. The absorbance at 595 nm was measured immediately after 5 min of shaking at room temperature. Protein concentrations were calculated from obtained absorption values using a cubic polynomial standard curve. Glucose concentration was determined using the background controls of the myrosinase assay (see above), and rates of absorbance increase were determined. Maximum rates of colour production were observed between 5 and 15 min. By relating sample maximum rates to those of the standard curve, glucose concentrations were obtained for each sample. Water contents of tissues were determined after freeze-drying of samples.

3.2.5 Statistical analyses

Control groups with and without bag treatment were analysed for effects on quantities of primary and secondary compounds by Mann-Whitney-U-tests. The effects of bag treatments with and without larvae on quantities of primary and secondary compounds were analysed separately for each organ and day of harvest by Kruskal-Wallis tests followed by multiple comparisons of ranks. A comparison of values from harvests on days one and two was achieved by Mann-Whitney-U-tests. Correlations between total myrosinase activites and p-OH-benzylglucosinolate levels were analysed by Pearson-Product moment correlations for each day and tissue separately (with three treatments: n=21 per correlation). Values for total myrosinase activity obtained from combined measurements of fractions were tested against values from the numerical addition of separately assessed values for both fractions by Wilcoxon tests for matched pairs.

3.3 Results

Since neither levels of primary and secondary metabolites, nor enzyme activities differed significantly between plants treated with muslin bags and non-treated plants in any of the Mann-Whitney-U-tests, in the following we will focus on the bag treatment as the more relevant control compared to feeding treatments.

3.3.1 Glucosinolate levels

The following seven glucosinolates were detected in *S. alba* in decreasing relative amounts: *p*-OH-benzylglucosinolate (about 90 % of total), benzylglucosinolate, 4-methoxy-indol-3-ylmethylglucosinolate, indol-3-ylmethylglucosinolate, and three minor aromatic glucosinolates not identified further. Leaves had higher glucosinolate concentrations than stems (Tables 3.1 and 3.2).

On day one, levels of total glucosinolates (sum of seven compounds) were higher in herbivore damaged leaves and adjacent younger stem parts than in those treated with bags only, irrespective of larval age (Table 3.1; Kruskal-Wallis test $P < 0.05$, multiple comparisons of ranks $P < 0.08$). The main component *p*-OH-benzylglucosinolate showed similar tendencies (Figure 3.3), but treatment effects were not significant. On day two, the effects declined.

In contrast, benzylglucosinolate concentrations of several organs differed strongly between treatments on day one (Table 3.2). Feeding by small and large *A. rosae* larvae led to more than nine-fold increases of benzylglucosinolate levels in damaged leaves and more than two-fold increases in systemic leaves and younger stems compared to the bag treatment. One day later (day two), overall benzylglucosinolate levels decreased, but significant differences were still present in damaged and younger leaves and also in younger stems, with highest levels in plants that had received a single large larva (Table 3.2). No significant effects were found on days three and five (data not shown).

Contrasting with these effects, a very strong induction of indol-3-ylmethylglucosinolate was found only in damaged leaves (Figure 3.1) but not in systemic tissues. After 24 hours of feeding, levels increased on average to 19-fold in leaves treated with single large larvae (multiple comparison with control $P < 0.01$) and to 12-fold in leaves treated with three small larvae ($P < 0.05$). In the following days, the concentration of indol-3-ylmethylglucosinolate gradually decreased in larvae-treated plants to levels of bag-treated plants.

Table 3.1: Levels of total [§] glucosinolates [μmol g^{-1} f.wt.] in leaves and stems of *Sinapis alba* cv. Salvo; mean ± SE. The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag. Bagged leaves without larvae served as controls (n = 7 plants per treatment).

		Treatment						
Organ		Bag		Three small larvae + bag		One large larva + bag		KW [†] P
Damaged leaf	d1	3.9 ± 0.4	a	5.0 ± 0.3	ab	5.5 ± 0.4	b [‡]	0.042
	d2	4.4 ± 0.1		4.4 ± 0.3		5.1 ± 0.3		n.s.
		n.s.		n.s.		n.s.		
Older leaf	d1	3.7 ± 0.4		3.5 ± 0.2		3.4 ± 0.3		n.s.
	d2	3.6 ± 0.2		3.1 ± 0.2		3.1 ± 0.1		n.s.
		n.s.		n.s.		n.s.		
Younger leaf	d1	5.1 ± 0.4		5.3 ± 0.2		5.6 ± 0.4		n.s.
	d2	5.1 ± 0.2		4.6 ± 0.1		5.2 ± 0.5		n.s.
		n.s.		n.s.		n.s.		
Older stem	d1	1.0 ± 0.2		1.4 ± 0.2		1.4 ± 0.2		n.s.
	d2	0.8 ± 0.1		0.9 ± 0.1		1.0 ± 0.2		n.s.
		n.s.		*		n.s.		
Younger stem	d1	1.8 ± 0.4	a	3.4 ± 0.5	b [‡]	3.4 ± 0.7	ab	0.047
	d2	1.7 ± 0.5		1.6 ± 0.3		2.0 ± 0.5		n.s.
		n.s.		*		n.s.		

Notes: [§] Total glucosinolates are the sum of seven individual compounds found: *p*-hydroxybenzylglucosinolate, benzylglucosinolate, indol-3-ylmethylglucosinolate, 4-methoxy-indol-3-ylmethylglucosinolate, three minor aromatic, not identified glucosinolates. [‡] Treatment effects were tested by Kruskal-Wallis-tests (KW) and subsequent multiple comparisons of ranks (significant differences are marked with different letters, $P<0.05$, or otherwise stated). [‡] Multiple comparisons of ranks were not significant with $P<0.05$, differences from control $P<0.08$. Differences between harvests on day one (d1) and two (d2) were tested by Mann-Whitney-U-tests. n.s. - not significant.

Table 3.2: Levels of benzylglucosinolate [$\times 10^{-1}$ μmol g^{-1} f.wt.] in leaves and stems of *Sinapis alba* cv. Salvo; mean ± SE. The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag. Bagged leaves without larvae served as controls (n = 7 plants per treatment).

		Treatment						
Organ		Bag		Three small larvae + bag		One large larva + bag		KW † *P*
Damaged leaf	d1	**0.6** ± 0.2	a	**5.0** ± 1.3	b	**5.9** ± 2.7	b	0.003
	d2	**0.6** ± 0.2	a	**2.7** ± 1.5	ab	**3.9** ± 1.1	b	0.034
		n.s.		n.s.		n.s.		
Older leaf	d1	**0.3** ± 0.1	a	**0.6** ± 0.1	ab	**1.9** ± 0.8	b	0.040
	d2	0.2 ± 0.1		0.6 ± 0.4		1.7 ± 0.8		n.s.
		n.s.		n.s.		n.s.		
Younger leaf	d1	2.1 ± 0.6	a	6.0 ± 1.6	ab	7.3 ± 2.3	b ‡	0.038
	d2	**1.3** ± 0.2	a	**4.4** ± 1.7	ab	**5.1** ± 1.5	b	0.041
		n.s.		n.s.		n.s.		
Older stem	d1	0.2 ± 0.1		0.6 ± 0.1		0.6 ± 0.0		n.s.
	d2	0.1 ± 0.0		0.5 ± 0.1		0.5 ± 0.2		n.s.
		n.s.		n.s.		n.s.		
Younger stem	d1	**0.6** ± 0.3	a	**1.7** ± 0.5	ab	**1.7** ± 0.3	b	0.018
	d2	**0.2** ± 0.0	a	**0.8** ± 0.2	b	**1.1** ± 0.4	b	0.008
		n.s.		n.s.		n.s.		

Notes: † Treatment effects were tested by Kruskal-Wallis-tests (KW) and subsequent multiple comparisons of ranks (significant differences are marked with different letters and values are highlighted in bold, $P<0.05$, or otherwise stated). ‡ Multiple comparisons of ranks were not significant with $P<0.05$, differences from control $P<0.08$. Differences between harvests on day one (d1) and two (d2) were tested by Mann-Whitney-U-tests. n.s. - not significant.

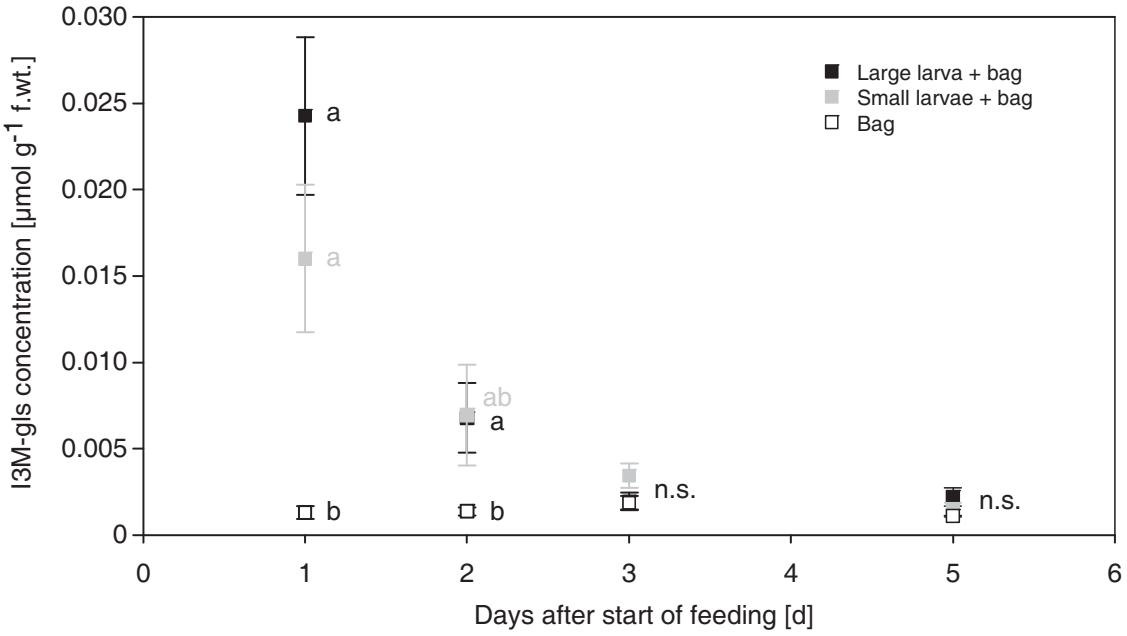

Figure 3.1: Indol-3-ylmethylglucosinolate levels (μmoles g^{-1} fresh weight, mean $\pm$ SE) in damaged leaves of *Sinapis alba* cv. Salvo. The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag for 24 hours. Bagged leaves without larvae served as controls (n = 7 plants per treatment). Treatment effects for each day were tested separately by Kruskal-Wallis tests and subsequent multiple comparisons of ranks (significant differences are marked with different letters, $P < 0.05$, n.s. - not significant).

3.3.2 Myrosinase activity

Effects on myrosinase activity varied between soluble and insoluble myrosinases (Figure 3.2). While activities of soluble myrosinases were induced only slightly due to larval feeding, a strong increase of activities of insoluble myrosinases could be detected compared to control plants treated with a bag on day 1. Stems were less affected and showed in general lower activities than leaves. In damaged leaves, activities of insoluble myrosinases significantly increased to about three-fold by treatment with single large larvae and groups of small larvae compared to bagged leaves. In systemic leaves, activities of insoluble myrosinases were induced less drastically than in damaged leaves. In almost all tissues, increases in activities of soluble and insoluble myrosinases were more influenced by feeding of single large larvae than of groups of small larvae. On day 2, myrosinase activities were lower than before. Significant increases of myrosinase activities remained only in systemic leaves of plants with previous feeding by single large larvae. While in older leaves only

soluble myrosinase activity was still affected on day two, in younger leaves both fractions showed increased activities compared to values of bag-treated plants. No significant effects were found on days three and five (data not shown).

Values for myrosinase activities of soluble and insoluble fractions tested together (= total myrosinase activity) were slightly but significantly reduced by $12.3 \pm 3.4\,\%$ (mean $\pm$ SE) compared to the numeric addition of the separately assessed values (Wilcoxon test, day 1: $Z = 5.02$, $P < 0.001$, day 2: $Z = 6.3$, $P < 0.001$).

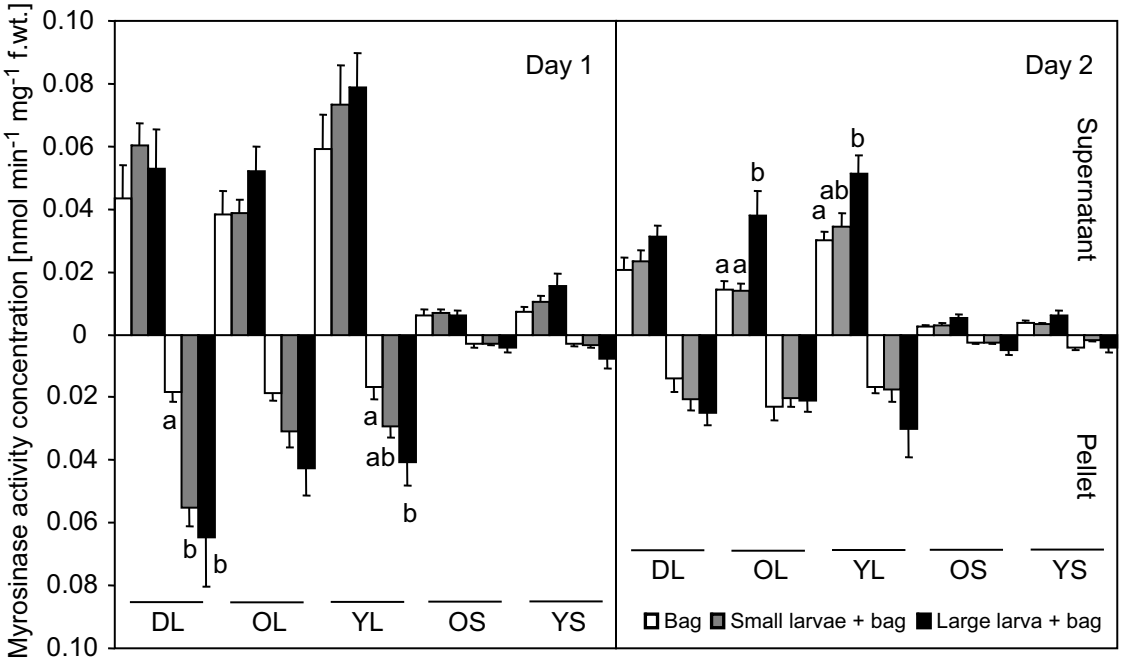

Figure 3.2: Levels of myrosinase activities (nmoles glucose min^{-1} mg^{-1} fresh weight, mean + SE) of soluble (supernatant, upper panel) and insoluble (pellet, lower panel) fractions in leaves and stems of *Sinapis alba* cv. Salvo. The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag for 24 hours. Bagged leaves without larvae served as controls (n = 7 plants per treatment). Treatment effects were tested by Kruskal-Wallis tests and subsequent multiple comparisons of ranks (significant differences are marked with different letters, $P < 0.05$). DL: damaged leaf; OL: older leaf; YL: younger leaf; OS: older stem; YS: younger stem.

3.3.3 Correlations between glucosinolate and myrosinase levels

We determined myrosinase activity by degradation of *p*-OH-benzylglucosinolate externally added as substrate to the assay. Thus, total myrosinase activity was correlated to the amount of this glucosinolate as found in the corresponding halves of the tissue

parts analysed by HPLC. Regarding all samples of day one, except plants with no treatment, a significant linear correlation was found for *p*-OH-benzylglucosinolate concentration with total myrosinase activity (Pearson's correlation coefficient: $R^2 = 0.28$, $F_{1,98} = 38.5$, $P < 0.001$). However, regarding each organ separately (Figure 3.3), no significant correlations were found between glucosinolate content and myrosinase activities of the 21 plants investigated per day. In damaged leaves, feeding by single large or groups of small larvae increased *p*-OH-benzylglucosinolate concentration and total myrosinase activity almost equally (50 % for total myrosinase activity and 40 % for *p*-OH-benzylglucosinolate). In contrast, in systemic leaves only myrosinase activities increased slightly after treatment with three small larvae, but strongly after feeding by one large larva (70 % for older leaves and 100 % for younger leaves). In stems, myrosinase activities and *p*-OH-benzylglucosinolate concentrations were induced after feeding by one large larva to similar degrees, with 40 % of both myrosinases and glucosinolates in older stems, and 90-100 % of both in younger stems. Since effects in all components described so far were strongest on day one, correlations for day two, three, and five are not shown.

3.3.4 Primary metabolites and water content

Stems showed in general between 30 and 50 % lower glucose and about 75 % lower protein levels than leaves, decreasing with age of tissue. Their water content (95 %) was higher than that of leaves, which ranged from 90 to 92 %. In leaves, responses were generally stronger when single large larvae had fed than by feeding of groups of small larvae. Feeding by single large larvae caused a significant increase of glucose levels: about two-fold both in the younger systemic (6.4 ± 0.6 nmoles glucose mg^{-1} fresh weight (f.wt.), mean $\pm$ SE, compared to 3.6 ± 0.4 nmoles mg^{-1} f.wt. in bagged leaves; multiple comparisons $P < 0.05$) and older leaves (4.3 ± 0.5 nmoles mg^{-1} f.wt. compared to 2.5 ± 0.4 nmoles mg^{-1} f.wt.; multiple comparisons $P < 0.05$). Protein levels were induced less strongly than glucose levels: in the younger systemic leaves only by single large larvae ($4.1 \pm 0.3\,\mu g\,mg^{-1}$ f.wt. compared to $3.2 \pm 0.2\,\mu g\,mg^{-1}$ f.wt. in bagged leaves; multiple comparisons $P < 0.05$) and in younger systemic stems only by groups of small larvae ($1.4 \pm 0.2\,\mu g\,mg^{-1}$ f.wt. compared to $1.2 \pm 0.3\,\mu g\,mg^{-1}$ f.wt.; $P < 0.05$). Water content was lower in damaged leaves when treated with a single large larva (90.2 ± 0.3 %) compared to the bagged controls (91.7 ± 0.3 %; multiple comparisons $P < 0.05$) and in systemic younger leaves (89.7 ± 0.2 % compared to 90.7 ± 0.3 %; $P < 0.05$). Almost all effects subsided on day two.

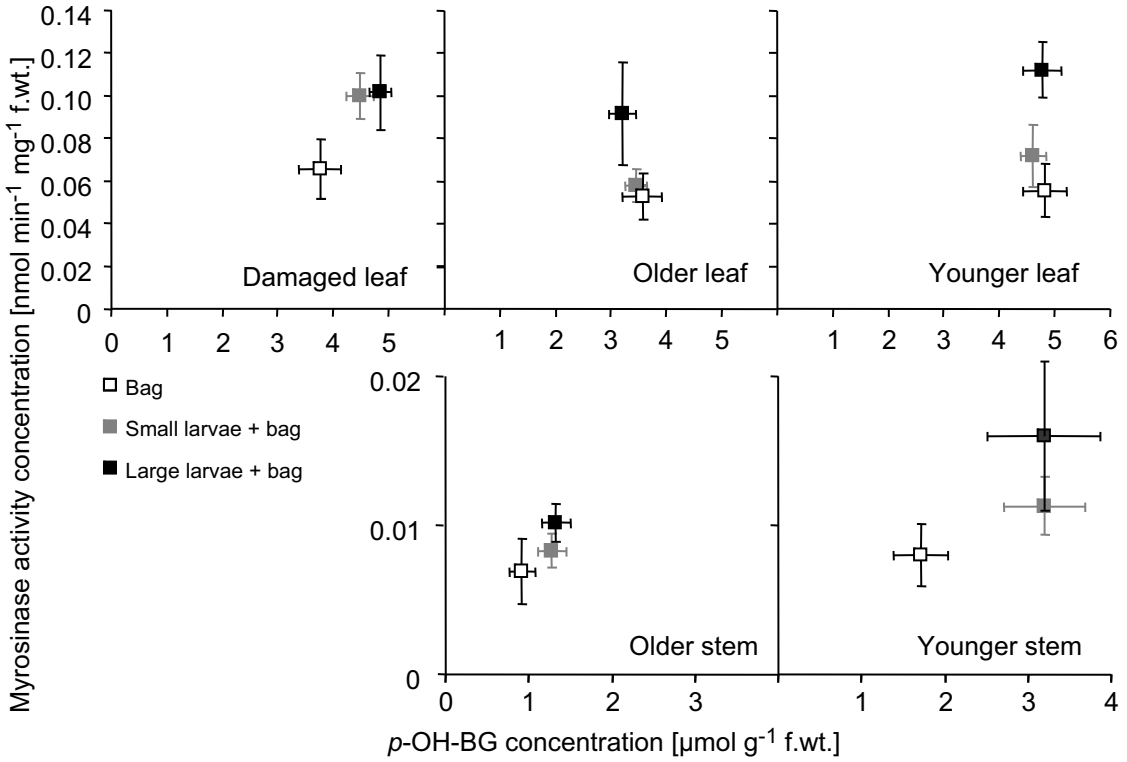

Figure 3.3: Correlations of p-OH-benzylglucosinolate (*p*-OH-BG, μmoles g^{-1} fresh weight, mean $\pm$ SE) and total myrosinase activity (nmoles glucose min^{-1} mg^{-1} fresh weight, mean $\pm$ SE) in leaves and stems of *Sinapis alba* cv. Salvo after 24 hours of feeding (day 1). The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag. Bagged leaves without larvae served as controls (n = 7 plants per treatment).

3.4 Discussion

The glucosinolate-myrosinase system of Brassicaceae is in general regarded as a defence system against antagonists (Blau *et al.*, 1978; Bennett and Wallsgrove, 1994; Wittstock and Gershenzon, 2002). "Optimal defence hypothesis" predicts that constitutive levels of defence should be high in tissues with high values for reproduction and/or with higher vulnerability (McKey, 1974, 1979). Considering value, we postulated that stems of *S. alba* are of higher value than leaves since damage to the single stem can impede flower production. Thus, stems should have higher levels of constitutive defence compounds and should be less likely induced than leaves. In contrast, we found, that constitutively (i.e. in undamaged plants) glucosinolate concentrations and myrosinase activities were lower in stems than in leaves. Regarding systemic tissues, both parameters tended to be induced in stems, while in systemic leaves only myrosinase activities were affected. Stem tissue is

normally not attacked by *A. rosae* larvae in the field or in the lab culture as long as leaf tissue is available (personal observation). Furthermore, nutritional quality of stems (i.e. glucose, protein and water levels) was found to be lower than that of leaves, and *S. alba* stems are more densely covered with trichomes. Thus, stems are likely less susceptible to attack than leaves. Therefore, consideration of vulnerability might be a better predictor for optimal defence in our study system. In a previous study on *Raphanus sativus* L., value for reproduction was shown to be a good predictor for the defensive properties and reactions upon damage of leaves and petals. Leaves were valued less important for reproduction and exhibited lower constitutive defence levels and stronger inducible responses than petals (Strauss *et al.*, 2004). The importance of value compared to vulnerability may increase with time of tissue appearance in a plant's ontogeny. As tissues age, their value to a plant should decrease (McKey, 1974; Strauss *et al.*, 2004). Conforming with this, we found the highest levels of glucosinolates and myrosinase activities in the youngest tissues of control plants. The decrease of glucosinolate and myrosinase activity levels from young to old leaves and stems is accompanied by a similar decrease of proteins, suggesting a lower need for protection in foods of low quality to a herbivore.

Our study demonstrates that the response in both myrosinases and glucosinolates is rather specific. In *S. alba* the response of insoluble myrosinases was even more pronounced after induction by *A. rosae* than the response of the soluble fraction (Figure 3.2). It is thus strongly recommended to evaluate both fractions of this enzyme in studies on Brassicaceae. While total glucosinolate levels increased only slightly, we observed a strong induction of benzylglucosinolate (Table 3.1) and indol-3-ylmethylglucosinolate (Figure 3.1) due to herbivore feeding. In previous studies of *S. alba* (and most other species of Brassicaceae), only one of the two parts of the glucosinolate-myrosinase system was considered. In mature *S. alba* plants mechanical damage with needles resulted in increased *p*-OH-benzylglucosinolate, benzylglucosinolate and indolylglucosinolate levels (Koritsas *et al.*, 1991), while in seedlings needle punctures or treatment with jasmonates had no effect on glucosinolates (Bodnaryk, 1992, 1994). Obviously, induction responses can differ dramatically in dependence of growth stage. To our knowledge, only two studies monitored glucosinolate and myrosinase levels simultaneously after herbivore or pathogen treatment (Siemens and Mitchell-Olds, 1998; Andréasson *et al.*, 2001b), which were conducted with seedlings of *Brassica* spp. Treatment with first-instar *Plutella xylostella* L. (Lepidoptera: Plutellidae) larvae caused an increase of soluble myrosinase activities and glucosinolate concentrations in *Brassica juncea* CZERN. cotyledons (Siemens and Mitchell-Olds, 1998). Treatment with *Leptosphaeria maculans* (fungal pathogen) resulted in increased glucosinolate levels in *B. juncea* cotyledons (Siemens and Mitchell-Olds, 1998) and reduced glucosinolate levels in *B. napus* cotyledons (Andréasson *et al.*, 2001b). Soluble myrosinase activities were not affected by the fungus in either of the plants. According to previous

studies and our work on the interaction of *S. alba* and *A. rosae*, it is apparent that patterns for glucosinolate and myrosinase induction vary according to the inducing organism. Furthermore, our study is the first showing that glucosinolate as well as myrosinase activity levels can be induced by herbivory in leaves and stems not only younger but also older than the damaged leaf.

Feeding by *A. rosae* larvae resulted in different plant responses in local compared to systemic tissue of *S. alba*. From the patterns, certain conclusions can be drawn for the interactions in this particular plant-insect-system: In damaged tissue, a local response occurred already within 24 hours. It encompassed increases of total glucosinolate concentrations, as well as total myrosinase activities. Both parts of the binary system increased to approximately the same degree compared to controls (Figure 3.3). Thus, for the herbivore, possible sequestration costs might be higher, due to a probably costly transport of glucosinolates such as *p*-OH-benzyl- and benzylglucosinolate into the haemolymph and an efficient inhibition of hydrolysis by myrosinases. On the other hand, possible benefits might result from a more efficient defence against the predators of larvae with higher levels of glucosinolates in the plant and, accordingly, the insect haemolymph (Müller *et al.*, 2002). Insect-induced increases of glucosinolates impaired the performance of the glucosinolate-sequestering aphid *Brevicoryne brassicae* (L.) on *Arabidopsis thaliana* (L.) Heynh. (Weber *et al.*, 1986; Bridges *et al.*, 2002; Mewis *et al.*, 2005). In this aphid-plant interaction, the induction response acts thus at least in part as genuine defence against a sequestering specialist. It remains to be seen, whether the response of *S. alba* induced by *A. rosae* has indeed negative effects on the sawfly, but it may affect other herbivores. Primary metabolites were only slightly changed in *S. alba* apart from glucose increases. Normally, nitrogen is the factor limiting performance of herbivores (White, 1993), thus, glucose increases are less likely to influence the nutritional situation of *A. rosae* larvae.

For the plant, a sequestering insect like *A. rosae* poses a specific problem, termed the "lethal plant defence paradox" (Price *et al.*, 1980; Malcolm and Zalucki, 1996). Firstly, these herbivores are generally not deterred by the plant's (putative) defence, and secondly, a sequestration of plant metabolites can even benefit their own fitness. An induction of high doses in response to feeding by young larvae, which are probably more sensitive, and rather moderate to low doses in response to large larvae might be favourable for the plant, as proposed by Malcolm and Zalucki (1996). In contrast to this proposal, our study showed that feeding by older larvae led to higher levels of glucosinolates and myrosinase activities compared to feeding by young larvae. On the other hand, induction responses found in this study were short-lived and could not be detected 24-48 or more hours after larval feeding. But different results might be obtained when larvae feed for longer periods. Similar patterns were found after initial damage for cardenolides (Malcolm and Zalucki,

1996) and after continuous feeding over several days for aristiolochic acids (Fordyce, 2001) in other plant-insect systems. In all of these cases, conspecific larvae feeding more than two days later on previously damaged plant tissue will probably not face changed metabolite levels.

In contrast to local responses addressed so far, in systemic leaves only total myrosinase activities, but not glucosinolate amounts, were increased by feeding of single large larvae (Figure 3.3). In such leaves, costs might exceed benefits for *A. rosae*. Within the gut of a sequestering insect, the plant myrosinase activity might "compete" against the glucosinolate uptake-efficiency of the insect. In case the myrosinases reach a higher affinity, glucosinolates would be hydrolysed to isothiocyanates and not be available for sequestration, and the isothiocyanates could negatively affect the herbivores (Agrawal and Kurashige, 2003). Increasing myrosinase activities would therefore reduce the sequestration rate and increase potential toxic effects. Older larvae show a higher mobility, while young larvae stay longer at their feeding sites (personal observation). Thus, older larvae are more likely to attack systemic tissues within a short time and thus would need to cope with increased myrosinase activities.

The different reactions triggered by groups of small and single large larvae could be a result of varying wound lengths or differences in larval physiology such as saliva composition (Zalucki *et al.*, 2002; Eichenseer *et al.*, 1999). In caterpillars of *Helicoverpa zea* (BODDIE) (Lepidoptera: Noctuidae), salivary glucose oxidase activity varies with developmental stage (Eichenseer *et al.*, 1999). Recently, this enzyme was shown to suppress induction of trypsin inhibitor titers after damage of tomato plants (Musser *et al.*, 2005). Possible underlying mechanisms for different induction patterns in *S. alba* are currently under investigation.

3.4.1 Conclusions

Our results have demonstrated that in induction studies on species of Brassicaceae, both parts of the glucosinolate-myrosinase system need to be evaluated, and within both parts, specific components show different responses in a space- and time-dependent manner. For a glucosinolate-sequestering herbivore, the fine-tuned changes in attacked pre-mature mustard plants can have dramatic impacts. Individual larvae may benefit from the local induction pattern, but larvae attacking the induced systemic leaves might be impaired by elevated myrosinase activities. Thus, in part the lethal plant defence paradox could be resolved by differential regulation of glucosinolates and myrosinases.

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Chapter 4

Specificity of plant responses in *Sinapis alba* L. and their effects on a specialist herbivore

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Abstract - The glucosinolate-myrosinase system of Brassicaceae is known to hold a defensive function in both a constitutive and an inducible fashion. Glucosinolates are sulphur and nitrogen containing metabolites which are hydrolysed upon tissue disruption by myrosinase enzymes. The resulting products are toxic for most herbivores. Nevertheless some insects evolved detoxification mechanisms which enable them to feed exclusively on Brassicaceae. Induction of plant chemical defences which deter or poison generalists might be ineffective against adapted specialist herbivores. We investigated the specificity of short term induction patterns of chemical defences in *Sinapis alba* damaged by a glucosinolate-sequestering specialist herbivore (turnip sawfly, *Athalia rosae*), a generalist herbivore (fall armyworm, *Spodoptera frugiperda*) or mechanical wounding (cork borer) and their effects on the behaviour of *A. rosae*. After 24 hours of damage to young leaves, local as well as systemic changes in glucosinolate and myrosinase levels were analysed. The intensity of the resulting changes was highest in damaged leaves. Induction responses in *S. alba* were dependent on the attacking herbivore and were distinct from a mere wound response. Specialist feeding and mechanical wounding evoked up to 3-fold increases in levels of both parts of the glucosinolate-myrosinase system, whereas generalist feeding induced up to 2-fold increases in glucosinolate levels only. The majority of constitutive and induced myrosinase activity was found in the insoluble fractions. Possible consequences for the plant-specialist-interaction were examined in behavioural tests with larvae and adult females of *A. rosae* on induced *S. alba* plants. Larval feeding and adult oviposition patterns were not modulated in relation to plant treatment. Thus specificity was found in the plant responses of *S. alba* in relation to the inducing agent, but it was not present in return in the effects on the behaviour of an adapted herbivore.

Key words: Behaviour, Brassicaceae, induction, insect resistance, mechanical wounding, vascular connectivity, glucosinolates, myrosinase.

4.1 Introduction

Plants defend themselves against herbivorous insects with a plethora of mechanical and chemical devices. Next to constitutive defence, plants can also respond to herbivore attack with increased metabolite production and enzyme activities (Dicke and Hilker, 2003). These induced defences are thought to have evolved as a cost saving strategy for plants growing under nutrient limited conditions (Zangerl, 2003). Specialist herbivores can show various countermeasures as adaptations to the specific constitutive metabolites of their host plants (Hartmann and Ober, 2000; Agerbirk *et al.*, 2006) and some might also be able to cope with induced defences. However, their performance and behaviour may well be impacted (Agrawal and Kurashige, 2003). In contrast, generalists will invest in more common detoxification strategies (Wadleigh and Yu, 1988; Schoonhoven *et al.*, 1998) but lack specific adaptations. Thus, from the plants' perspective, there exists a dilemma: While they defend themselves chemically against a variety of generalists, non-deterred specialists can use these metabolites as host finding and oviposition cues. Furthermore, highly adapted specialists even use plant toxins for their own protection against members of the third trophic level by sequestration ("lethal plant defence paradox"; Fordyce, 2001). For the plant, it might be thus adaptive to respond differently to attack depending on the inducing organism, e.g. with regard to the degree of specialization of the feeding insect (Baldwin and Preston, 1999; Schwachtje *et al.*, 2006).

In Brassicaceae, glucosinolates and their hydrolysing enzymes, the myrosinases, form a constitutive defence system against herbivores and microorganisms (Halkier and Gershenzon, 2006). Glucosinolates consist of a thioglucoside moiety linked to a variety of amino acid-derived side chains (Bones and Rossiter, 2006). Myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147) occur in various isoforms which are either soluble under non-denaturing conditions or insoluble due to complex formation with myrosinase-binding proteins (MBPs; Rask *et al.*, 2000). The myrosinase subunits of the insoluble complexes are still active and a strongly modified protocol for the quantification of insoluble as well as soluble fractions of myrosinase has been proposed (Martin and Müller, 2007). A variety of specialist insects developed means of circumvention and detoxification of the glucosinolate-myrosinase system (Bones and Rossiter, 2006; and references therein) . Larvae of the turnip sawfly *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae), which feed on different species of Brassicaceae, sequester certain glucosinolates of their host plants within their hemolymph (Müller *et al.*, 2001; Müller and Wittstock, 2005) and can use these for their own defence (Müller *et al.*, 2002; Müller and Brakefield, 2003).

Glucosinolates and myrosinases were also shown to be inducible in many ways (e.g. Pontoppidan *et al.*, 2005; Martin and Müller, 2007; and references therein). Feeding by *A. rosae*

larvae induced short-term increases in levels of glucosinolates and myrosinase activities in damaged leaves of *Sinapis alba* L. cultivar Salvo (Martin and Müller, 2007). However, the specificity of this induction effect with regard to the inducing agent and its ecological relevance for the glucosinolate-sequestering herbivore remained unclear. In general, the specificity of a plant's induction response is known to be dependent on the inducing agent and on the feeding mode of the herbivore (Walling, 2000; Dicke and Hilker, 2003). Furthermore, the vascular architecture can constrain induction patterns within a plant (Orians, 2005). In Brassicaceae, studies comparing induction responses to three agents, mechanical damage, specialist and generalist feeding, have to date only focused on gene expression studies (Reymond *et al.*, 2004). In pair wise comparisons, changes in gene expression and in chemical and mechanical defences were found (Agrawal, 2000; Reymond *et al.*, 2004; De Vos *et al.*, 2006; Widarto *et al.*, 2006). However, effects on myrosinases were mainly studied in isolation from glucosinolates (but see: Siemens and Mitchell-Olds, 1998; Andréasson *et al.*, 2001b). Specialist behaviour and performance was shown to be affected to different degrees by induced responses of plants (Agrawal, 2000; De Vos *et al.*, 2006). None of these insects investigated with regard to its behaviour uses plant defences for its own protection.

The aim of this study was to investigate the specificity of induction responses of *S. alba* we described recently (Martin and Müller, 2007) and to characterize its ecological relevance for larvae and for adult females of the glucosinolate-sequestering specialist *A. rosae*. Specificity of response was investigated at different levels. At the level of induction mode, plants were treated in three different ways with either mechanical wounding, feeding by generalist caterpillars or by the specialist larvae of *A. rosae*. At the plant level, we used a different cultivar of *S. alba* than in the previous study. We determined glucosinolate concentrations and myrosinase activities (soluble and insoluble fractions) in local as well as adjacent older and younger leaf tissues after 24 hours of induction on *S. alba* cv. Silenda plants. To investigate potential directions for signal transport, connectivity of phloem bundles between these tissues was followed with a dye. Furthermore, subsequent behavioural studies with larvae and with adult females of *A. rosae* were performed in order to evaluate the consequences of specific induction responses of the plant on the specialist. This way a triple comparison of induction effects of a generalist, a specialist and wounding was investigated with respect to both induced plant response and induced resistance against a specialist herbivore.

4.2 Methods and materials

4.2.1 Planting and induction experiment

Seeds of *Sinapis alba* L. cv. Silenda (Kiepenkerl, Norken, Germany) were sown in mid January 2005 and kept in a climate chamber at 25 °C, 16:8 hours light-dark-cycle, and 70 % relative humidity (light source: Osram L 58 W/25 Universal White, 4150 lumens, Osram, Munich, Germany). Seven days later, the seedlings were transferred to individual pots (diameter: 9 cm; height: 7 cm) containing unfertilized soil (peat, pH 6). After 21 days, when five to six true leaves had developed, plants received one of the following five treatments on the second youngest leaf to investigate the specificity of the plant's response: (a) one male larva of the specialist *A. rosae* (six days after egg hatch, third larval instar; larval weight 32.7 ± 1.6 mg, mean $\pm$ SE, $n = 8$) enclosed with the leaf in a muslin bag (8 x 10 cm, 1 mm mesh width); (b) one caterpillar of the generalist *Spodoptera frugiperda* J. E. SMITH (Lepidoptera: Noctuidae; seven days after egg hatch, third larval instar; caterpillar weight 21.7 ± 1.5 mg, mean $\pm$ SE, $n = 8$) in a bag; (c) mechanical damage made by three holes (one in the morning at 10 a.m., one in the afternoon at 4 p.m. and one the next morning at 8 a.m.) punched with a cork borer (diameter: 1.5 cm) and the leaf enclosed in a bag; (d) a bag only; (e) no treatment. Effects of herbivore damage on myrosinase gene expression had been shown to be more resembled by cutting with scissors than by crushing with forceps (Pontoppidan *et al.*, 2005). Therefore we used a cork borer as a cutting tool to remove equivalent amounts of leaf tissue. Seven to eight replicates per treatment were prepared. Plants of all treatments were placed in random order in the climate chamber. Sawfly larvae were taken from a laboratory culture established from a field collection in Germany in the previous autumn and kept on *S. alba*. Caterpillars of *S. frugiperda* were obtained from Bayer CropScience AG (Monheim, Germany) as eggs and were reared on an artificial diet based on kidney beans modified from King and Leppla (1984).

Insects and muslin bags were removed after 24 hours and photos were taken of treated leaves to determine the pattern and degree of damage. Samples for chemical analyses were taken from damaged leaves as well as the flanking upper and lower leaves. Leaves were cut longitudinally, weighed, frozen in liquid nitrogen, and stored at -80 °C for later analysis. This allowed us to analyse both glucosinolate and myrosinase levels from the same leaves.

4.2.2 Analysis of glucosinolates

Frozen samples were freeze-dried, weighed, and pulverized in a mill (Retsch, MM301, Haan, Germany). Glucosinolates were extracted in 80 % methanol after addition of 20 μL

of a 5 mM solution of 2-propenylglucosinolate (Merck, Darmstadt, Germany) as internal standard. Glucosinolate analysis was performed by conversion to desulphoglucosinolates as described elsewhere (Agerbirk *et al.*, 2001). For analysis of desulphoglucosinolates, samples were subjected to HPLC analysis on a 1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector. Elution was accomplished on a Supelco C-18 column (Supelcosil LC-18, 250 x 4.6 mm, diameter 5 μm, Supelco, Bellefonte, Pennsylvania, USA) with a gradient (solvent A: water, solvent B: methanol) of 0-5 % B (10 min), 5-38 % B (24 min), followed by a cleaning cycle. Desulphoglucosinolates were identified by comparison of retention times and UV spectra to those of purified standards and identities had been further confirmed by LC-MS (Müller *et al.*, 2001; Müller and Martens, 2005). Peaks were quantified by the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, applying the response factors as used by Brown *et al.* (2003) and Martin and Müller (2007).

4.2.3 Analysis of myrosinase activity

Myrosinase activity was determined by photometric quantification of released glucose from the externally added substrate *p*-OH-benzylglucosinolate (obtained from glucosinolates.com, Denmark), which is the most dominant glucosinolate in *S. alba*. The myrosinase extraction protocol followed (Müller and Martens, 2005; Martin and Müller, 2007). Frozen leaf material was pulverized in a mill, extracted in buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice, centrifuged and supernatants were purified *via* ion-exchange columns (DEAE Sephadex A-25 swollen in acetic acid buffer; Sigma-Aldrich, St. Louis, Missouri, USA) to eliminate internal glucosinolates. These purified extracts served as source to determine soluble myrosinase activities. The remaining pellets were re-suspended in buffer, and two further tests were performed. Half of each pellet suspension was diluted with buffer to determine the activity of insoluble myrosinases. The other half was centrifuged and supernatants were discarded. The resulting pellets were re-suspended in half of the corresponding purified extract. Thereby, a suspension was obtained with a reconstituted ratio of soluble and insoluble myrosinases alike the crude, unpurified extract but without interfering internal glucosinolates. Soluble protein concentrations of purified extracts were determined according to Bradford (1976), and were adjusted to protein concentrations of 200 μg mL^{-1}. Both suspensions were diluted the same way as the purified extracts.

Activities of soluble and insoluble fractions of myrosinases were determined individually and together for each leaf sample on 96-well microplates. The three enzyme preparations were each added to four wells. Two wells served as test wells to which 2 mM *p*-OH-benzylglucosinolate-phosphate buffer was added as substrate to measure kinetics of glucose

release. Two additional wells served as respective background controls which received only phosphate buffer to measure levels of internal glucose and light scattering. Glucose was detected by addition of 50 μL freshly mixed colour reagent, including glucose oxidase, peroxidase, 4-aminoantipyrine and phenol (Trinder, 1969; Kunst *et al.*, 1984; Siemens and Mitchell-Olds, 1998) and quantified by reading the absorbance at 492 nm on a Multiskan EX (Thermo Labsystems, Vantaa, Finland) for 45 minutes at room temperature (22 – 24 °C). Means of the two replicate measurements were calculated after subtraction of the means of the two background controls. A glucose dilution series was included in each assay. The rates of colouration which can be impacted by ascorbic acid (Kleinwächter and Selmar, 2004), were not affected by ascorbate under our assay conditions (Chapter 2). The resulting enzyme kinetics were analysed for a linear range of myrosinase activity. Whereas the specific activity (Units mg^{-1} protein) is used to characterize an enzyme's activation status (Moss, 1984; Stranger and Mitchell-Olds, 2005), the concentration of myrosinase activity in relation to tissue fresh weight is a measure of the plant tissue's capacity to produce harmful hydrolysis products. Myrosinase activity concentrations are given in nanomoles of glucose released per minute and per milligram of fresh weight in this study, as we focussed on the ecological effects.

4.2.4 Analyses of soluble protein and water contents

Protein concentrations were obtained from purified extracts of the myrosinase assay. Soluble protein concentration was measured in duplicate of two dilutions according to Bradford (1976). Water contents of tissues were determined after freeze-drying of samples used for glucosinolate analysis.

4.2.5 Behavioural experiments with *Athalia rosae*

To investigate the behavioural response of larvae of the specialist *A. rosae* to metabolite changes in plant tissues evoked by different induction modes, *S. alba* plants were induced as described above with treatments a, b, c, and d. Twenty replicates were prepared per treatment. After 24 hours of induction, bags and insects were removed from the plants and each plant received one male *A. rosae* larva (four days after egg hatch, third larval instar; larval weight 16.3 ± 1.3 mg, mean $\pm$ SE, $n = 80$). Larvae were placed on the soil of the pot near the base of the plant and were allowed to move freely to and on the plants. Time until first feeding and feeding sites at the first and nine subsequent time points (90, 215, 285, 350, 405, 635 minutes after start of the test on the same day and 1265, 1340, 1430 minutes after start of the test on the next day) were recorded within 24 hours. To

determine oviposition behaviour of *A. rosae* on differently treated *S. alba*, plants were induced as described above with fourteen replicates per treatment. After 24 hours of induction and after the removal of bags and insects, plants were placed in large muslin bags ($20 \times 60 \times 10$ cm, mesh width 1 mm) and each received one naïve (without oviposition experience), unmated female *A. rosae* (four days after hatching from pupae, female weight 23.1 ± 0.4 mg, mean $\pm$ SE, n = 52) for 24 hours. Numbers of eggs were counted for each leaf separately. Locations of larval feeding and egg deposition within 24 hours on differently induced plants were grouped in two categories in behavioural tests. Category I included the treated leaf and all younger ones in the upper plant region, because induction effects were strongest in these tissues. Category II included all older leaves below the treated leaf, where induction effects were less pronounced. A Preference Index of Categories (PI_c) was calculated per female according to the following equation:

$$PI_c = \frac{(\text{number of eggs on category I}) - (\text{number of eggs on category II})}{\text{total number of eggs}}$$

The extreme values of 1 and -1 represent preferences for categories I and II, respectively.

4.2.6 Plant vascular connectivity

For the determination of the vascular connections of the second youngest leaf of *S. alba*, nine plants were subjected to phloem staining with Rhodamine B modified from (Orians *et al.*, 2000). Plants were wounded on the second youngest leaf with a cork borer (three holes, no time delay, diameter 5 mm). A droplet of 25 μL of a 0.25 % solution of Rhodamine B in nanopure water was applied to each of the wounded areas and was sealed with parafilm and silicone grease (Wacker, Munich, Germany) to minimize evaporation. After 24 hours of stain relocation in the phloem of the plant, treated leaves as well as the adjacent upper and lower leaves and stem parts were harvested. Samples were freeze-dried and pulverized in a mill. Rhodamine B was removed from plant material by three subsequent extractions with 400 μL of nanopure water for leaves and 200 μL for stems. Supernatants were separated from pellets by centrifugation for two times at room temperature for 10 min at 16,060 $\times$ g. Supernatants were analysed for Rhodamine B content by reading the absorbance at 570 nm on a Multiplate EX photometer. A standard dilution series of Rhodamine B was included on each microplate.

4.2.7 Statistical analyses

Differences in damage patterns and in induction patterns of water contents, soluble protein, glucosinolate and myrosinase activity concentrations between treatments in organs

of identical age were analysed by one-way ANOVA followed by HSD tests for imbalanced number of subsets. Variance homogeneity was examined by Levené-tests and data were log- or squareroot-transformed where needed. Control groups without and with bag treatment were analysed for effects of the muslin bag on induction responses by T-tests. Treatment effects on the number of damage sites were analysed by Mann-Whitney-U-tests which were tie and Bonferroni corrected.

In behavioural assays, the distribution of feeding sites of *A. rosae* larvae was grouped in two categories (see above) and homogeneity of subsets between the four plant treatments was analysed by χ^2-tests (4×2). Plant treatment effects on time until first feeding of larvae, on egg deposition sites (PI_c), and on numbers of eggs laid by adults were analysed by one-way ANOVA followed by HSD tests for imbalanced number of subsets. Numbers of eggs within one treatment were examined for differences between both categories by Wilcoxon tests for matched pairs.

Differences of percentages in Rhodamine B dye distribution were analysed using Wilcoxon tests for matched pairs. To analyse whether weight and dye content of a particular organ were correlated, Pearson-Product moment correlations were executed over all tissues, over only leaves or only stems.

4.3 Results

Induction patterns of plants treated with muslin bags only and non-treated plants did only differ in protein concentration and the three myrosinase activity concentrations (soluble, insoluble and the combination of both) in local leaf tissue, which were up to 20 % reduced due to bag treatment. All other traits in local tissue and all traits measured in systemic tissues were not significantly different between these plant treatments. In the following we will focus on the bag treatment as the more relevant control compared to damage treatments.

4.3.1 Damage patterns

Within 24 hours the caterpillars of the generalist *S. frugiperda* consumed almost the same amount of leaf tissue compared to the specialist *A. rosae* larvae but produced a 1.7-fold higher number of damage sites (generalist - 10.5 ± 1.1, specialist - 6.1 ± 0.6, mean $\pm$ SE, $n = 8$; Mann-Whitney-U-test: $Z = -2.6$, $P = 0.020$; Figure 4.1). Thereby, the total wound length caused by the generalist was nearly twice as long as that caused by the specialist (total wound length was calculated as the sum of the perimeters of all damage sites;

ANOVA: $F = 16.47$, $df = 2$, $P < 0.001$; HSD for imbalanced number of subsets $P = 0.002$). Damage by mechanical wounding was very similar to feeding by *A. rosae* with respect to wound length and area but not to number of damage sites which were significantly different (Mann-Whitney-U-test: $Z = -3.1$, $P = 0.004$).

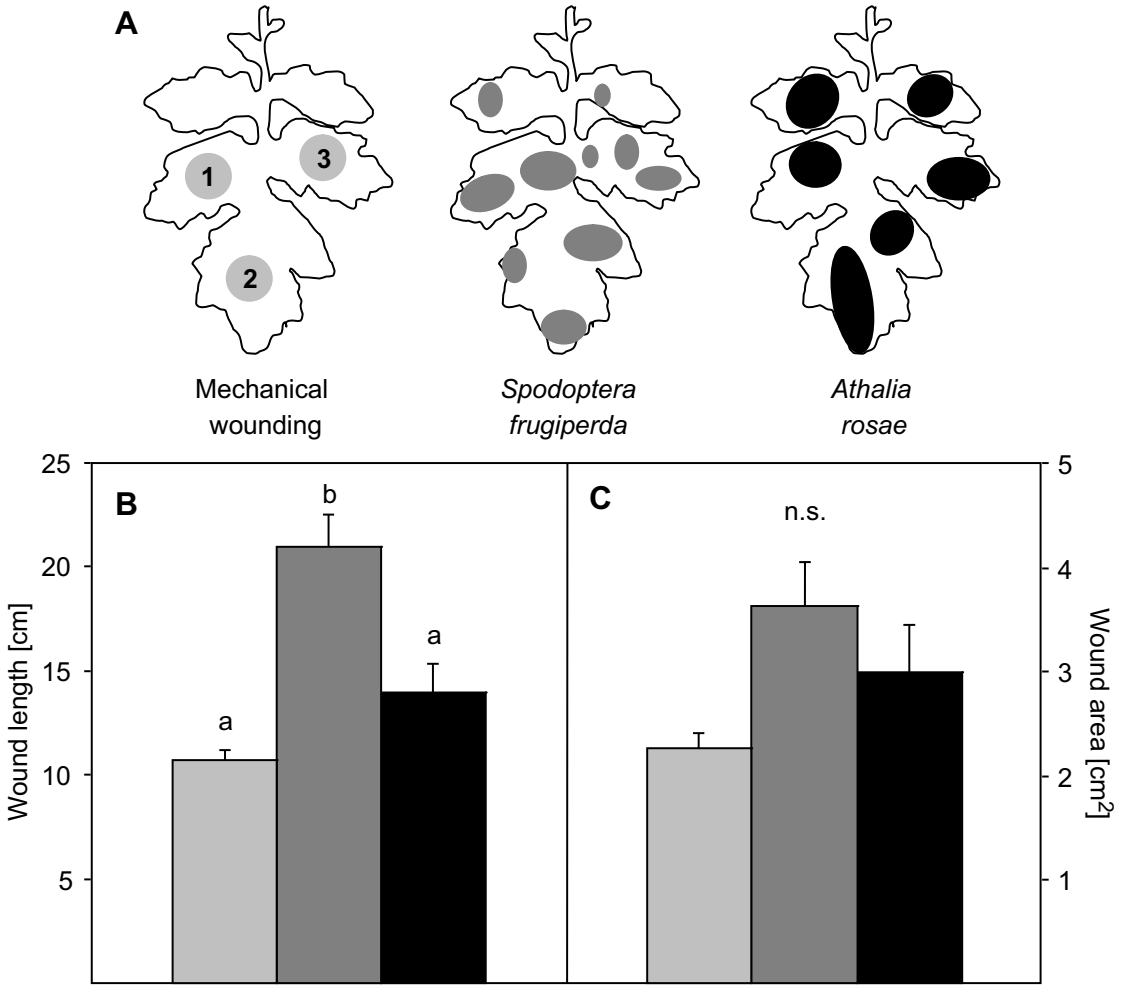

Figure 4.1: Damage treatments of *Sinapis alba* cv. Silenda leaves: (A) damage patterns after mechanical wounding (three holes at different time points: 10 a.m.; 5 p.m.; 8 a.m., 3 hrs before harvest), feeding by *Spodoptera frugiperda* or *Athalia rosae*; Wound length (B) and removed tissue area (C) were determined by photo analysis compared to standards, letters above bars indicate significant differences (ANOVA, HSD tests: $P < 0.01$, n.s. - not significant), mean ± SE, n = 6-8.

4.3.2 Glucosinolate concentrations and myrosinase activities

The following eight glucosinolates were detected according to their retention times and UV-spectra in *S. alba* cv. Silenda leaves in decreasing relative amounts: *p*-OH-benzylglucosinolate (85 - 95 %), benzylglucosinolate (5 - 14 %), 4-methoxy-indol-3-ylmethylglucosinolate, indol-3-ylmethylglucosinolate, phenylethylglucosinolate, and three minor putative aromatic glucosinolates not identified further. The sum of all six minor compounds represents only about 1 % of the sum of all glucosinolates (= total glucosinolates). The capacities of the leaf tissues to hydrolyse glucosinolates were tested as myrosinase activity concentrations against the main glucosinolate *p*-OH-benzylglucosinolate. Total myrosinase activity of a tissue consists of soluble and insoluble fractions which were tested together. The constitutive concentrations of total glucosinolates and total myrosinase activity were highest in tissues of younger leaves and decreased more than 2-fold with leaf age. Upon damage treatment, concentrations of glucosinolates in locally induced leaves increased significantly about 80 % irrespective of inducing agent (Figure 4.2). In contrast, significant increases by three times in concentrations of total myrosinase activity were found in damaged leaves treated with mechanical wounding or specialist feeding only, but not in those leaves treated with generalist caterpillars. In systemic tissues, increases were more pronounced in younger than in older leaves revealing the same patterns as in damaged leaves, but differences were not significant due to high variation of individual values compared to differences in damaged tissue.

Individual glucosinolates showed in detail different responses (Table 4.1, Figure 4.3). Whereas the absolute increase in concentration was highest for the main glucosinolate compound *p*-OH-benzylglucosinolate, indol-3-ylmethylglucosinolate concentrations were increased with the highest relative factor compared to bag treated controls. In damaged leaves, *p*-OH-benzylglucosinolate concentrations increased by a factor of 1.4, 1.6, or 1.9 due to mechanical, generalist, or specialist damage, respectively, compared to the bag control, whereas the corresponding factors for indol-3-ylmethylglucosinolate were 2.4, 3.8, and 3.7. Significant differences of individual glucosinolates compared to the control were found only for insect treatments, but not for mechanical wounding in damaged leaves. The pattern of benzylglucosinolate induction was different from all the others: in damaged leaves wounding resulted in the highest increase (13.2 ± 4.6 % of total glucosinolates, mean $\pm$ SE, n = 7) though not significant, *S. frugiperda* feeding in intermediate increases (11.3 ± 2.4 %) and *A. rosae* feeding had almost no effect (6.0 ± 1.7 %) compared to bag treatment (7.4 ± 1.9 %).

Overall, increases of soluble myrosinase activity concentrations were low (Figure 4.3). In contrast, concentrations of insoluble myrosinase activities increased significantly in

damaged leaves compared to controls by a factor of 3.3 or 4.4 due to mechanical wounding or specialist feeding, respectively. Generalist feeding had no effect on either soluble or insoluble myrosinase activity concentration in any of the leaves tested.

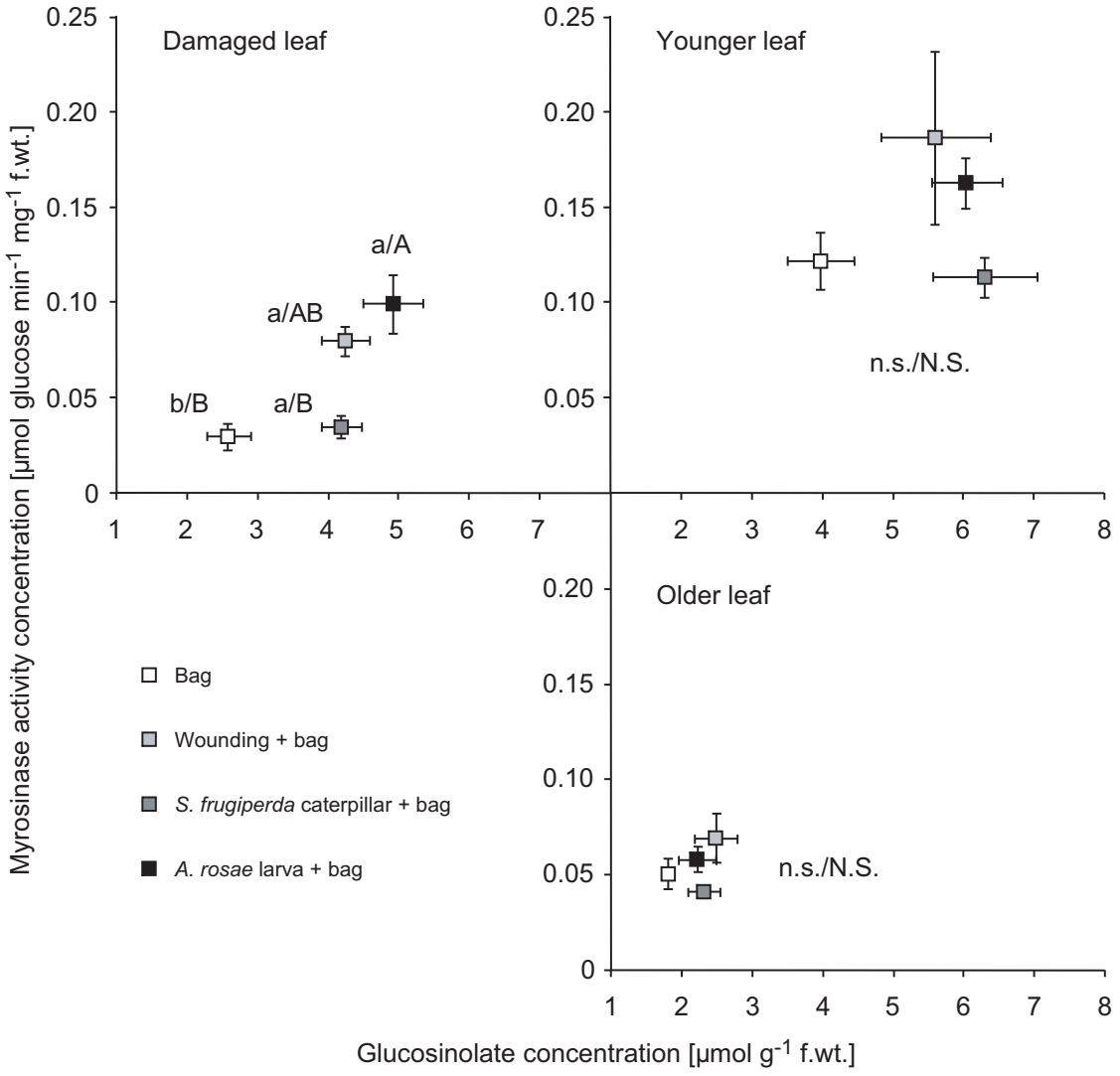

Figure 4.2: Correlations of total glucosinolate concentration (sum of eight individual compounds, μmol g^{-1} fresh weight, mean $\pm$ SE) and total myrosinase activity concentration (μmol glucose min^{-1} g^{-1} fresh weight, mean $\pm$ SE) in leaves of *Sinapis alba* cv. Silenda. The second youngest leaf was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hours. Bagged leaves without any further treatment served as controls (n = 6-8 per treatment). Letters above bars indicate significant differences (ANOVA, HSD tests: $P < 0.05$, n.s. - not significant). Lower case letters refer to glucosinolate concentrations, upper case letters to myrosinase activity concentrations.

Table 4.1: Levels of the most dominant glucosinolates in leaves *Sinapis alba* cv. Silenda. The second youngest leaf (DL) was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hours. Bagged leaves without any further treatment served as controls. DL - damaged leaf; OL - older leaf; YL - younger leaf. Mean ± SE, n = 6-8. Note different scales of single compounds!

Organ	Bag		Wounding + bag		*S. frugiperda* +bag		*A. rosae* +bag		ANOVA *F*	ANOVA *P*
					p-OH-benzylglucosinolate [μmol g^{-1} f.wt.]					
DL	**2.36** ± 0.24	a	**3.59** ± 0.27	ab	**3.66** ± 0.24	b	**4.55** ± 0.37	b‡	**8.92**	**<0.001**
OL[2]	1.71 ± 0.07		2.20 ± 0.26		2.07 ± 0.16		2.08 ± 0.23		0.95	n.s.
YL	3.70 ± 0.40		4.98 ± 0.73		5.58 ± 0.65		5.65 ± 0.49		2.35	n.s.
					Benzylglucosinolate [*10^{-1}μmol g^{-1} f.wt.]					
DL	2.15 ± 0.77		6.12 ± 2.38		4.88 ± 1.15		3.13 ± 1.12		1.33	n.s.
OL[1]	0.91 ± 0.46		2.79 ± 1.07		2.41 ± 0.87		1.30 ± 0.47		1.37	n.s.
YL	2.61 ± 0.90		5.69 ± 1.69		6.74 ± 1.44		3.43 ± 1.10		2.15	n.s.
					Indol-3-ylmethylglucosinolate [nmol g^{-1} f.wt.]					
DL[1]	**4.09** ± 1.38	a	**9.94** ± 2.45	ab	**15.73** ± 3.47	b	**15.18** ± 1.98	b	**5.50**	**<0.01**
OL[2]	1.10 ± 0.37		1.76 ± 0.59		0.87 ± 0.15		1.75 ± 0.42		1.08	n.s.
YL[2]	7.75 ± 1.82		17.65 ± 5.52		14.38 ± 1.63		14.34 ± 2.66		2.03	n.s.
					4-Methoxy-indol-3-ylmethylglucosinolate [nmol g^{-1} f.wt.]					
DL[1]	**13.58** ± 2.30	a	**14.66** ± 2.26	a	**19.82** ± 3.19	ab	**32.02** ± 5.44	b	**5.30**	**<0.01**
OL[1]	4.27 ± 0.57		5.71 ± 0.96		6.41 ± 1.02		8.61 ± 1.90		1.64	n.s.
YL[1]	**13.44** ± 1.40	a	**18.84** ± 2.41	ab	**30.80** ± 6.90	b†	**30.80** ± 6.08	b†	**3.41**	**<0.05**

Notes: Treatment effects were tested by one-way ANOVA followed by HSD tests for imbalanced numbers of subsets (significant differences are marked with different letters and values are highlithted in bold, $P<0.05$, or otherwise stated, n.s. - not significant). Differences from control in HSD test: $^{\dagger}P<0.08$; ‡ $P<0.001$. Data were square root[1]- or log[2]-transformed to ensure variance homogeneity. Back-transformed data means and standard errors are shown.

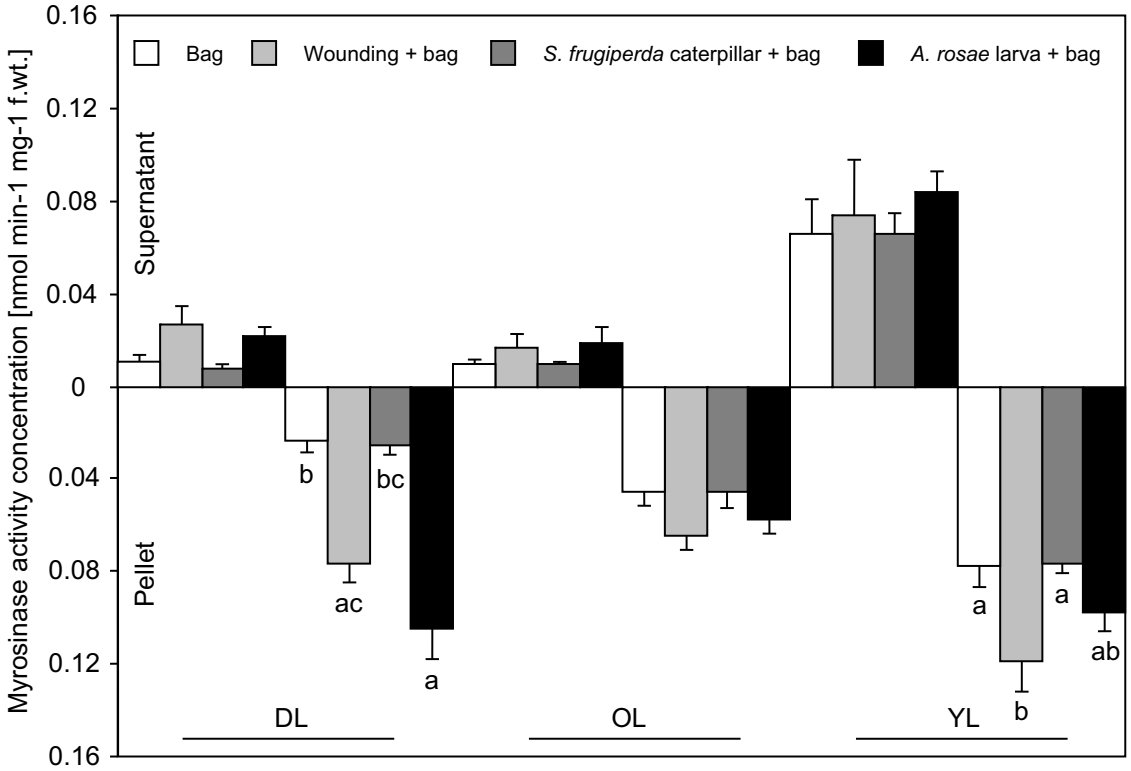

Figure 4.3: Levels of myrosinase activity concentrations (μmol glucose min^{-1} g^{-1} fresh weight, mean $\pm$ SE) of soluble (supernatant, upper panel) and insoluble (pellet, lower panel) fractions in leaves of *Sinapis alba* cv. Silenda. The second youngest leaf was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hours. Bagged leaves without any further treatment served as controls (n = 6-8 per treatment). Letters above bars indicate significant differences (ANOVA, HSD tests: $P < 0.05$). DL: damaged leaf; OL: older leaf; YL: younger leaf.

4.3.3 Soluble protein and water content

Protein concentrations were strongly influenced by treatment in damaged leaves (ANOVA: $F = 4.16$, $df = 3$, $P = 0.016$, data were square-root transformed to ensure variance homogeneity). Both mechanical wounding (4.9 ± 0.3 mg g^{-1} fresh weight, mean $\pm$ SE) and feeding by *A. rosae* larvae (4.7 ± 0.9 mg g^{-1} f.wt.) induced soluble protein concentrations by a factor of 1.7 compared to bag treated leaves (2.9 ± 0.4 mg g^{-1} f.wt.), but *S. frugiperda* caterpillar feeding had no effect (3.3 ± 0.4 mg g^{-1} f.wt.). Differences to controls were only significant for mechanical wounding with $P = 0.030$ (HSD tests). In systemic leaves, *A. rosae* feeding had stronger effects than the other two damage treatments compared

to bag treated controls, but none of the differences was significant (ANOVA: $F = 2.38$, $df = 3$, $P = 0.092$). Decreases in water content (range: 88.1 to 90.7 %) were found only due to mechanical wounding, but differences were very small and not significant for any organ compared to bag treated controls.

4.3.4 Behavioural experiments with *Athalia rosae*

The proportion of larvae feeding on younger tissues in the upper plant region was low in the beginning and increased during the 24 hours-test from about 25 % to 50 % of larvae feeding in category I. There were no significant differences in attack pattern on differently treated plants at any of the time points tested (χ^2-tests). Time to first feeding as a measure of whole plant acceptance was not significantly affected by plant treatment (ANOVA: $F = 0.51$, $df = 3$, $P = 0.674$). In the 24 hours test period, each female laid on average 39.2 ± 1.7 eggs (mean $\pm$ SE, n = 52), with no significant differences between plant treatments (ANOVA, $F = 1.01$, $df = 3$, $P = 0.397$). The mean distribution of eggs laid in categories I and II were similar for all treatments except for mechanical wounding where overall significantly more eggs were laid on older than on younger leaves (Figure 4.4). Females did not show any preference for one of these categories within plants treated with bags only ($PI_c = -0.05 \pm 0.14$, mean $\pm$ SE, n = 14; Wilcoxon test: $Z = 0.38$, $P = 0.706$), with *S. frugiperda* caterpillars ($PI_c = -0.07 \pm 0.12$, n = 14; Wilcoxon test: $Z = 0.06$, $P = 0.950$) or *A. rosae* larvae ($PI_c = -0.08 \pm 0.12$, n = 10; Wilcoxon test: $Z = 0.30$, $P = 0.767$). However, a clear preference for category II leaves was found in plants treated with mechanical wounding ($PI_c = -0.39 \pm 0.16$, n = 14; Wilcoxon test: $Z = 2.76$, $P = 0.006$). No significant effects due to treatment of plants were found for PI_c of individual females (ANOVA, $F = 1.87$, $df = 3$, $P = 0.147$).

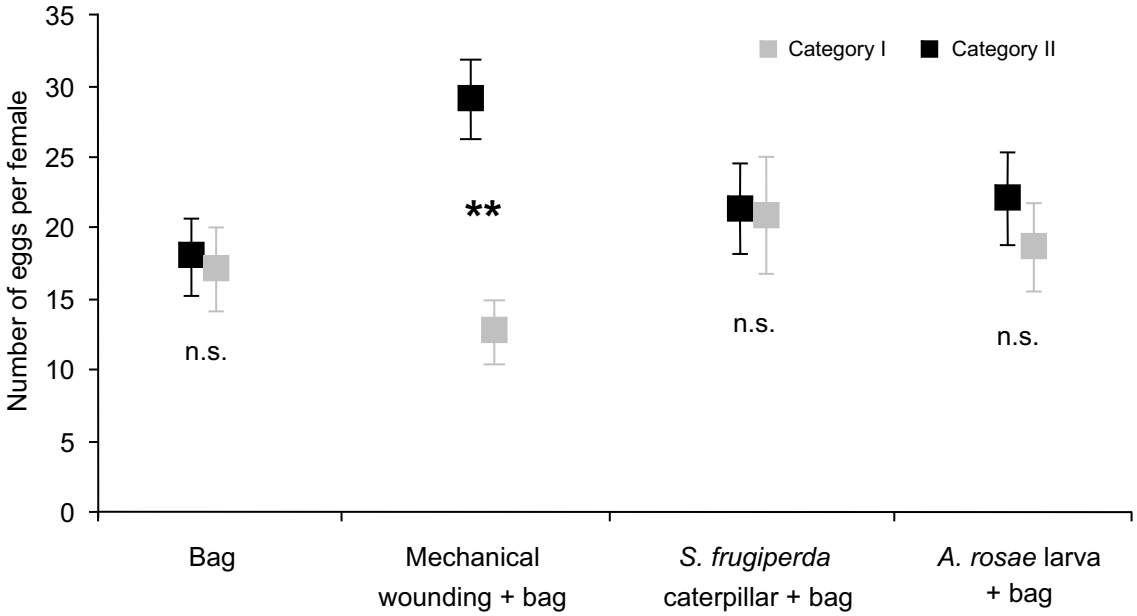

Figure 4.4: Number of eggs per female (mean ± SE) on category I (induced and younger) and II (all older) leaves of *Sinapis alba* cv. Silenda in a 24 hours test. The second youngest leaf was pretreated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hours. Bagged leaves without any further treatment served as controls (n = 14 per treatment). One female *A. rosae* was enclosed in large muslin bags with one plant for 24 hours. Differences in mean number of eggs per category were tested by Wilcoxon tests for matched pairs (** - $P < 0.01$, n.s. - not significant).

4.3.5 Plant vascular connectivity

Rhodamin B content was higher in leaves than in stems. Signals of stem tissue samples were below the detection limit in many cases. The recovery rate of applied dye was $81 \pm 6\,\%$ (mean ± SE, n = 9). Some of the dye was incorporated into the hydrophobic sealing grease and could therefore not be solubilized by nanopure water. The distribution of dye between the five organs was $35.1 \pm 2.7\,\%$ (mean ± SE, n = 9) in treated leaves, $40.7 \pm 1.8\,\%$ in younger leaves, $19.4 \pm 2.8\,\%$ in older leaves, $4.5 \pm 0.6\,\%$ in younger stems and in older stems $7.5 \pm 1.3\,\%$ of the recovered amount of Rhodamine B per plant. The dye accumulated in treated and younger leaves, with significant differences between older leaves and treated leaves (Wilcoxon test: $Z = 2.07$, $P = 0.038$) and between older and younger leaves (Wilcoxon test: $Z = 2.54$, $P = 0.011$). We found an overall significant correlation between organ fresh weight and accumulated dye per organ within all tissues (Pearson's correlation coefficient: $R^2 = 0.29$, $F_{1,32} = 13.6$, $P < 0.001$), but not within leaves or stems only.

4.4 Discussion

4.4.1 Specificity according to mode of induction

Plants of *S. alba* cv. Silenda treated with mechanical wounding, generalist or specialist feeding induced total glucosinolate concentrations to a similar extent, yet their composition varied. Mechanical wounding and feeding by *A. rosae* larvae on *S. alba* leaves did induce increases in protein and myrosinase activity concentrations mostly to the same extent, whereas no significant increase in those parameters could be found in the leaves damaged by *S. frugiperda* (Figure 4.2). The plants' responses to different inducing agents roughly reflected the differences in wounding patterns. The damage patterns (Figure 4.1) after mechanical wounding and specialist *A. rosae* feeding were similar, but damage inflicted by generalist caterpillars of *S. frugiperda* resulted in about 2-times longer wound perimeters, a measure for dose of damage.

A dose-dependency of damage was shown previously for the induction of indolic and aliphatic glucosinolates upon mechanical wounding (Koritsas *et al.*, 1991; Bodnaryk, 1992) and upon application of the wound-induced plant hormone jasmonic acid (Bodnaryk, 1992; Doughty *et al.*, 1995). Probably, also dose-dependent influences exist for induction of aromatic glucosinolate concentrations or myrosinase activities in *S. alba*. But many plant responses are dependent on the concentration, place and mode of action of elicitors present in the saliva or oviposition secretion of a given herbivore (Felton and Eichenseer, 1999; Hilker and Meiners, 2006; and references therein). The salivary compounds are not always eliciting an increase of metabolites. Glucose oxidase or pH-effects influencing methanol release were shown to inhibit plant responses otherwise occurring upon mechanical wounding (von Dahl *et al.*, 2006). We were not able to detect glucose oxidase activity in head or body extracts of *S. frugiperda*, nor in extracts of *A. rosae* using the method described by Eichenseer *et al.* (1999; data not shown). Other elicitors, i.e. fatty acid-amides, were also reported for caterpillars of *S. frugiperda* (Spiteller and Boland, 2003). Furthermore, the temporal pattern of wounding can be of importance for the induction, as has been shown for the release of volatiles in lima beans due to feeding by *Spodoptera littoralis* (BOISD.) (Mithöfer *et al.*, 2005). We applied mechanical damage three times interspaced as to mimic not only spatial but also broad-scale temporal pattern of herbivore feeding. Nevertheless, we are aware that herbivore feeding differed from our mechanical treatment, because plant tissue was removed in small bits by the herbivores but in larger bulks by cork borer wounding.

Within a plant individual, the stronger response to damage in treated and younger systemic tissue compared to older systemic tissue in *S. alba* cv. Silenda can be well explained

by the tissue connectivity which was visualized by the transport of the dye Rhodamine B. The amount of dye recovered after 24 h was about twice as high in treated and younger than in older leaves. The relocation of dye between the leaves was not correlated to organ biomass and is thought to be due to source-sink relationships modulating bulk phloem flows (Orians, 2005). Similar relationships of induction responses between differently aged leaves were found also in other systems (Taipalensuu *et al.*, 1997a,b; Orians *et al.*, 2000; Viswanathan and Thaler, 2004). The differences in strength of response between local and systemic younger leaf tissues could be based on a "signal" delay in systemic tissue due to transportation processes.

4.4.2 Specificity according to plant cultivar

Variation in quantity and quality of plant defences, i.e. glucosinolates and myrosinase activity in this case, is widely known to occur between species, plant individuals and different organs (Brown *et al.*, 2003; Charron *et al.*, 2005a). Crop production by humans, for which a diversity of cultivars was bred, adds another level of specificity to this scheme (for *Sinapis alba* see: Hopkins *et al.*, 1998a; Schuster-Gajzágó *et al.*, 2006). However, only a few studies compared these cultivars with respect to induction properties of glucosinolates and even fewer to those of myrosinase activity (Ludwig-Müller *et al.*, 1997; Hopkins *et al.*, 1998b; Andréasson *et al.*, 2001b). The *S. alba* cultivar Silenda used in this study and the *S. alba* cultivar Salvo previously investigated (Martin and Müller, 2007) differed with respect to constitutive glucosinolate and myrosinase activity concentrations, but moreover also with respect to induction responses to feeding by larvae of *A. rosae*. Upon induction with six-day-old larvae, local responses in damaged leaves were stronger in the cultivar Silenda than in Salvo for total glucosinolate concentrations as well as for all three myrosinase activity concentrations (soluble, insoluble and combination of both). The systemic response of glucosinolate concentrations was stronger in the cultivar Silenda, but changes of myrosinase activity concentrations were stronger in leaves of the cultivar Salvo. Thus, we were able to show that specificity of short-term induction response upon feeding by a glucosinolate-sequestering specialist herbivore varies strongly with respect to cultivar, as was also shown for studies of long-term induction effects of the non-sequestering specialist *Delia floralis* (FALL.) (Hopkins *et al.*, 1998b) and of pathogens (Andréasson *et al.*, 2001b; Ludwig-Müller *et al.*, 1997) in other Brassicaceae.

4.4.3 Consequences for interaction

The defensive properties of differently induced plants and their acceptability were tested against subsequent attacks of *A. rosae* larvae and egg-laying females. Larvae usually prefer

to move to the upper plant parts (personal observation). When placed at the bottom of a plant pot, movement of larval feeding pattern was comparable on all differently pretreated plants. Therefore, larvae seem to be unaffected by different plant induction states, specifically of the upper plant region where changes are more pronounced. Females distributed their eggs on young and old plant parts similarly on most plant treatments (Figure 4.4). However, marginal behavioural modifications were observed in response to mechanically wounded plants, where females laid fewer eggs on damaged and younger systemic than on older leaves. These plants in particular showed differences in glucosinolate profiles, especially in benzylglucosinolate concentrations, compared to the other treatments. Females of *A. rosae* were shown to respond differently to isothiocyanates, the hydrolysis products of glucosinolates, with varying side-chains (Barker *et al.*, 2006) but it is not clear whether altered glucosinolate profiles in *S. alba* might have influenced choices for egg deposition sites in our bioassays. In other systems, side chain modifications of glucosinolates led to modulations in disease resistance (Brader *et al.*, 2006) and in specialist feeding damage (Giamoustaris and Mithen, 1995), but not in generalist feeding and performance (Giamoustaris and Mithen, 1995; Burow *et al.*, 2006). Overall, the observed induction responses in *S. alba* did not serve as rapid plant protection against *A. rosae* larvae or egg-laying females, regardless of the inducing agent. However, long-term induction responses were not investigated in this study, which might reveal different aspects of resistance against a sequestering herbivore in the field. In a former study, almost no differences were found in the long-term development of larvae and adults of *A. rosae* raised on *Brassica juncea* CZERN. lines with differing glucosinolate and myrosinase contents (Müller and Sieling, 2006). In contrast, other non-sequestering specialists were found to have a reduced performance in long-term (Agrawal, 2000) and short-term trials (De Vos *et al.*, 2006) on induced plants. As a conclusion, the glucosinolate-sequestering herbivore *A. rosae* seems highly tolerant to variation in the glucosinolate-myrosinase system evoked either by rapid induction changes or by permanent modifications in different inbred lines.

Considering the arms race between plants and herbivores, induction responses in *S. alba* do not seem to benefit the plant, but rather its opponents: the sequestering specialist might profit from increased glucosinolate levels and thus increased protection due to sequestration. The generalist herbivore suppresses induction responses of hydrolysing enzymes and might obtain less toxic food this way. Therefore, in *S. alba*, the lethal plant defence paradox (Fordyce, 2001) is present for the cultivar Silenda, since increased glucosinolates mean benefits for the sequestering herbivore (Müller *et al.*, 2002). The selective increase due to *A. rosae* feeding of only myrosinase activity concentrations in systemic leaves of the cultivar Salvo (Martin and Müller, 2007) might have a protective function for the plant. In *B. juncea*, *A. rosae* larval feeding reduced glucosinolate and myrosinase activity levels within 24 hours (Müller and Sieling, 2006). This shows that not only the lethal plant

defence paradox seems to be solved to different extents in plants, but also that there is no universal short-term response of plants to one herbivore.

4.4.4 Conclusions

The induction responses found for the glucosinolate-myrosinase system in *S. alba* showed some specificity due to the inducing agent. Glucosinolates and myrosinases revealed different induction patterns pointing at separate regulation mechanisms. Insect derived elicitors and the redistribution of a potential signal *via* the phloem seem likely to be involved in this specificity. Nevertheless, these differences on the plant's side were not mirrored in behavioural responses of larvae or adult females of *A. rosae*.

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Chapter 5

Matching plant defence syndromes with preference and performance of a specialist herbivore

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Abstract - Plants use different strategies to fend off opponents. These strategies may comprise chemical or mechanical defences or avoidance mechanisms based on phenological, nutritional, or ecological features. According to the plant defence theory these traits are predicted to covary across taxa and were shown to be grouped into several syndromes. Specialist herbivores, on the other hand, tolerate, circumvent, detoxify or even recruit components of the plants' chemical weapons. Their development on different plant species might mirror the putative defence syndromes of their host species. This hypothesis was tested by investigating various plant traits of seven species of Brassicaceae with regard to nutritive values and potential defence properties taking also leaf age into account. We found no bivariate trade-offs between the plant traits, but strong positive correlations between water, soluble protein, nitrogen content and specific leaf area and between trichome densities and proteinase inhibitor activities. With a hierarchical cluster analysis, plants could be assembled in three distinct groups with either low nutritional quality or higher nutritional quality together with chemical or mechanical defences. Although young and old leaves highly differed in quantities of defences within one plant species, they always grouped within the same cluster. Development of neonate larvae until adulthood on young and old leaves of each of these plant species and adult oviposition preference were measured in the oligophagous sawfly species *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae). This specialist herbivore sequesters part of the plant defence for its own protection against antagonists. Several performance and preference parameters were used for a cluster analysis on the insect side. Clusters forming plant syndromes matched very well, but not entirely, with clusters determined from the insect parameters of this highly adapted specialist. As revealed in regression analyses, larval performance and adult preference were more influenced by mechanical than by chemical defence traits as predicted for a specialist herbivore.

Key words: *Athalia*, Brassicaceae, chemical ecology, host plant quality, insect life-history parameters, leaf age, macroevolution, microevolution, phytochemistry, plant defence traits, plant-insect interactions, plasticity.

5.1 Introduction

The understanding of plant-herbivore interactions and the factors that structure these relationships are of high interest to ecologists (e.g. Cornell and Hawkins, 2003). Plants, on the one hand, use a wide variety of strategies to fend off opponents (Stamp, 2003). These strategies may comprise chemical or mechanical defences or avoidance mechanisms based on phenological, nutritional or ecological characteristics, which can influence various trophic levels. Herbivores, on the other hand, tolerate, circumvent, detoxify or even recruit defence components (for Brassicaceae specialists see: Müller and Wittstock, 2005; Ratzka *et al.*, 2002).

In terms of a plant's fitness, it has long been the understanding that one sufficiently effective defence trait should trade off against a second, redundant one, especially when limited nutrients could otherwise be used for growth and reproduction (Agrawal, 2007). This view has some drawbacks, i.e. it does not account for (1) abiotic and biotic selective forces acting on the same plant traits, for (2) a community of differently specialised opponents requiring different defences, or for (3) possible synergism of defence traits. Recently, a novel multivariate approach was used to incorporate macroevolutionary patterns across taxa in a theory on plant defence strategies (Agrawal, 2007): the "plant defence syndrome hypothesis" rejects the prediction that any two plant defences are redundant, but it predicts that defence syndromes, e.g. tolerance and resistance traits, can trade off if they are true alternative strategies (for examples see: van der Meijden *et al.*, 1988; Fineblum and Rausher, 1995; Agrawal and Fishbein, 2006). In the "defence syndrome triangle", postulated by Agrawal and Fishbein (2006), plant defences were grouped according to the traits edibility and defence status. In a study on 23 milkweed species (*Asclepias* spp. L.), no bivariate tradeoffs were found among the tested defence traits, but significant negative correlations were detected between nutritional quality (C:N ratio) and defence. These inter-correlating plant characteristics were then grouped into three syndromes by cluster analysis. Plant species of the "low nutritional quality" syndrome did not invest in mechanical or chemical feeding barriers, but had a low nutritive value. The "nutrition and defence" syndrome grouped plant species where high edibility was balanced by equally high mechanical or chemical defences. The "tolerance/escape" syndrome, defined for fast-growing plant species in resource rich environments which have very high nutritious values but rather low defence levels, was not found within the studied milkweeds. Furthermore, Agrawal and Fishbein (2006) could attribute short-term larval performance of the specialist *Danaus plexippus* L. (Lepidoptera: Danaidae), i.e. growth rate and mortality, to an array of single plant traits, but not to trait clustering. The generality of the described defence syndromes remained yet unclear.

To test for transferability of these plant defence syndromes and match these with insect parameters, we used seven species of Brassicaceae and a specialised insect. The most prominent chemical defence in Brassicaceae is the glucosinolate-myrosinase system, also called the "mustard oil bomb". Glucosinolates are nitrogen- and sulphur-containing secondary plant metabolites (mustard oil glucosides) which are hydrolysed by myrosinase enzymes (Bones and Rossiter, 2006). The resulting breakdown products (mustard oils) display varying toxicity against an array of organisms (Renwick, 2002; see also references therein). Next to this well-known chemical weapon, mechanical barriers against herbivores such as trichomes are also found among Brassicaceae (Handley *et al.*, 2005). Furthermore, some species use digestibility reducers, namely proteinase inhibitors, to diminish the nutritive value of their tissues (Broadway and Colvin, 1992). Variation of these plant traits was shown qualitatively and quantitatively across taxa and across leaf ages (for the glucosinolate-myrosinase system see: Rask *et al.*, 2000; Fahey *et al.*, 2001; for trichomes see: Agrawal, 1999b; Beilstein *et al.*, 2006; for nutritional characteristics see: Lambdon and Hassall, 2005).

The turnip sawfly *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) feeds exclusively on Brassicaceae (Riggert, 1939; Oishi *et al.*, 1993). The larvae are able to concentrate glucosinolates of their host plants within their haemolymph (Müller *et al.*, 2001). Furthermore, the sequestered glucosinolates are used by the larvae for their own protection against invertebrate predators (Müller *et al.*, 2002; Müller and Brakefield, 2003). Adults of this species still contain glucosinolates that were incorporated in the larval stage into the haemolymph (Müller and Sieling, 2006). Larval performance did only differ slightly on inbred homozygous lines of *Brassica juncea* Czern. with different combinations of glucosinolate and myrosinase levels (Müller and Sieling, 2006). However, when presenting a wider range of variation in defence traits as well as nutrient levels by offering three plant species of Brassicaceae, developmental times and adult weights differed significantly between groups fed on either *Barbarea stricta* Andrz., *Brassica nigra* (L.) W.D.J. Koch or *Sinapis alba* L. (Müller and Arand, 2007).

From the insect's perspective, host plant quality is generally determined by a suite of different traits (Scriber and Slansky, 1981; Raubenheimer and Simpson, 1999; Awmack and Leather, 2002). The composition encompasses micro-, and macronutrients, as well as secondary putative defensive chemicals and leaf morphology. Furthermore, in herbivorous species, especially with non-feeding adult stages, host quality strongly determines oviposition behavior of females and reproduction success (Awmack and Leather, 2002). Because host quality can affect larval and also adult performance parameters to highly different degrees, a profound evaluation of plant suitability can only be made when considering many insect life-history parameters and their correlations (Scheirs *et al.*, 2003; Moreau *et al.*, 2006).

The aim of this study was to investigate if plant defence syndromes determined exclusively from the plant traits might match with the individual performance and preference of a highly adapted specialist herbivore. Therefore, we investigated on the plant side how several plant traits concerning nutritive values and defensive properties were correlated in leaf tissue of seven species of Brassicaceae. A cluster analysis was applied to verify if the same "plant defence syndromes" could be found in Brassicaceae as Agrawal and Fishbein (2006) determined in the Asclepiadaceae. As plant quality is highly variable within a plant due to leaf age, we tested the "defence syndrome triangle" by investigating leaves of different age and hence comparing macro- with microevolutionary plant trait associations. We chose seven species which belong to five tribes according to Al-Shehbaz *et al.* (2006). Therefore we assume that evolutionary constraints due to phylogenetic conservatism should be unlikely and that the obtained data across taxa are statistically independent. For leaves of different age which share the same genetic background, the dynamic processes of differentiation and growth could shape nutrition and defence traits phenotypically in different directions (Wallace and Eigenbrode, 2002; Kursar and Coley, 2003; Lambdon *et al.*, 2003; Valkama *et al.*, 2004). Therefore despite genetic dependence, we assume that phenotypic plasticity due to aging of leaves provides statistic independence.

On the insect side, neonates of *A. rosae* were reared to adulthood on young and old leaves of these seven species of Brassicaceae. An array of several performance and preference parameters was measured and investigated for correlations in a further cluster analysis. This enabled to match plant clustering to insect clustering. As this herbivore is well adapted, chemical defences should have a less severe to no impact on insect development and oviposition compared to mechanical defence. Moreover, being a food specialist, this herbivore should also be forced to be a nutrient generalist due to limited accessibility of suitable food in natural environments (Raubenheimer and Simpson, 1999). A regression of defence traits across taxa and leaves of different age with preference and performance of *A. rosae* was used to determine common resistance mechanisms exerted by plant traits and their importance for the fitness of a specialist herbivore.

5.2 Materials and methods

5.2.1 Plant and insect material

Young and old leaves of pre-flowering to flowering plants of six plant species were collected every other day from late April to early June 2004 in the Botanic Garden of the University of Würzburg: *Alliaria petiolata* (M. Bieb) Cavara & Grande (garlic mustard, Thlaspideae), *Armoracia rusticana* P. Gaertn., B. Mey. & Scherb. (horseradish,

Cardamineae), *Bunias orientalis* L. (Turkish rocket, Anchonieae), *Cardamine heptaphylla* (Vill.) O. E. Schulz (seven-leaflet bittercress, Cardamineae), *Cardamine pentaphyllos* (L.) Crantz (five-leaflet bittercress, Cardamineae), and *Lunaria rediviva* L. (perennial honesty). Additionally, glasshouse grown plants of *Brassica rapa* L. em. Metzg. ssp. *chinensis* (L.) Hanelt, cultivar Cantonner Witkrop (=Granat) (chinese cabbage, Brassiceae) were used (seeds were obtained from a local garden market). Nomenclature follows the SysTax-Database (`www.biologie.uni-ulm.de/systax`; Mansfeld and Hanelt, 2001; Wisskirchen and Adolphy, 1998). Tribe associations are given after common names in brackets, but *Lunaria* species could not be related to any accepted tribe yet (Al-Shehbaz *et al.*, 2006; Beilstein *et al.*, 2006). Four of the species are native and three are alien to Germany according to the Flora Europaea (Tutin *et al.*, 1993). Of the alien species, *Ar. rusticana* has been cultivated already for a long time and is now naturalized in many sites, *Br. rapa* ssp. *chinensis* has been cultivated only in recent decades and *B. orientalis* was introduced probably in the 18^{th} century and has received weed status over the last years (Tutin *et al.*, 1993). Plant life cycle characteristics were taken from the Flora Europaea (Tutin *et al.*, 1993).

Neonates (less than one day old) and naïve adult females (3 to 5 days after emergence from pupa, without oviposition experience) of *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) were taken from a laboratory culture. This was established from a field collection around Würzburg, Germany, in the previous autumn and insects were kept on *Sinapis alba* L. (white mustard, cultivar Salvo; seeds were a kindly gift from Mommersteeg International, formerly Advanta Seeds, Kapelle, Netherlands).

5.2.2 Leaf chemistry

To analyse different parameters of plant chemistry, samples were taken from young and old leaves of the same plant individuals of the above mentioned seven plant species. Leaf discs (diameter 1.8 cm) were cut avoiding the main veins. Samples were weighed, frozen in liquid nitrogen and stored at −80 °C until later analysis. Sets of samples were prepared in quadruplicate for all analyses. The determination and presentation of leaf chemistry parameters focuses on fresh weight (f.wt.) of tissues as a reference, because insects are faced with the concentrations of molecules thus ingested.

C:N-element-analysis

Frozen samples were freeze-dried, weighed, and pulverized in a mill (Retsch, MM301, Haan, Germany). Total carbon and nitrogen content of samples were quantified by decomposition of substances by oxidative combustion (CHN-O-Rapid, Heraeus, Hanau, Germany).

Glucosinolate analysis

Frozen samples were freeze-dried, weighed, and pulverized in a mill (see above). Glucosinolates were extracted in 80 % methanol after addition of 20 μL of a 5 mM solution of internal standard solution (Table 5.1). Glucosinolates were converted to desulphoglucosinolates and analysed by HPLC (1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1040M diode array detector) as described earlier (Martin and Müller, 2007), but using a slightly modified gradient (solvent A: water, solvent B: methanol) of 0-5 % B (10 min), 5-45 % B (28 min), followed by a cleaning cycle. Quantification was derived from the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, considering response factors for different side chains.

Myrosinase activity and soluble protein concentrations

Myrosinase activity was determined by photometric quantification of released glucose from the externally added substrate (Table 5.1). The myrosinase extraction protocol followed (Martin and Müller, 2007). For three plant species, activity was only found in the soluble myrosinase fraction (*B. orientalis*, *C. heptaphylla*, and *L. rediviva*), whereas three others contained myrosinase activity in soluble and insoluble fractions (= measured as a mixture of both fractions; *A. petiolata*, *Ar. rusticana*, and *C. pentaphyllos*). In samples of *Br. rapa* ssp. *chinensis* no myrosinase activity could be detected within 24 hours. Soluble protein concentrations were determined according to Bradford (1976).

Proteinase inhibitors

Frozen leaf material was pulverized in a mill (see above), extracted in 150 μL buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice and centrifuged. Soluble protein concentration of the resulting supernatant was measured (see above) and trypsin inhibitor concentrations were determined in a radial-diffusion assay according to (Cipollini and Bergelson, 2000). In most samples, the margins of the diffusion zones were diffuse. Quantification of proteinase inhibitor activity is therefore given as category unit per leaf disc (0: no inhibition to 6: inhibition which is equivalent to 0.14 nmol of soybean trypsin inhibitor (TI) applied per well, 7: more than 0.14 nmol of TI). Exact values could be determined in a few samples with defined margins using a calliper. *L. rediviva* had 32.9 ± 1.7 nmol g^{-1} f.wt. (mean $\pm$ SE, n = 4) TI in young leaves and 54.6 ± 27.0 nmol g^{-1} f.wt. (mean $\pm$ SE, n = 4) TI in old leaves; *B. orientalis* had 20.4 nmol g^{-1} f.wt. (mean, n = 2) TI in young leaves and 27.8 nmol g^{-1} f.wt. (mean, n = 2) TI in old leaves.

Table 5.1: Glucosinolates used as internal standards for glucosinolate analysis and as substrate in myrosinase enzyme activity assays for sample processing of the seven plant species. For the former, a glucosinolate not naturally occurring in the plant species was chosen. For the latter, the dominant glucosinolate type of the respective plant species was used. Species code in brackets refer to species names and leaf ages (y = young; o = old) in the following. Abbreviation: gls = glucosinolate.

Plant species	Species code	Internal standard for glucosinolate analysis	Glucosinolate substrate used for myrosinase activity assay
Alliaria petiolata (M. Bieb) Cavara & Grande	(Apy/-o)	benzyl-gls †	2-propenyl-gls ‡
Armoracia rusticana P. Gaertn., B. Mey. & Scherb.	(Ary/-o)	benzyl-gls	2-propenyl-gls
Brassica rapa L. em. Metzg. ssp. *chinensis* (L.) Hanelt	(Bry/-o)	2-propenyl-gls	indol-3ylmethyl-gls †
Bunias orientalis L.	(Boy/-o)	2-propenyl-gls	*p*-hydroxybenzyl-gls §
Cardamine heptaphylla (Vill.) O. E. Schulz	(Chy/-o)	2-propenyl-gls	4-methylsulfinybutyl-gls §
Cardamine pentaphyllos (L.) Crantz	(Cpy/-o)	2-propenyl-gls	*p*-hydroxybenzyl-gls
Lunaria rediviva L.	(Lry/-o)	2-propenyl-gls	4-methylsulfinybutyl-gls

Notes: Glucosinolates were obtained from † Phytoplan, Heidelberg, Germany, ‡ Merck, Darmstadt, Germany, § Glucosinolates.com, Kopenhagen, Denmark.

5.2.3 Leaf morphology and water content

Trichome density was determined from transparent nail polish imprints of ab- and adaxial leaf sides. Fragments were viewed under a dissecting microscope. Numbers of trichomes were counted on 9 or 36 mm^2 of surface area. Water contents of tissues were determined after freeze-drying of samples used for glucosinolate and C:N-element analysis. Specific leaf areas (SLA) were obtained by relating leaf disc area (2.54 cm^2) to dry weights of samples.

5.2.4 Insect performance parameters

40 mated and 40 unmated females of *A. rosae* were allowed to oviposit in separate groups on *S. alba* plants for 24 hours. Because sex is determined by arrhenotoky in this species, unfertilized eggs develop into males and fertilized eggs into females (Lee *et al.*, 1998). Larvae emerged after 8 days. Within 20 hours of hatching, they were distributed into four groups for each of the seven plant species: (a) male larvae (n = 30) on young leaves, (b) male larvae (n = 30) on old leaves, (c) a mixture of male and female larvae (n = 50) on young leaves and (d) a mixture of male and female larvae (n = 50) on old leaves. Larvae were placed on moist tissue paper in 2 or 3 L plastic containers ($200 \times 200 \times 65/95$ mm; Gerda, Schwelm, Germany) with gauze ventilation in the lid and fed *ad libitum* with cut leaves of the seven plant species. Leaves were supplied with water by transferring the petiole into floral water tubes with stoppers to prevent desiccation and were replaced at least every other day. Insects were kept in a climate chamber at 25 °C, 16:8 hours light-dark-cycle, and 70 % relative humidity (light source: Osram L 58 W/25 Universal White, 4150 lumens, Osram, Munich, Germany) throughout all trials. Duration of larval development until and body weights at the final, non-feeding instar, the eonymph, were determined. Eonymphs were transferred to soil containing cups (diameter 4 cm, height 8 cm) for pupation. Duration until emergence from pupae, body weights and sex of adults were recorded. Male adults were frozen immediately after weighing.

To quantify the body composition of male adults, water content was determined after 24 hours of drying at 70 °C in an oven. 10 dried males of each rearing group were subjected to C:N element analysis as described above for plant samples. All other dried males were pulverized in 2 mL Eppendorf tubes in a mill (see above) and fat was extracted in three steps with 1 mL of n-hexane each (Roth, Karlsruhe, Germany). The remaining solvent in the body tissue was allowed to evaporate overnight and the dried tissue was weighed. This tissue was then subjected to glucosinolate analysis as described above for plant samples.

5.2.5 Insect preference bioassays

To test for innate preferences of *A. rosae* for young or old leaves of the seven plant species, unmated, 3 to 5 days-old females, which were reared on white mustard plants as larvae, were used. Each female was placed separately into a Petri dish (diameter 5.5 cm) lined with moist filter paper and was offered two leaf squares (2×2 cm) of different age from one plant species. Leaf squares were placed on small pieces of foam ($5 \times 5 \times 2$ mm) to allow access to the leaf edges needed for oviposition (Lee *et al.*, 1998). 17 replicates were prepared per plant species. Numbers of eggs were counted after three hours.

To test for consequences of larval host plant species on preferences of adults, 15 to 22 unmated, 3 to 5 days-old females from group (c) (see 5.2.4) of all plant species were used, where available. Sufficient numbers of female adults had not developed from *B. orientalis*. Every female was confronted in no-choice tests with one medium-aged leaf disc (diameter 2.4 cm) of one plant species for two hours in Petri dishes as described above and numbers of deposited eggs were counted afterwards. Tests were conducted so that every female was exposed to all seven plant species in random order within two days. Numbers of accepted host plants were counted for each female. Leaf squares and discs were used instead of whole plants to mainly test effects of chemical and mechanical cues on oviposition choice but to exclude interrelationships with visual cues of different plant architecture and leaf size.

5.2.6 Statistical analyses

All following analyses were executed using SPSS software (version 14.0, Chicago, IL, USA).

Plant trait analyses

Plant data were obtained from young and old leaves of seven species (datasets: $n = 2_{age} \times 7_{species}$; each set $n = 4_{replicates}$). Each of the ten plant traits was tested for normal distribution of data by Kolmogorov-Smirnov-tests. Multifactorial ANOVA were used to determine the effects of species, leaf age, and the interaction term leaf age × species on observed variances (Appendix B, Table B.3). Homogeneity of variances was tested according to Levené. Pairwise correlations of plant traits were conducted on raw data ($n = 56$) and on plant species means for young and old leaves respectively ($n = 14$). Pearson product moment coefficients were calculated and obtained P-values were Bonferroni-corrected (45 single comparisons). A subsequent principal component analysis was performed on the means of two separate sets of highly correlated variables for a reduction of data. Species

means for young and old leaves were transformed to Z-scores (mean = 0, SD = 1) to obtain comparable scales for individual variables (C:N ratio, glucosinolate and myrosinase activity concentrations) and principal components factors. A hierarchical cluster analysis was conducted using squared Euklidian distances and Ward's method for linkage to identify which of the plant datasets group together. Outliers were tested by single linkage. A discriminant function analysis was subsequently performed to test for contribution of single plant traits and factors to effective separation of clusters. Differences between the clusters in mean individual plant traits were tested with unifactorial ANOVA followed by Tukey-HSD tests. Qualitative plant traits (alien versus native status, regrowth capacity likely or not, perennial versus annual/biennial lifecycle) were analysed for distribution between clusters by contingency tables (χ^2-tests).

Insect performance and preference parameter analyses

Insect developmental data were obtained from rearing larvae in groups on young and old leaves of seven species (groups: $n = 2_{sex} \times 2_{age} \times 7_{species}$; each set $n = 30/50_{replicates}$ at start). Each insect parameter was tested for normal distribution of data by Kolmogorov-Smirnov-tests for male and female insects separately. Multifactorial ANOVA were used to determine the effects of species, leaf age, and the interaction term leaf age × species on observed variances of developmental parameters for male and female insects separately (Appendix B, Table B.6). Homogeneity of variances was tested according to Levené. T-tests for independent samples were used to assess the difference between mortality rates of males (groups a and b, see Insect performance parameters) and the mixture of male and female larvae (groups c and d). Pairwise correlations of developmental parameters for male and female insects were conducted on raw data (n = 108 to 512) and on means and mortalities of rearing groups on young and old leaves respectively (n = 11 to 14). Pearson product moment coefficients were calculated and P-values were Bonferroni-corrected (males: 18 single comparisons on raw data and 28 on means and mortalities; females: 6 single comparisons on means and mortalities). Mean adult parameters of males and females and mortality rates of males and the mixture of both sexes were both sexes were correlated by Pearson product moment correlation. Egg numbers laid on different plant species by naïve females were obtained in choice tests between leaves of different age within one species, thus data are not independent. However, periods for oviposition in bioassays were very short (3 hours), so that any unnatural egg laying pressure was avoided (Odendaal and Rausher, 1990). Hence, a near-natural choice behavior was displayed. Moreover, egg numbers differed highly between plant species and leaves of different age. This allowed for approximation of data independence (overall minimum value: no eggs; maximum value: 29 eggs per female). Therefore, we included these data in correlation and cluster analysis

of means and mortalities. Multivariate ANCOVA were used to assess effects of species and leaf age as predictors and female weight as a covariate on egg numbers of naïve females. Similarly, the egg deposition data for experienced females were used in multivariate ANCOVA to assess effects of larval rearing species as predictor and adult female weight as covariate on the total numbers of eggs laid in seven consecutive bioassays. Furthermore, the numbers of accepted host plants were compared in multivariate ANCOVA with larval rearing species as predictor and adult female weight and total numbers of eggs as covariates. Pearson product moment correlation coefficients were determined for the variable female weight and total egg number or numbers of accepted host plants, separately. For a complete cluster analysis containing all 14 rearing groups, missing developmental data were substituted by mean values of the respective variables for the four groups reared on *B. orientalis* and one group reared on *L. rediviva*, where mortality was 100 %. Egg numbers and overall mortality rates were obtained for all groups. Insect parameter means and mortalities of rearing groups were transformed to Z-scores (mean = 0, SD = 1) to obtain comparable scales. A hierarchical cluster analysis was conducted using squared Euklidian distances and Ward's method for linkage. Outliers were tested by single linkage. A discriminant function analysis was subsequently calculated to test for contribution of single insect parameters to effective separation of clusters. Differences between the clusters in mean insect parameters were tested with unifactorial ANOVA followed by Tukey-HSD tests and T-tests where only two clusters included sufficient rearing groups. Homogeneity of variances was tested according to Levené.

Insect parameter regression on plant traits

Multiple regression analysis was used to assess the effects of single plant traits on sawfly performance using mortality rates and means of plant traits and insect parameters. Data were transformed to Z-scores to obtain comparable scales and therefore also comparable coefficients. Probit models were used for mortality rates with mean plant traits. Linear regression models were applied to means of total duration times and adult weights for male and female insects separately, as well as to body composition parameters of male adults and egg numbers of naïve females.

5.3 Results

5.3.1 Plant defence syndromes

The observed variation of plant trait means was higher across species than between leaves of different age (Table 5.2, Appendix B). The following plant traits were not normally distributed: myrosinase activity concentrations, trichome densities on both leaf sides, and proteinase inhibitor activities (raw data, $n = 56$; all $P < 0.01$). In 45 pairwise comparisons, eleven correlations of plant trait pairs were significant after Bonferroni correction (Table 5.3). The condensation of data in the analysis by using means instead of individual values changed dimensions of correlation coefficients and reduced the number of significant correlations to eight. Two major groups of correlations with high coefficients ($r \sim 0.7$ to 0.9) we want to stress here. First, water content, soluble protein concentration, nitrogen content per fresh weight, and SLA were all strongly correlated using raw data and most of them also correlated strongly using means (Table 5.3). Second, trichome densities on both leaf sides and trypsin inhibitor activities were highly correlated using raw data and means (Table 5.3). Means of water, soluble protein and nitrogen content of fresh weight and SLA were combined to obtain the factor "nutrition" ($n = 14$; eigenvalue: 3.48; communalities - water: 0.868; protein: 0.899; nitrogen: 0.959; SLA: 0.751). 87 % of original variability between species and leaf ages of the four variables was incorporated in the factor values based on regression (component loadings - water: -0.931; protein: 0.948; nitrogen: 0.979; SLA: -0.867). Trypsin inhibitor activities and trichome densities of both leaf sides were combined to obtain a factor which can be described as "mechanical defence" ($n = 14$; eigenvalue: 2.69; communalities - trypsin inhibitor activity: 0.914; abaxial trichome density: 0.893; adaxial trichome density: 0.88). 90 % of original variability between species and leaf ages of the three variables was incorporated in the factor values based on regression (component loadings - trypsin inhibitor activity: 0.956; abaxial trichome density: 0.945; adaxial trichome density: 0.938). For reasons of clustering efficiency and to avoid incorporating redundant information, these factor values were used for further analyses. The hierarchical cluster analysis using single linkage method did not reveal any outliers. Thus, using Ward's method we found three clusters at a combined distance of 11. Cluster 1 contained both young and old leaves of *Ar. rusticana*, *C. heptaphylla*, and *C. pentaphyllos*. Cluster 2 contained both young and old leaves of *A. petiolata* and *Br. rapa* ssp. *chinensis*. Cluster 3 contained both young and old leaves of *B. orientalis* and *L. rediviva* (Figure 5.1). Within the three clusters almost all single variables and factors displayed smaller variances compared to the overall variances with the exceptions of myrosinase activity concentration in cluster 1 (72 % increased variance) and C:N ratio in cluster 2 (9 % increased variance). A subsequent discriminant function analysis revealed that the standardized

canonical discriminant function coefficients for myrosinase activity concentration differed least between the two functions and thus contributed least to the separation of clusters (Table 5.4). To function 1 predominantly the two principal component factors "nutrition" and "mechanical defence" and C:N ratio contributed the most, while onto function 2 mainly glucosinolate and myrosinase activity concentrations were loaded. According to these functions plant clusters were effectively separated with a minimum of 98 % correct classification.

Unifactorial ANOVA on single variables revealed that significant differences were found for all plant traits except for water content, SLA and myrosinase activity concentration between the clusters (Table 5.5). Cluster 1 was formed from plants with a high nitrogen content of fresh weight and medium C:N ratio together with high glucosinolate concentrations. In contrast, cluster 2 included plants with a low nitrogen content and high C:N ratio and lower glucosinolate concentrations. In cluster 3 plants were grouped with high nitrogen content and low C:N ratio together with high densities of trichomes and high proteinase inhibitor activities.

Distribution of qualitative plant traits (alien versus native status, regrowth capacity likely or not, perennial versus annual/biennial lifecycle) between clusters differed significantly only for lifecycle characteristics ($n = 7,\ \chi^2 = 7.00,\ df = 2,\ P = 0.030$). The two annual/biennial species included in the study, *A. petiolata* and B. rapa ssp. chinensis, were found exclusively in cluster 2. Clusters 1 and 3 contained only perennial species. For regrowth capacity only a marginal distribution inhomogeneity ($n = 7,\ \chi^2 = 3.73,\ df = 2,\ P = 0.154$) was found: the two *Cardamine* species in cluster 1 normally develop only three to five leaves and one inflorescence per growing season, while all five other species are able to produce several secondary shoots, inflorescences and leaves.

Table 5.2: Plant leaf traits of Brassicaceae. Ranges for species and leaf age means followed by percentage variation.

Plant trait	species means			leaf age means		
	minimum	maximum	variation †	minimum	maximum	variation
Water content [% of f.wt.‡]	80.04	90.21	13	83.59	85.25	2
Specific leaf area [cm^2 mg^{-1}]	0.23	0.63	174	0.43	0.46	7
Soluble protein content [μg mg^{-1} f.wt.]	1.86	6.00	223	3.34	4.14	24
Nitrogen content [‰ of f.wt.]	3.64	9.40	158	6.38	7.03	10
C:N ratio	8.73	13.15	51	10.64	10.70	1
Glucosinolate concentration [μmol mg^{-1} f.wt.]	2.09	13.97	568	6.87	8.39	22
Myrosinase activity concentration [pmol min^{-1} mg^{-1} f.wt.]	0 §	82.36	Infinite	16.63	20.81	25
Adaxial trichome density [no. hairs mm^{-2}]	0	3.55	Infinite	0.97	1.28	32
Abaxial trichome density [no. hairs mm^{-2}]	0	10.89	Infinite	2.00	2.43	22
Trypsin inhibitor activity [category unit per leaf disc]	0.13	5.63	4,231	1.96	2.36	20

Notes: † Variation is given as percentage of minimum-maximum-difference in relation to minimum value; ‡ f.wt. - fresh weight; ‡ No myrosinase activity could be detected within 24 hours.

Table 5.3: Pairwise correlations of plant leaf traits among young and old leaves of seven species of Brassicaceae.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) Water content		**0.68** ***	**-0.61** ***	**-0.74** ***	-0.12	**-0.51** **	-0.12	-0.13	0.06	0.05
(2) Specific leaf area	*0.72* †		-0.76 ‡	**-0.78** *	0.03	**-0.47** *	0.06	-0.20	0.05	-0.09
(3) Soluble protein content	***-0.83*** *	***-0.53*** **		**0.77** ***	-0.41	0.33	0.10	0.30	0.09	0.11
(4) Nitrogen content	**-0.92** ***	***-0.55*** **	**0.94** ***		**-0.49** **	0.42	0.16	0.27	0.08	0.12
(5) C:N ratio	0.12	0.03	-0.57	*-0.48*		-0.08	-0.08	-0.29	-0.24	-0.30
(6) Glucosinolate concentration	*-0.62*	*-0.66*	0.54	0.55	-0.14		0.23	0.06	-0.16	0.00
(7) Myrosinase activity concentration	-0.08	0.09	0.04	0.10	-0.13	0.22		-0.29	-0.27	-0.19
(8) Adaxial trichome density	-0.15	-0.24	0.38	0.35	-0.56	0.08	-0.31		**0.80** ***	**0.72** ***
(9) Abaxial trichome density	0.07	0.06	0.14	0.13	-0.47	-0.23	-0.29	***0.82*** *		**0.75** ***
(10) Trypsin inhibitor	0.05	-0.13	0.26	0.16	-0.55	0.07	-0.27	***0.85*** **	***0.87*** **	

Notes: Pearson product moment correlation coefficients (r) above the diagonal are calculated from individual plant samples (n = 56); those below are calculated from plant species means for young and old leaves respectively (n = 14); significant correlations after Bonferroni correction (45 comparisons in total) are highlighted in bold; italic coefficients designate correlations changed in significance level due to data reduction by formation of means; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; † $P < 0.2$; ‡ $P < 0.1$. For units of plant traits see Table 5.2.

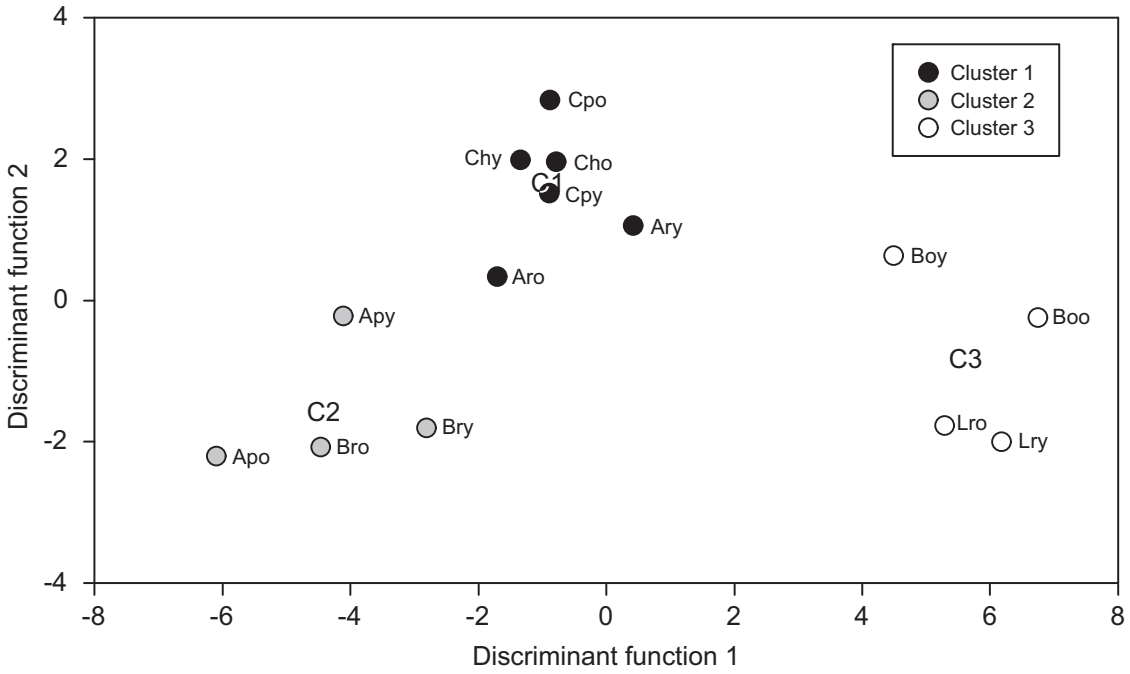

Figure 5.1: Plant clustering according to the two discriminant functions; C1 - centroid of cluster 1, C2 - centroid of cluster 2, C3 - centroid of cluster 3; three letter abbreviations refer to plant species means for young and old leaves.

Table 5.4: Coefficients of discriminant function analysis of clustering results using mean plant traits of young and old leaves of seven species of Brassicaceae.

Plant trait	Function 1	Function 2
C:N ratio	-0.696	-0.077
Glucosinolate concentration	0.163	0.940
Myrosinase activity concentration	0.223	0.648
"Nutrition"	1.168	-0.116
"Mechanical defence"	1.391	-0.214
Eigenwert of function	19.06	2.59
Explained variability [%]	88	12

Notes: For units of plant traits see Table 5.2.

Table 5.5: Relationship between means of all plant leaf traits and clustering of young and old leaves of seven species of Brassicaceae (mean ± SE).

Plant trait	Cluster 1 (n = 6)	Cluster 2 (n = 4)	Cluster 3 (n = 4)	ANOVA F	ANOVA P
Water content	83.29 ± 1.04	87.33 ± 2.03	83.22 ± 1.27	2.58	0.121
Specific leaf area	0.40 ± 0.05	0.59 ± 0.05	0.38 ± 0.09	2.96	0.094
Soluble protein content	3.91 ± 0.45 ab	**2.27** ± 0.30 a	**4.95** ± 0.71 b	6.09	0.017
Nitrogen content	**7.13** ± 0.58 a	**4.61** ± 0.67 b	**8.17** ± 0.75 a	6.09	0.017
C:N ratio	10.85 ± 0.38 ab	**12.03** ± 0.80 a	**9.06** ± 0.19 b	6.71	0.012
Glucosinolate concentration	**10.64** ± 1.13 a	**3.00** ± 1.45 b	7.75 ± 1.56 ab	8.32	0.006
Myrosinase activity concentration ‡†	36.82 ± 15.17	4.24 ± 2.78	6.07 ± 0.76	2.70	0.111
Adaxial trichome density ‡	**0.35** ± 0.22 a	**0.14** ± 0.11 a	**3.28** ± 0.71 b	18.60	<0.001
Abaxial trichome density ‡†	**0.02** ± 0.01 a	**0.68** ± 0.60 a	**7.05** ± 2.35 b	10.24	0.003
Trypsin inhibitor activity ‡	**1.25** ± 0.44 a	**1.00** ± 0.67 a	**4.69** ± 0.54 b	13.46	0.001

Notes: † No variance homogeneity was found. ‡ Not normally distributed. Tukey HSD-tests were used as Post-hoc tests; letters indicate significant differences at $P < 0.05$; significantly different means are highlighted in bold. For units of plant traits see Table 5.2.

5.3.2 Insect performance and preference

Means of insect developmental parameters varied more distinctly between host plant species than leaf age classes (Tables 5.6 and 5.7, Appendix B). The following insect parameters were not normally distributed for males (n = 108 to 512) or females (n = 196): developmental times of the larva, the pupa and complete developmental times of both sexes, and water content of adult males. Overall, larval developmental times of females were a little bit longer, but their pupal times were about one day shorter than those of males. Females were about 30 % heavier as larvae and as adults compared to males (Tables 5.6 and 5.7). Mortality rates could be obtained only on males and a mixture of sexes (see 5.2.4). There were no differences between any of the mortality rates obtained from males alone or from a mixture with females (T-tests for independent groups: larval mortality, n = 28, $T = 0.23$, $P = 0.82$; pupal mortality, n = 22, $T =$ -0.92, $P =$ 0.37; total mortality, n = 28, $T = 0.08$, $P = 0.93$; variances were homogenous). Therefore, we included mortalities of the mixture of sexes as a substitute in the variation and correlation analysis of female developmental parameters (Tables 5.7 and 5.9).

Developmental parameters of larval stages determined the integrated total outcome until adult hatching and showed higher variation of values than those parameters of pupal stages (Tables 5.6 and 5.7). Larval mortality rates were highly correlated with total mortality rates (n = 14; males: r = 0.99, $P < 0.001$; mixture of sexes: r = 0.99, $P < 0.001$). Using raw data, larval developmental times were strongly correlated with total developmental times (males: n = 488, r = 0.86, $P < 0.001$; females: n = 196, r = 0.90, $P < 0.001$) and eonymph weight determined adult weight (males: n = 487, r = 0.86, P r = 0.86, $P < 0.001$; females: n = 196, r = 0.93, $P < 0.001$). In the following we will hence focus on the integrated total outcome.

In 18 comparisons of male developmental parameters using raw data 13 correlations were significant after Bonferroni correction (Table 5.8). Total developmental times displayed a highly significant, negative correlation with insect adult weights, but the correlation coefficient of -0.48 was only of medium strength. Significant correlations of adult weight with the different body composition parameters revealed that heavier males had relatively less water, and thereby more non-fat dry matter, higher glucosinolate concentrations and higher nitrogen contents. Fat content was not correlated to adult weight, but to non-fat dry matter. Using group developmental means increased possible comparisons in total to 28 since mortalities and all body composition parameters could be included. Overall eleven significances were lost by data reduction of using means instead of raw data. Water content correlations with non-fat dry matter and nitrogen content remained, stressing the significance of water content for male body composition.

Using raw data of female development, total developmental times displayed again a highly significant, negative correlation with insect adult weights and the correlation coefficient of -0.55 was, as in males, only of medium strength (Table 5.9). When using means of rearing groups the number of possible comparisons increased to six of which only one was significant after Bonferroni-correction at $P < 0.05$. Total developmental times of females correlated positively with total mortality rates of the mixtures of sexes. A similar relationship, though not significant, was also found for male insects. Total mortality rates showed marginal, negative correlations with adult weight and egg numbers of naïve females.

Male and female mean parameters and mortality rates of males and both sexes in the different rearing groups were strongly correlated (mortality rates: $r = 0.94$, $n = 14$, $P < 0.001$; developmental times: $r = 0.93$, $n = 11$, $P < 0.001$; and adult weights: $r = 0.94$, $n = 11$, $P < 0.001$). Since mortality rates did not differ between males only and the mixture of sexes (see above), the mean overall mortality rates of males only and in mixture with females were included in the following analyses. For reasons of clustering efficiency and to avoid incorporating redundant information a subset of insect parameters was used: overall mortality rate, female adult weights, egg numbers of naïve females, as well as fat content and glucosinolate concentration of males. The hierarchical cluster analysis using single linkage method did not reveal any outliers. Thus, using Ward's method we found three clusters at a combined distance of 12. Cluster 1 contained insect groups reared on both young and old leaves of *A. petiolata*, *Ar. rusticana*, and *C. heptaphylla*. Cluster 2 contained insects groups reared on both young and old leaves of *Br. rapa* ssp. *chinensis* and *C. pentaphyllos*. Cluster 3 contained insects groups reared on both young and old leaves of *B. orientalis* and of *L. rediviva* (Figure 5.2). Within the three clusters almost all single variables displayed smaller variances compared to the overall variances with the exceptions of egg numbers and male fat content in cluster 2 (122 % and 78 % increased variance, respectively). A subsequent discriminant function analysis revealed that all parameters contributed meaningfully to the separation of clusters (Table 5.10). To function 1 predominantly the overall mortality rates, egg numbers of naïve females, and male glucosinolate concentrations and fat content contributed, while function 2 was dominated by female adult weights. According to these functions, insect clusters were effectively separated with 100 % correct classification of rearing groups into the three clusters. Unifactorial ANOVA revealed that significant differences depending on the clusters were found for mortality rates of males alone and in a mixture with females, as well as for female developmental times and adult weights (Table 5.11). Due to high variation of values especially in cluster 2, egg numbers in the three clusters did not differ significantly, but naïve females laid fewer eggs on plants in cluster 3 than on those in clusters 1 and 2. T-tests comparing all other traits between clusters 1 and 2 revealed differences for most other parameters except for male fat and water content and male developmental times.

In summary, cluster 1 was formed from insects with high adult weights of both sexes and high glucosinolate concentrations and nitrogen contents in males. In contrast cluster 2 included insects with low adult weights of both sexes and low glucosinolate concentrations and nitrogen contents in males. Both clusters showed low mortality and developmental times. In cluster 3 insects were grouped with high mortality rates. The remaining insects in three groups reared on leaves of *L. rediviva* showed prolonged developmental times and lower adult weights of females and males compared to clusters 1 and 2. Furthermore, males were containing remarkably more fat per weight than in the other clusters.

A multifactorial ANCOVA on egg numbers of naïve females assuming data independence revealed that plant species had a stronger influence on oviposition preference compared to leaf age, i.e. 60 % of variance was explained by the first and only 6 % by the latter. The covariate female weight was not significant as a predictor ($n = 238$, ANCOVA: $df = 8$, $F = 48.1$, η^2 (eta squared) $= 0.63$; species effect $df = 6$, $F = 57.5$, $P < 0.001$, η_p^2 (partial eta squared) $= 0.60$; leaf age effect $df = 1$, $F = 13.5$, $P < 0.001$, $\eta_p^2 = 0.06$; covariate female weight $df = 1$, $F = 0.2$, $P = 0.669$, $\eta_p^2 = 0.001$; variances were not homogenous).

For experienced females, the total numbers of eggs that were laid in seven consecutive bioassays were significantly influenced by the species on which larvae were raised, but not by female weight ($n = 123$, ANCOVA: $df = 6$, $F = 6.1$, $\eta^2 = 0.24$; rearing species effect $df = 5$, $F = 6.3$, $P < 0.001$, $\eta_p^2 = 0.21$; covariate female weight $df = 1$, $F = 0.1$, $P = 0.752$, $\eta_p^2 = 0.001$; variances were not homogenous). In contrast, neglecting plant species, we found a significant positive correlation between total numbers of eggs and female weight ($r = 0.73$, $P < 0.001$). The numbers of accepted host plants were significantly influenced by the species on which larvae were raised, and even 5-times stronger by the total numbers of eggs laid within the bioassays, but not by female weight ($n = 123$, ANCOVA: $df = 7$, $F = 30.7$, $\eta^2 = 0.65$; rearing species effect $df = 5$, $F = 2.8$, $P = 0.020$, $\eta_p^2 = 0.11$; covariate total numbers of eggs $df = 1$, $F = 124.6$, $P < 0.001$, $\eta_p^2 = 0.52$; covariate female weight $df = 1$, $F = 2.8$, $P = 0.098$, $\eta_p^2 = 0.024$; variances were homogenous). In contrast, neglecting plant species and total egg numbers, we found a significant positive correlation between female weight and numbers of accepted host plants ($r = 0.41$, $P < 0.001$).

Table 5.6: Male insect developmental parameters on young and old leaves of seven species of Brassicaceae. Ranges with regard to species and leaf age means are followed by percentage variation.

Insect parameter	species means			leaf age means		
	minimum	maximum	variation †	minimum	maximum	variation
Larval mortality [%]	5	100	1,900	28	38	36
Pupal mortality [%]	2	17	750	6	8	33
Total mortality [%]	7	100	1,329	33	42	27
Larval developmental time [d]	8.6	13.0	51	9.9	10.1	2
Pupal developmental time [d]	12.4	13.2	6	12.5	12.6	1
Total developmental time [d]	21.0	26.0	24	22.4	22.7	1
Eonymph weight [mg]	22.3	32.1	44	28.4	28.4	0
Adult weight [mg]	8.6	11.6	35	9.9	10.1	2
Water content of adult f.wt. ‡ [%]	76.0	82.1	8	77.8	78.4	1
Non-fat dry matter content of adult f.wt. [%]	6.6	15.5	135	13.4	14.6	9
Fat content of adult f.wt. [%]	5.4	10.7	98	7.4	8.2	11
Gls. concentration § [μmol mg^{-1} adult f.wt.]	2.9	7.3	152	5.9	5.9	1
C:N ratio	4.1	4.7	14	4.4	4.4	0
Nitrogen content [‰ of adult f.wt.]	22.5	26.8	19	24.7	25.7	4

Notes: † Variation is given as percentage of minimum-maximum-difference in relation to minimum value; ‡ f.wt. - fresh weight; § Gls. - glucosinolate concentration.

Table 5.7: Female insect developmental parameters on young and old leaves of seven species of Brassicaceae. Ranges with regard to species and leaf age means are followed by percentage variation.

Insect parameter	species means			leaf age means		
	minimum	maximum	variation †	minimum	maximum	variation
Larval mortality ‡ [%]	2	100	4,900	28	32	14
Pupal mortality ‡ [%]	4	24	500	9	10	11
Total mortality ‡ [%]	6	100	1,567	35	38	9
Larval developmental time [d]	9.1	13.5	48	9.9	10.8	9
Pupal developmental time [d]	11.0	12.4	13	11.4	11.5	1
Total developmental time [d]	20.6	25.4	23	21.3	22.3	5
Eonymph weight [mg]	30.9	47.5	54	37.7	40.4	7
Adult weight [mg]	12.1	20.3	68	15.7	16.8	7
Egg numbers of naïve females §	0.1	15.8	15,700	5.3	7.4	40

Notes: † Variation is given as percentage of minimum-maximum-difference in relation to minimum value; ‡ Mortality rates could only be obtained from a mixture of both sexes; § Egg numbers of naïve females were obtained in leaf-age-choice tests, but periods of bioassays were very short (3 hours) and egg numbers were highly different between plant species and leaf ages and thus allowed for approximation of data independence (overall minimum value: no eggs; maximum value: 29 eggs per female).

Table 5.8: Pairwise correlations of developmental traits of male insects reared on young and old leaves of seven species of Brassicaceae.

	Mortality	Time	Weight	Water	Dry matter	Fat	Gls.	Nitrogen
Total developmental time	0.62		**-0.48** ***	**0.34** ***	**-0.25** ***	-0.10	-0.12	**-0.33** *
Adult weight	0.48	*-0.67*		**-0.48** ***	**-0.48** ***	-0.13	**0.21** **	**0.47** ***
Water content	0.43	*0.78*†	*-0.80*‡		**-0.82** ***	-0.15	**-0.29** ***	**-0.73** ***
Non-fat dry matter content	-0.56	*-0.72*	*0.67*	**-0.83** *		**-0.45** ***	**0.18** *	n.d.
Fat content	0.28	-0.01	0.03	-0.07	*-0.49*		0.14†	n.d.
Glucosinolate concentration	-0.16	-0.27	*0.74*	*-0.72*	*0.51*	-0.07		n.d.
Nitrogen content	-0.34	*-0.71*	*0.73*	***-0.90*** **	0.68	0.15	0.73	

Notes: Pearson product moment correlation coefficients (r) above the diagonal are calculated from developmental raw parameters (n = 108 to 512); those below are calculated from developmental mean parameters of insects reared on young and old leaves of seven species (n = 11 to 14); significant correlations after Bonferroni correction (18 comparisons using raw data, 28 comparisons using means) are highlighted in bold; italic coefficients designate correlations changed in significance level due to data reduction by formation of means; *** $P < 0.001$; ** $P < 0.01$ and * $P < 0.05$; † $P < 0.2$; ‡ $P < 0.1$; n.d. - not determined due to different subsets of insects analyzed. For units of insect parameters see Table 5.6.

Table 5.9: Pairwise correlations of developmental traits of female insects reared on young and old leaves of seven species of Brassicaceae.

	Mortality[§]	Time	Weight
Total devlopmental time	**0.87** **		**-0.55** ***
Adult weight	-0.67[†]	*-0.67*[†]	
Egg numbers[#]	-0.53	-0.71[‡]	0.27

Notes: Pearson product moment correlation coefficients (r) above the diagonal are calculated from developmental raw parameters (n = 196); those below are calculated from developmental mean parameters of insects reared on young and old leaves of seven species (n = 12 to 14); significant correlations after Bonferroni correction (6 comparisons using means) are highlighted in bold; italic coefficients designate correlations changed in significance level due to data reduction by formation of means; *** $P < 0.001$; ** $P < 0.01$ and * $P < 0.05$; [†] $P < 0.2$; [‡] $P < 0.1$. [§] Mortality rates could only be obtained from a mixture of both sexes; [#] Naïve females were used in choice bioassays with young and old leaves of one species (see Materials and Methods). For units of insect parameters see Table 5.7.

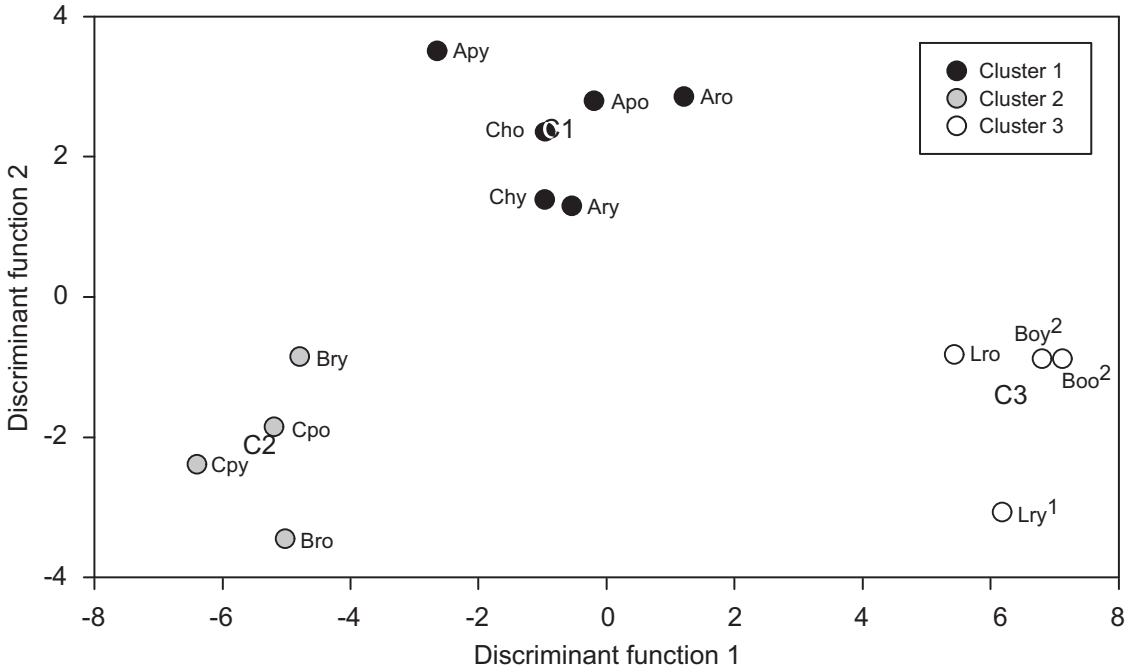

Figure 5.2: Insect clustering according to the two discriminant functions; C1 - centroid of cluster 1, C2 - centroid of cluster 2, C3 - centroid of cluster 3; three letter abbreviations refer to plant species means for young and old leaves; [1] three or [2] two missing values were substituted with the parameter mean (n = 11/12) for female weight in two groups (Boo and Boy) and fat content and glucosinolate concentration of males in three groups (Boo, Boy and Lry).

Table 5.10: Coefficients of discriminant function analysis of clustering results using mean parameters of insects reared on young and old leaves of seven species of Brassicaceae.

Insect parameter	Function 1	Function 2
Overall mortality rates	1.444	-0.241
Egg numbers of naïve females	-1.110	0.011
Females adult weight	0.139	0.861
Male fat content	1.981	0.320
Male glucosinolate concentration	1.083	0.240
Eigenwert of function	25.47	5.44
Explained variability [%]	82	18

Notes: For units of insect parameters see Tables 5.6 and 5.7.

Table 5.11: Relationship between means of insect parameters and clustering of insects reared on young and old leaves of seven species of Brassicaceae (mean ± SE).

Insect parameter	Cluster 1 (n = 6)	Cluster 2 (n = 4)	Cluster 3 (n = 4/2/1 †)	ANOVA F	ANOVA P	T-test T	T-test P
Total mortality of both sexes	**0.13** ± 0.03 a	**0.26** ± 0.05 a	**0.82** ± 0.15 b	20.56	<0.001		
Total mortality of males	**0.16** ± 0.07 a	**0.16** ± 0.04 a	**0.92** ± 0.08 b	38.79	<0.001		
Egg numbers of naïve females	8.99 ± 1.63	7.90 ± 4.72	0.79 ± 0.29	2.76	0.107		
Total dev. time of females	**21.14** ± 0.20 a	**22.22** ± 0.63 a	**25.41** ± 0.59 b	18.32	0.001		
Adult weight of females	**19.12** ± 0.54 a	**12.67** ± 0.75 b	**12.42** ± 0.80 b	34.44	<0.001		
Total dev. time of males	21.83 ± 0.35	23.13 ± 0.54	26.04			-2.12	0.067
Adult weight of males	**10.81** ± 0.32 a	**8.63** ± 0.27 b	8.64			4.80	0.001
Water content of males ‡	**76.40** ± 0.35 a	**80.46** ± 0.73 b	82.14			-5.04	0.006
Non-fat dry matter content of males	15.06 ± 0.34	12.43 ± 0.98	6.58			2.55	0.068
Fat content of males	8.24 ± 0.15	7.11 ± 1.03	10.66			1.08	0.356
Glucosinolate concentration of males	**6.98** ± 0.28 a	**3.60** ± 1.05 b	5.79			3.76	0.006
Nitrogen content of males	**26.67** ± 0.30 a	**23.54** ± 0.88 b	22.53			3.37	0.032

Notes: No variance homogeneity was found for water, fat, and nitrogen content of males. † insect parameters 1 to 3 (n = 4), 4 and 5 (n = 2), 6 to 12 (n = 1); ‡ Not normally distributed. Tukey HSD-tests were used as Post-hoc tests; letters indicate significant differences at $P < 0.05$; significantly different means are highlighted in bold. For units of plant traits see Tables 5.6 and 5.7.

5.3.3 Multiple regression analyses on plant and insect traits

Conducting probit analyses on total mortality rates revealed that the best results were obtained when the factor "mechanical defence" was replaced by its individual plant traits in a model also including C:N ratio, glucosinolate, myrosinase activity concentrations, and the factor "nutrition" (Table 5.12). The resulting separate models using males only or the mixture of sexes were very similar, but a combined model showed no parallelism (model fit: $\chi^2 = 35.79,\ df = 19,\ P = 0.011$; test for parallelism: $\chi^2 = 13.778,\ df = 1,\ P < 0.001$). The model for male mortality rates was less well fitted to the original observations than the mixture's model (male $\chi^2 = 12.99,\ df = 6,\ P = 0.043$; mixture $\chi^2 = 9.01,\ df = 6,\ P = 0.173$). The coefficients for adaxial trichome density, trypsin inhibitor activity and the factor "nutrition" were positive, while C:N ratio, glucosinolate concentration and abaxial trichome density coefficients were negative. Thus increases in the first three plant traits should increase the probability of death of an insect, while a decrease of mortality probability is likely followed by increases in the latter three plant trait values. Myrosinase activity concentration had no significant influence on total mortality rates.

Linear regression models for total developmental times, adult weights, male fat content, male glucosinolate concentration, and egg numbers of naïve females were fitted on the mean plant traits C:N ratio, glucosinolate and myrosinase activity concentration, together with the two principal component factors "mechanical defence" and "nutrition". A complete model and a reduced model were calculated for all insect parameters. Total developmental times were mainly influenced by C:N ratio, myrosinase activity and "mechanical defence" and those variables explained more than 91 % of the observed variability (Table 5.13). According to the signs of the coefficients, longer developmental times could thus be attributed mostly to higher trichome densities and trypsin inhibitor activities and a little less to lower C:N ratios for both sexes. Higher myrosinase activity was found to increase developmental time at least for males. Adult weights of insects regressed strongly on C:N ratio and also slightly on the factor "nutrition" and both explained 71 % of variability for males and 87 % for females. Whereas for males only the coefficient for C:N ratio was significantly different from zero, for females both coefficients revealed significant influences. In alternative reduced models for males in which the factor "nutrion" was substituted by one of its single variables, only water and nitrogen content of fresh weight displayed coefficients significantly different from zero. Thus, increases in C:N ratio and "nutrition" parameters resulted in an increase of adult weight. The multiple linear regression models for fat content of male adults displayed a high negative influence of myrosinase activity concentration and a slightly positive one of "mechanical defence" on fat accumulation of male adults. The reduced model explained 82 % of the variability of male fat content. The glucosinolate concentration of adult male insects was strongly influenced by C:N ratio,

and a little less also influenced by "mechanical defence" and "nutrition". All coefficients showed significant positive relationships in the reduced model and explained 86 % of data variability. Egg numbers of naïve females regressed negatively on myrosinase activity and the factors "mechanical defence" and "nutrition". The reduced model could explain only 62 % of the observed variability in egg numbers. In alternative reduced models with one single parameter of the factor "nutrition", especially nitrogen content of fresh weight displayed coefficients significantly different from zero. Glucosinolate concentration of the plant tissue never had a significant influence on insect development or egg deposition.

Table 5.12: Probit analysis of for mortality rates using the sum of dead larvae and pupae of male insects only and in mixture with females in rearing groups depending on plant traits (Z-scores); whole model predictability male $\chi^2 = 12.99,\ df = 6,\ P = 0.043$; mixture $\chi^2 = 9.01,\ df = 6,\ P = 0.173$; Test for parallelism: $\chi^2 = 13.778,\ df = 1,\ P < 0.001$.

Plant trait	Slope	SE	Z-value	P
(a) males				
C:N ratio	-0.40	0.13	-3.01	0.003
Glucosinolate concentration	-1.07	0.30	-3.60	<0.001
Myrosinase activity concentration	0.10	0.12	0.83	0.406
Adaxial trichome density	2.43	0.82	2.96	0.003
Abaxial trichome density	-1.81	0.54	-3.37	0.001
Trypsin inhibitor activity	0.50	0.34	1.45	0.147
"Nutrition"	1.06	0.23	4.58	<0.001
Constant	0.03	0.21	0.15	0.880
(b) females				
C:N ratio	-0.50	0.09	-5.41	<0.001
Glucosinolate concentration	-0.76	0.13	-6.01	<0.001
Myrosinase activity concentration	0.02	0.07	0.27	0.787
Adaxial trichome density	1.48	0.30	4.90	<0.001
Abaxial trichome density	-1.63	0.22	-7.28	<0.001
Trypsin inhibitor activity	0.75	0.21	3.61	<0.001
"Nutrition"	0.52	0.11	4.81	<0.001
Constant	-0.22	0.08	-2.86	0.004

Notes: All slopes correlate with each other except for C:N ratio: (a) $r \sim 0.9$, (b) $r \sim 0.7$.

Table 5.13: Linear regression for developmental parameters depending on plant traits (Z-scores).

Insect parameter	Model	R^2	ANOVA			Standardized coefficients			
			F	df	P	C:N ratio	Myrosinase	"Mech. defence"	"Nutrition"
(a) male									
Total developmental time	complete	0.98	41.41	5	<0.001	-0.39 **	0.42 **	0.67 **	
	reduced	0.97	87.34	3	<0.001	-0.38 **	0.43 **	0.69 **	
Adult weight	complete	0.73	2.63	5	0.156	0.76 ‡			0.27 $^{n.s.}$
	reduced	0.71	9.98	2	0.007	0.80 **			0.36 †
Fat content of adults	complete	0.85	5.74	5	0.039		-0.73 *	0.36 †	
	reduced	0.82	18.72	2	0.001		-0.76 **	0.35 ‡	
Glucosinolate concentration	complete	0.89	8.20	5	0.019	0.82 **		0.49 *	0.53 ‡
of adults	reduced	0.86	14.02	3	0.002	0.81 **		0.50 *	0.69 **
(b) female									
Total developmental time	complete	0.93	14.79	5	0.003	-0.38 **	0.29 ‡	0.68 **	
	reduced	0.91	27.69	3	<0.001	-0.41 **	0.26 ‡	0.67 **	
Adult weight	complete	0.89	8.18	5	0.019	0.77 **			0.48 ‡
	reduced	0.87	26.67	2	<0.001	0.80 ***			0.55 **
Egg numbers of naïve females	complete	0.68	3.32	5	0.065		-0.57 *	-0.43 †	-0.49 †
	reduced	0.62	5.44	3	0.018		-0.57 *	-0.53 *	-0.35 †

Notes: : Complete models were established on the plant traits C:N ratio, glucosinolate and myrosinase activity concentration and the factors "mechanical defense" and "nutrition". Reduced models contained only those plant traits or factors for which regression coefficients are displayed. In complete models no other coefficients were significantly different from zero than those displayed; asterisks indicate levels of significance for standardized regression coefficients: *** $P < 0.001$; ** $P < 0.01$ and * $P < 0.05$; † $P < 0.2$; ‡ $P < 0.1$; n.s. - not significant.

5.4 Discussion

5.4.1 Plant defence syndromes

One central aim of this study was to evaluate whether plant traits connected with general defence against herbivorous insects in Brassicaceae could be grouped into syndromes. Using seven species of Brassicaceae and analysing leaves of two different ages, we found no bivariate trade-offs between any two defences. Within this subset of Brassicaceae, the nutritious value of a plant tissue was predominantly protected by trichomes and proteinase inhibitors (88 % of variation, discriminant function 1; Table 5.4), whereas chemical defences, i.e. glucosinolate and myrosinase activity concentrations, were more or less expressed independently (12 % of variation, discriminant function 2). In *Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz ssp. *petrea* and *Brassica nigra* glucosinolates and trichomes were also not correlated phenotypically and only weakly on the genetic level (Traw, 2002; Clauss *et al.*, 2006). Therefore, chemical and mechanical defences are likely to represent two independent, but not mutually exclusive strategies across taxa which are comprised of several traits (for a critical meta-analysis see: Koricheva *et al.*, 2004).

Accordingly three clusters of plant defence traits were found that resembled those found by Agrawal and Fishbein (2006): high nitrogen containing plants with chemical defences were grouped in cluster 1, whereas those with mechanical defences were found in cluster 3 (Figure 5.1, Table 5.5). In plants of cluster 2, nitrogen content was low and also low levels of mechanical or chemical defences were found. Throughout all clusters, water content was high, where nitrogen content was low (principal component factor "nutrition"). Low water content is usually attributed to "hard to eat" characteristics of a plant (Scriber and Slansky, 1981). Thus plants with low water content were placed in the "low nutritional quality" syndrome (Agrawal and Fishbein, 2006). But high water contents per fresh weight ($\sim$80 to 90 %) indicate higher dilutions of metabolites which result in compensatory feeding of insects (Slansky and Wheeler, 1992). Water content of plants might therefore show a biphasic relation to insect performance with an optimum below which feeding barrier characteristics are of higher importance (Coley *et al.*, 2006) and above which nutrition dilution rather is the prime cause for insect resistance. Also strong associations of proteinase inhibitor activity with trichome densities (principal component factor "mechanical defence") and with the nutritional status of a tissue (discriminant function 1) were found. Within the syndrome triangle, these digestibility reducing proteins were hypothetically expected to be associated with the "low nutritional quality" syndrome. But it might be more adaptive for a plant to develop high titers of proteinase inhibitors when there is a lot worthy to be protected, i.e. high contents of nitrogen or proteins (Broadway and Colvin, 1992). In addition, proteinase inhibitors could also act synergistically with trichomes,

because the first might impede actual nitrogen acquisition, whereas the latter might cause tissue damage in the gut, whose repair is nitrogen demanding (Raubenheimer and Simpson, 1999). As in the milkweeds (Agrawal and Fishbein, 2006) also in the current analysis on Brassicaceae the "tolerance/escape" syndrome was not found and no association of theoretical regrowth capacity with any of the other clusters could be confirmed by contingency table analysis. A measure for plant growth was not included. This or the choice of plant species might have restrained the power of detection in the analysis.

Plant species composition in clusters 2 and 3 was very heterogeneous and seemed not to be influenced by phylogenetic history, i.e. tribe association. But in cluster 1 both *Cardamine* species and *Ar. rusticana* were grouped. According to Al-Shehbaz *et al.* (2006) these species belong to one tribe, the Cardamineae, which lack trichomes and mostly occur in wet habitats (Beilstein *et al.*, 2006). Therefore, the observed clustering behavior can be either due to phylogenetic constraints or due to adaptive evolution because trichomes can function as light and transpiration protection not needed in wet habitats (Roy *et al.*, 1999).

Alien status of plant species with regard to Germany was not associated with one particular cluster. Because one weed and two cultured plant species were used, different strategies might have led to a bias. Weedy plants are expected to employ novel weapons whereas plants bred for cultivation might not need those (Renwick, 2002).

In cluster analysis, young and old leaves of one species were always grouped close together contesting the hypothesized statistical independence of leaf age classes (Figure 5.1). However, observed differences between leaves of different age were substantial in all three clusters, especially in cluster 2 where age particularly reinforced the "low nutritional quality" syndrome. Because most variation was based on the nutritional characteristics and mechanical defence compared to chemical defence, this bias might have restrained the investigation on leaf age effects. Chemical defences can be remobilized from aging leaves (for glucosinolates see: Chen and Andréasson, 2001), but trichomes once built are not accessible for nutrient recovery or relocation to developing tissue.

5.4.2 Insect performance and preference

Several life-history traits for *A. rosae* were measured to evaluate which determine insect fitness most and how they correlate. Insect performance and preference parameters were inter-correlated on the univariate and multivariate levels. Host mediated insect fitness was largely influenced by mortality rates, fat accumulation, glucosinolate sequestration, and oviposition of naïve females (82 % variation, discriminant function 1; Table 5.10), whereas

adult weight was less important and more or less independent from these parameters (18 % variation, discriminant function 2). Additionally, developmental times and adult weights were negatively correlated for *A. rosae*. In contrast, larval developmental time was found to be positively correlated with pupal mass in other insects, e.g. in *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) when reared on different grape cultivars of unknown host quality (Moreau *et al.*, 2006).

As in the analysis of plant traits, three clusters were found for the development and preference of *A. rosae* (Figure 5.2, Table 5.11). The first one could be ranked the best with low mortality, short developmental times, high numbers of eggs, and high glucosinolate concentration, but low nitrogen content in males. The second was equal to the first in many aspects, but adults were lighter and had lower glucosinolate concentrations and nitrogen contents in males. The third cluster was largely determined by high mortality, long developmental times and low egg numbers. The overall adult weights were low and similar to those in the second cluster. Adult weight is frequently cited as a predictor of female fecundity and/or fertility, i.e. realized egg deposition (Awmack and Leather, 2002; Tammaru *et al.*, 2002), but these parameters can also be independent from adult weight (Moreau *et al.*, 2006). Concerning egg deposition of experienced *A. rosae* females adult body weight was not important for the total numbers of eggs laid within the bioassay period, nor for the numbers of accepted hosts. The total number of eggs laid was only influenced by the larval host plant species, whereas the number of accepted host plants increased concomitantly with total amount of eggs produced by the females as described previously for pipevine swallowtail butterfly females (Odendaal and Rausher, 1990). We did not determine female fat content, but the overall correlation of male and female performance parameters was remarkably high to assume similar results for both sexes. Experienced females laid the fewest mean egg numbers, accepted the lowest number of host plants and male larvae accumulated the lowest fat content on *C. pentaphyllos* (Appendix B). In contrast, females reared on *L. rediviva* with a similar body weight laid about three times more eggs on a medium number of hosts and males accumulated a twice as high percentage of fat. In pine sawflies about half of total body fat is allocated to the egg load (Herz and Heitland, 2002). Host plant effects on female fecundity and host selectivity could thus be mediated by modulation of acquisition of storage fat.

The choice of oviposition site determines the female's contribution to the next generation (Moreau *et al.*, 2006) and is for many insect species important because young larval instars are not very mobile (Scheirs and De Bruyn, 2002). We found an overall correlation of preference of naïve females with performance parameters which diverged only for one plant species. Larval development on *Br. rapa* ssp. *chinensis* was intermediate, but females readily accepted this plant for egg deposition. Due to the low numbers of eggs on

some plants we did not include egg mortality in the presentation of data, but the lowest egg mortality was recorded on *Br. rapa* ssp. *chinensis* leaves. A weak negative correlation between egg numbers laid by naïve females and egg mortality was found ($n = 12$; $r = -0.58$, $P < 0.05$). Thus adult preference did largely reflect larval performance, but egg performance seemed to be of some additional importance.

5.4.3 Matching of plant and insect clusters

Comparing plant and insect derived clustering, plant clusters matched with insect clusters for most of the 14 groups. Moreover, leaves of both age classes within a plant species were grouped closely in both analyses. As predicted for a food specialist, insect development was better in general on chemically (plant cluster 1) than on mechanically (plant cluster 3) defended plants. But cluster affiliations of two plant species were interchanged with regard to plant and insect clustering (*A. petiolata* and *C. pentaphyllos*). The nutritional needs of *A. rosae* and the mechanism by which the larvae inactivate myrosinase to sequester intact glucosinolates (Müller and Wittstock, 2005) are still unclear. But the poor performance on *C. pentaphyllos* which has the highest myrosinase activities could be due to increased costs for the larvae to inhibit myrosinases or to negative effects of developing breakdown products. Potential nutritional deficits might be compensated for by the larvae only in the absence of mechanical plant defence, because *A. petiolata* turned out to be a highly suitable host for the sawflies, but *Br. rapa* ssp. *chinensis* plants, that are rich in trichomes, provided less valuable food.

Variability was largely determined by one discriminant function in both plant and insect clustering. Plants predominantly varied in nutritional quality and mechanical defence, while insect fitness was dominated by mortality rates, fat accumulation and glucosinolate sequestration of males, and oviposition of naïve females. In regression analyses, mortality rates of larvae and pupae were mostly increased by the predominant plant characteristics. However, all slopes depending on plant traits were strongly correlated except for C:N ratio, so that it remains unclear whether abaxial trichomes were effectively causing a reduction in mortality probability or whether the ultimate reason was some correlated plant trait.

Increased mortality rates were clustered together with longer developmental times (insect cluster 3) and the latter similarly regressed on nutritional quality and mechanical defence properties of plant tissues (Table 5.13). The availability of nitrogen for growth can be more important for early instars because of higher metabolic activities and growth rates (Raubenheimer and Simpson, 1999; Zalucki *et al.*, 2002). Severe deficits in protein could thus be lethal for early instars, but in later instars prolonged growth can compensate for these effects (Raubenheimer and Simpson, 1999). As a by-product of prolonged feeding,

carbohydrates and also secondary defensive metabolites are over-ingested (Slansky and Wheeler, 1992). As a consequence for a sequestering specialist, fat and glucosinolates accumulate in body tissues. In addition, fat and glucosinolate storage were also dependent on adult weight (insect cluster 1). Hence, the amount of sequestered glucosinolates was mostly determined by C:N ratio, as was found for adult weight, but also by mechanical defence (Table 5.13). Fat accumulation was largely decreased by myrosinase activity, but also marginally increased by mechanical defence.

Next to the above described array of plant defensive traits, Brassicaceae can also contain other defences (Hegnauer, 1964; Renwick *et al.*, 2001; Cipollini and Gruner, 2007). For example, alkaloids and phenylpropanoids were reported for some species of Brassicaceae, including *B. orientalis* and *L. rediviva* (Dietz and Winterhalter, 1996; Brock *et al.*, 2006). However, the explained variability expressed as the R^2 values in regression analyses (Table 5.13) was very high for all insect parameters except for egg numbers of naïve females. It is therefore very likely that the defensive plant traits chosen for this study were highly important for the performance of *A. rosae*. Agrawal and Fishbein (2006) did not find differences in caterpillar growth of *D. plexippus* examined for five day periods on different Asclepiadaceae. It is probably highly important to follow the complete larval development as well as adult fitness parameters to be able to group larval parameters and match these with plant defence syndromes.

5.4.4 Conclusions

The concept of plant defence syndromes grouping plant traits within a triangle formed a reasonably good framework for this study. Edibility and feeding barrier characteristics of Brassicaceae could be confirmed as important trait associations for cluster formation. We suggest a revised association of water content and proteinase inhibitors within the triangle. Within the selected plant species two syndromes in three independent clusters could be confirmed with a bias for nutritional and mechanical defence strategies. Plant defences of Brassicaceae were more variable due to species than to leaf-age classes. This can indicate differences between macro- and microevolutionary driving forces. Whether this pattern is consistent in other species and whether it is adaptive to factors other than herbivory or due to genetic or physiological constraints of leaves of different age needs to be explored further.

The performance and preference parameters recorded for the oligophagous sawfly *A. rosae* were also clustered in three groups which highly overlapped with those of plants. Larval performance and adult preference were more influenced by general mechanical defence than by chemical defence as predicted for a specialist herbivore that uses plant chemical defences for its own protection. Nutritional deficits of plant quality seemed to be compensated by

the sawfly in the absence of mechanical defence. Thus this food specialist is very likely a nutrition generalist. In summary, defence syndromes of Brassicaceae matched with performance and preference of the specialist herbivore *A. rosae*, but displayed constraints which are likely caused by limited capacities for compensation of nutritional deficits and chemical "detoxification" by the insect.

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Chapter 6

Discussion

Two general fields of interest were chosen to illuminate the role of the glucosinolate-myrosinase system for interactions of Brassicaceae with the turnip sawfly: variation within individual plants and between plant species. Several bioassays with the turnip sawfly accompanied the investigations for plant variation, so that their importance for the interaction could be assessed. An enzyme assay for soluble and insoluble isoforms was established (Chapter 2) to reliably investigate the variation of myrosinase activity within plants and their importance for the turnip sawfly. The short-term induction responses presented in Chapters 3 and 4 focussed on variation within plant individuals primarily of the glucosinolate-myrosinase system in white mustard. A long-term approach evaluating the importance of variation between plant species was concerned with interactions of several defensive plant traits (Chapter 5).

6.1 Variation within plant individuals and short-term effects

Which responses are triggered by feeding of sequestering *A. rosae* larvae in tissues and organs around the damage site of two different cultivars of *S. alba* and how long do they last?

The immediate, physiological responses of plants to changing environmental conditions are described as induction (Karban and Baldwin, 1997; Agrawal, 1999a). Due to metabolic and environmental costs, the rapid production of defensive compounds is thought to be advantageous for plants in contrast to continuously high defence levels (Agrawal, 2000; Cipollini *et al.*, 2003b). This way allocation from growth to defence only takes place in

larger amounts when and where needed. This strategy of saving costs is widespread among plants, including many Brassicaceae species (for examples see Chapters 3 and 4). However, a detailed study using a glucosinolate-sequestering insect is presented here for the first time. Moreover, the induction processes of both parts of the glucosinolate-myrosinase system were determined. The short-term physiological changes in *S. alba* evoked by 24 hours of feeding by larvae of *A. rosae* included increases in glucosinolate AND myrosinase activity levels, and furthermore in glucose and protein levels (Chapters 3 and 4). We focussed on short-term responses within 24 hours of the surrounding tissues which are the most likely to be attacked next. In *S. alba* we found very strong responses in the surrounding leaves and stems. These changes are likely to be important for resistance against an immediate attack (see below). The defensive properties of *S. alba* leaves differed between the two cultivars, Salvo and Silenda. Non-induced plants of the latter cultivar had higher myrosinase activities, whereas those of the cultivar Salvo had higher glucosinolate concentrations in leaf tissues. The composition of glucosinolates in leaves of *S. alba* was very similar in, but the ratio of soluble and insoluble fractions of myrosinase activities differed between control plants of the two cultivars. Sinalbin (*p*-hydroxybenyzlglucosinolate) was the dominating single compound followed by benzylglucosinolate. Soluble myrosinases were about twice as active as insoluble myrosinases in the cultivar Salvo. In Silenda control plants the opposite was determined. Induction responses to *A. rosae* feeding were in general stronger in the cultivar Silenda compared to Salvo. However, upon induction single compounds were differently increased in the two cultivars, e.g. indol-3-ylmethylglucosinolate stronger in Salvo and sinalbin stronger in Silenda. Insoluble increased stronger than soluble myrosinase activity without cultivar differences.

In former studies, a sap-sucking sequesterer [*Brevicoryne brassicae* (L.)] induced increases in aliphatic glucosinolate concentrations in *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia, but myrosinase activities were not measured (Mewis *et al.*, 2005, 2006). In *Brassica napus* the same herbivore increased the expression of myrosinase binding and associated proteins, but reduced myrosinase gene expression (Pontoppidan *et al.*, 2003). Induction responses in *S. alba* were until now only triggered by plant hormones (Bodnaryk, 1994; Bennett *et al.*, 1997) or mechanical wounding (Koritsas *et al.*, 1991; Bodnaryk, 1992). Here, time gaps varied from 24 hours to 10 days between treatment and harvest (Bodnaryk, 1992; Koritsas *et al.*, 1991). However, plants need immediate defences and glucosinolates and myrosinases are produced only a few hours after onset of the attack (Du *et al.*, 1995; Pontoppidan *et al.*, 2005). When single leaves were treated, systemic reactions were monitored only in younger tissue mostly at distances of at least two leaves to the damage site (Bartlet *et al.*, 1999; Agrawal, 1999b; Traw and Dawson, 2002; Traw and Bergelson, 2003; Shelton, 2005; Widarto *et al.*, 2006). Direct neighbouring tissues were monitored rarely (Koritsas *et al.*, 1991; Pontoppidan *et al.*, 2005). Cultivar differences were found for

Chinese cabbage in response to a pathogen (Ludwig-Müller *et al.*, 1997) and for oilseed rape in response to insect herbivory (Hopkins *et al.*, 1998b), after mechanical wounding of leaves (Koritsas *et al.*, 1991), or after pathogen infection (Andréasson *et al.*, 2001b) in long-term induction studies. Soluble myrosinase activity and expression of myrosinase binding proteins (MBPs) were only determined in one of these studies (Andréasson *et al.*, 2001b).

Defence levels of *S. alba* were furthermore influenced by tissue age and organ (Chapters 3 and 4). Higher constitutive concentrations of both components of the defence system were found in young *versus* old and in leaf *versus* stem tissues. Increases in the defence system after feeding of *A. rosae* were also more vigorous in the constitutively highly defended tissues. Both components of the glucosinolate-myrosinase system increased in stems and damaged leaves of the cultivar Salvo, whereas in systemic leaves only myrosinase activity was induced. In all leaves of the cultivar Silenda both components were increased after sawfly herbivory. Glucose and protein levels were less influenced by herbivory, but stronger by age of tissue and organ. Again higher levels were found in young *versus* old and leaf *versus* stem tissue. From the plant's perspective, value and vulnerability of a tissue should be significant predictors for its defensive properties as described in the "optimal defence hypothesis" (McKey, 1974, 1979). We hypothesized that stems are of higher structural value for a plant compared to leaves, because of their vascular transport function. Therefore, stems should display high constitutive defences and low induction. However, these tissues had much lower glucose and protein levels and higher water contents. Furthermore, stems are densely covered with trichomes. Because they are hard to eat (trichomes) and confer little reward to the herbivore (low nutrition), they are less likely to be attacked by herbivores. Hence, the low constitutive defensive properties of stems might reflect the impact of low vulnerability. In contrast, a leaf's defence should be determined by its value which is shaped by tissue age and its appearance in a plant's ontogeny (Lambdon and Hassall, 2005). In accordance younger leaves of *S. alba* were stronger defended both constitutively and by induction than older ones. Similar relationships of tissue age and defence status were also found in several other species (Clossais-Besnard and Larher, 1991; Porter *et al.*, 1991; Brown *et al.*, 2003; Lambdon *et al.*, 2003). The value of a leaf correlates with its actual and future photosynthetic activity and is thus high in young leaves (Lambdon and Hassall, 2005). In contrast to the comparison of stem and leaf tissue in *S. alba*, defence properties in *Raphanus sativus* L. could be well predicted by value differences of vegetative and generative tissues, i.e. leaves and petals (Strauss *et al.*, 2004). The trade-off between constitutive and induction defence proposed in the "optimal defence hypothesis" was found in *R. sativus* comparing vegetative and generative tissues (Strauss *et al.*, 2004), but not in generative tissues of *S. alba* (Chapters 3 and 4). Thus, organ comparisons and age effects are likely shaped to different extents by

value and vulnerability of a tissue. The connections between, and the scales for these two properties need to be explored further. Considering only one of them in the framework of the "optimal defence hypothesis" is likely to lead to misinterpretations of induction results.

The vascular architecture can be an important mechanistic factor which shapes systemic responses in individual plants (Orians, 2005). The connectivity among leaves can restrain the distribution of an elicited signal or even glucosinolates within a plant. The dye Rhodamine B was applied onto damaged leaves and allowed to spread for 24 hours within white mustard plants (Chapter 4). The highest proportion of recovered dye accumulated in the younger leaves, shortly followed by the damaged ones. In older leaves only about half the amount compared to the other two tissues was found. In stem tissue only very low amounts of dye were found. The induction reactions of glucosinolate and myrosinase activity levels within leaves of the cultivar Silenda roughly corresponded to the distribution pattern of the dye. The stronger induction effects in local leaves compared to higher-dye-accumulating systemic younger leaves however might reflect a reaction delay due to transportation of a putative signal or of newly synthesised glucosinolates from the damage site. The dye-relocation process was not correlated to organ biomass and is thought to be due to source-sink relationships modulating bulk phloem flows within a plant individual (Orians, 2005). In younger systemic leaves of Solanaceae, dye accumulation positively correlated with proteinase inhibitor induction after mechanical wounding (Orians *et al.*, 2000), and with herbivore performance reduction after conspecific damage (Viswanathan and Thaler, 2004). In contrast to our activity measurements in *S. alba*, wound-induced expression of myrosinase binding and associated proteins were stronger in older than in younger systemic leaves of *B. napus* (Taipalensuu *et al.*, 1997a,b). In almost all tissues of investigated Brassicaceae species glucosinolate biosynthesis was detected, but with differences in activity levels between and among generative and vegetative tissues (Du and Halkier, 1998; Grubb *et al.*, 2004). The connection between dye accumulation and induction properties in tissues of Brassicaceae could thus be mediated by elicitor-inducible biosynthetic activity (Mikkelsen *et al.*, 2003; Bednarek *et al.*, 2005) or import of glucosinolates into the tissue from other organs (Chen and Halkier, 2000; Chen and Andréasson, 2001).

All of the reactions to 24 hours of feeding by *A. rosae* described above for *S. alba* cv. Salvo were short-lived and lasted at most for two days. Similarly, expression levels of myrosinase binding proteins in *B. napus* decreased almost immediately after 24 hours of damage by *Plutella xylostella* (Pontoppidan *et al.*, 2005). However, shorter damage periods led to longer-lasting induction responses (Bodnaryk, 1992, 1994; Pontoppidan *et al.*, 2003, 2005). The decrease in levels of glucosinolates and myrosinase activity immediately after

the damage ceased is probably favourable for two reasons. Since production of defensive metabolites is connected to allocation costs from growth (Lewis *et al.*, 2006), high levels of defence are only produced where and when needed. A time-limited increase would therefore allow for subsequent normal growth which might compensate for damage inflicted by herbivory. In the *S. alba* cv. Salvo with low induced increases in glucosinolates, organ biomass growth rates over the five-day experiment period were not affected in damaged and older leaves, but even increased in younger leaves (Appendix A, Table A.1). In the *S. alba* cv. Silenda with higher increases of induced glucosinolates, biomasses of almost all investigated organs were reduced after 24 hours of *A. rosae* feeding compared to bag treated control plants (Figure A.2). These growth differences could be the result of short-term costs of induction responses. Additionally, very high defence levels are posing also an ecological complicacy, because specialist herbivores use exactly these metabolites as host recognition cues. These and other ecological costs, e.g. deterrence of pollinators, can be highly important for plant-insect interactions in their natural environments where also abiotic stresses need to be integrated to yield a complete picture (Strauss *et al.*, 2004; Reifenrath and Müller, 2007).

How are induction responses in *S. alba* influenced by insect specialism, insect instar, and damage mode?

Specialist insects pose a problem for chemically defended plants. The metabolites that effectively deter or poison generalists are rendered harmless by specialists and are frequently exploited as host finding and oviposition cues (Renwick, 2002). Furthermore, specialists that sequester these metabolites are also protected against higher trophic levels (Price *et al.*, 1980). According to this "lethal plant defence paradox" increases in these metabolites, e.g. upon induction, should proportionally increase the benefits of these insects. As a solution to this plant dilemma, it was proposed that only younger, susceptible instars should encounter vigorous increases, but not older, resistant instars (Malcolm and Zalucki, 1996). In *S. alba* cv. Salvo these suggestions were not confirmed. Glucosinolates increased in damaged leaves and were not influenced by insect instar. The higher glucosinolate levels could lead to higher levels of sequestered glucosinolates within the larvae and would thus reinforce predator protection. However, it is not yet clear which costs are connected to sequestration by transport into the haemolymph. Furthermore, there is a turn-over of glucosinolates within the body which is possibly costly and might be enhanced by increased glucosinolate levels in the diet (Müller and Wittstock, 2005). In contrast to a sap-sucking sequesterer (*B. brassicae*), the leaf-chewing sawfly larvae are confronted with glucosinolates AND myrosinases. The selective increase of only myrosinases in systemic leaves of cultivar Salvo plants could represent one mechanism of dealing with the "lethal plant defence paradox". The inactivation of myrosinases might also be costly to the insect.

Very high levels of hydrolytic activity of plant tissues influenced performance and especially fat accumulation of the turnip sawfly in a long-term study (Chapter 5). However, the capacities for detoxification and their costs still remain unclear.

In evolutionary terms it might be adaptive for a plant to respond differently to attack with regard to the organisms' degree of specialization. Hence, specialist feeding should trigger plant responses which are distinct from those evoked by generalist feeding and mechanical damage. In *Nicotiana attenuata* Torr. ex Wats. (Solanaceae), specialist feeding induced a nutrient recovery response directed to the root for storage (Schwachtje *et al.*, 2006). It is argued that after herbivory this store can be used effectively in regrowth of damaged or lost organs. In *S. alba* cv. Silenda only specialist *A. rosae* feeding and mechanical wounding increased both components of the glucosinolate-myrosinase system (Chapter 4). Feeding by *Spodoptera frugiperda*, a generalist, evoked responses only in glucosinolate levels. Local and systemic responses differed only in intensity, but the patterns due to damage mode were the same. The plant's response to *A. rosae* feeding and mechanical wounding were very much alike, but glucosinolate composition varied between these damage modes. Furthermore, organ biomasses after 24 hours of treatment were reduced by herbivory of both insect species, but not by mechanical wounding (Appendix A, Table A.1). Significant differences in organ biomasses between plants treated with *A. rosae* feeding or only bags were found in damaged (corrected for lost tissue) and younger leaves. Herbivore treated plants had significant lower leaf biomasses than those treated with mechanical wounding irrespective of herbivore species. In view of an evolutionary arms race between herbivores and plants, the patterns of the defence system seem to benefit the opponent rather than the plant. The sequestering specialist could profit from increased glucosinolate levels and hence increased protection due to sequestration. The generalist might obtain food with lower defence levels by suppressing plant defence responses that otherwise occur upon mechanical damage. The lower defence levels might be more tolerable due to only moderately effective detoxification mechanisms (Blau *et al.*, 1978). However, the reduced organ weights which occurred only after herbivory could be a tolerance response on the plant's side. As in *N. attenuata* (Schwachtje *et al.*, 2006), also in *S. alba* nutrient relocation mechanisms could be used to postpone growth in attacked plant parts to a time point when the herbivore has left.

Why is the elicited response of the plant different between young and old instars, generalists and specialists?

Induction responses in general need an elicitor triggering changes in gene expression and plant physiology (Walling, 2000). Next to decomposing plant tissue, insect saliva is one potential source for these molecules. Hormones of plant origin are for example jasmonic acid, salicylic acid, hydrogen peroxide, or ethylene, which can induce increases in the

glucosinolate-myrosinase system, e.g. (Bodnaryk, 1994). Additionally, some elicitors were already found in herbivore saliva. Fatty acid amides, glucose oxidase, and pH value modulate plant responses (Musser *et al.*, 2002; Spiteller and Boland, 2003; von Dahl *et al.*, 2006). However, they can also suppress induction events. Glucose oxidase, which was first detected in the caterpillar saliva of the generalist *Helicoverpa zea* (BODDIE) (Lepidoptera: Noctuidae) (Eichenseer *et al.*, 1999; Musser *et al.*, 2002), was shown to inhibit induction responses otherwise occurring upon mechanical damage (Musser *et al.*, 2002, 2005; Bede *et al.*, 2006). Among some other lepidopterans, two sister species of *S. frugiperda* were investigated to date for the presence of glucose oxidase. Whereas for the saliva of *S. exigua* (HÜBNER) caterpillars enzyme activity was reported (Merkx-Jacques and Bede, 2004; Bede *et al.*, 2006), *S. litura* (FABRICIUS) caterpillars apparently have no glucose oxidase activity in their saliva (Zong and Wang, 2004). In head or body tissue extracts of *S. frugiperda* or *A. rosae*, no glucose oxidase activity could be detected (data not presented). The pH value and buffer capacity of solutions applied onto wounded tissues were shown to modulate methanol release in *N. attenuata* by which plant proteinase inhibitor concentrations were reduced (von Dahl *et al.*, 2006). When larvae of *S. frugiperda* and *A. rosae* were induced to feed on pH-indicator paper moistened with a glucose solution, the saliva residues of both species were strongly alkaline (pH $\sim$ 9). 24 hours after feeding the coloration of *S. frugiperda* fed indicator paper was however stronger compared to *A. rosae* fed ones (Figure 6.1). Buffer capacity or amount of secreted saliva could thus be species-specific. So far, no studies have been conducted with any of these herbivore-derived single compounds as elicitors and plants containing the glucosinolate-myrosinase system. In other systems, fatty acid amides of *S. frugiperda* can trigger the release of volatile organic compounds (VOCs) from the plant (Alborn *et al.*, 1997). In general, VOCs are involved in indirect defence of plants recruiting predators and parasitoids of herbivores (Walling, 2000). However, mimicking the temporal damage pattern of *Spodoptera littoralis* (BOISDUVAL) with mechanical wounding alone can elicit the same release of VOCs in lima beans (Mithöfer *et al.*, 2005). Thus, not only the mode of mechanical damage (scissors/cork borer *versus* forceps, Pontoppidan *et al.*, 2005), but also the timing of these inflictions are crucial to simulate herbivore feeding. Mechanical damage was applied with a cork borer at three times over the 24 hour-period to mimic the broad-scale pattern of damage (Chapter 4). However, herbivores remove plant tissue in smaller bits compared to the larger bulks cut by cork borer wounding.

A dose-dependency is known for the induction of indolic and aliphatic glucosinolates after mechanical wounding and after exogenous application of jasmonic acid (Bodnaryk, 1992, 1994). Indolic glucosinolates increased steadily to a continuous maximum, whereas aliphatic glucosinolates were induced up to a certain amount of damage and further increases led to reduced induction responses (Koritsas *et al.*, 1991). The length of a wound is

a measure for dose of damage. *S. frugiperda* caterpillars inflicted longer wound perimeters compared to *A. rosae* larvae. Both herbivores removed the same amounts of tissue and therefore generalist feeding led to a damage pattern with a large perimeter-to-area ratio. If a dose-dependency is responsible for the different myrosinase induction responses of generalist and specialist, a relationship with a maximum of induction at medium damage levels similar to the responses for aliphatic glucosinolates should be found in further studies. The feeding patterns of three small larvae compared to one large larva of *A. rosae* were not recorded by photographs. The estimated amount of removed tissue was similar, but it is very likely that the wounding pattern differed. Three small larvae should produce more and smaller damage sites with a large perimeter-to-area ratio at one time. Furthermore, saliva composition and thus elicitor concentrations could be different in young *versus* old instars as was shown for larval stages of *Helicoverpa zea* (Eichenseer *et al.*, 1999). Instar differences were shown to modulate photosynthesis and transpiration rates (Tang *et al.*, 2006), as well as glucosinolate accumulations (Widarto *et al.*, 2006) in Brassicaceae. At present, no conclusive solution can be given whether different elicitors or damage patterns caused the observed induction responses in white mustard. Further research on putative elicitors, damage patterns, and their working mechanisms on the plants' side is needed. The identification of herbivore-derived elicitors and their involvement in plant signalling cascades will be most important. In Chinese cabbage, *B. napus*, and *S. alba* jasmonic acid (JA) and salicylic acid (SA) induced classes of glucosinolates differently (Bennett *et al.*, 1997; Ludwig-Müller *et al.*, 1997). Furthermore, myrosinase enzymes and their binding proteins were expressed after wounding and after the application of several plant hormones (Taipalensuu *et al.*, 1997a,b). However, SA had no impact on the expression of either myrosinases or their binding proteins. Investigations of the differences and analogies to plant-pathogen interactions will very likely promote the mechanistic understanding of herbivore-evoked induction responses in plants.

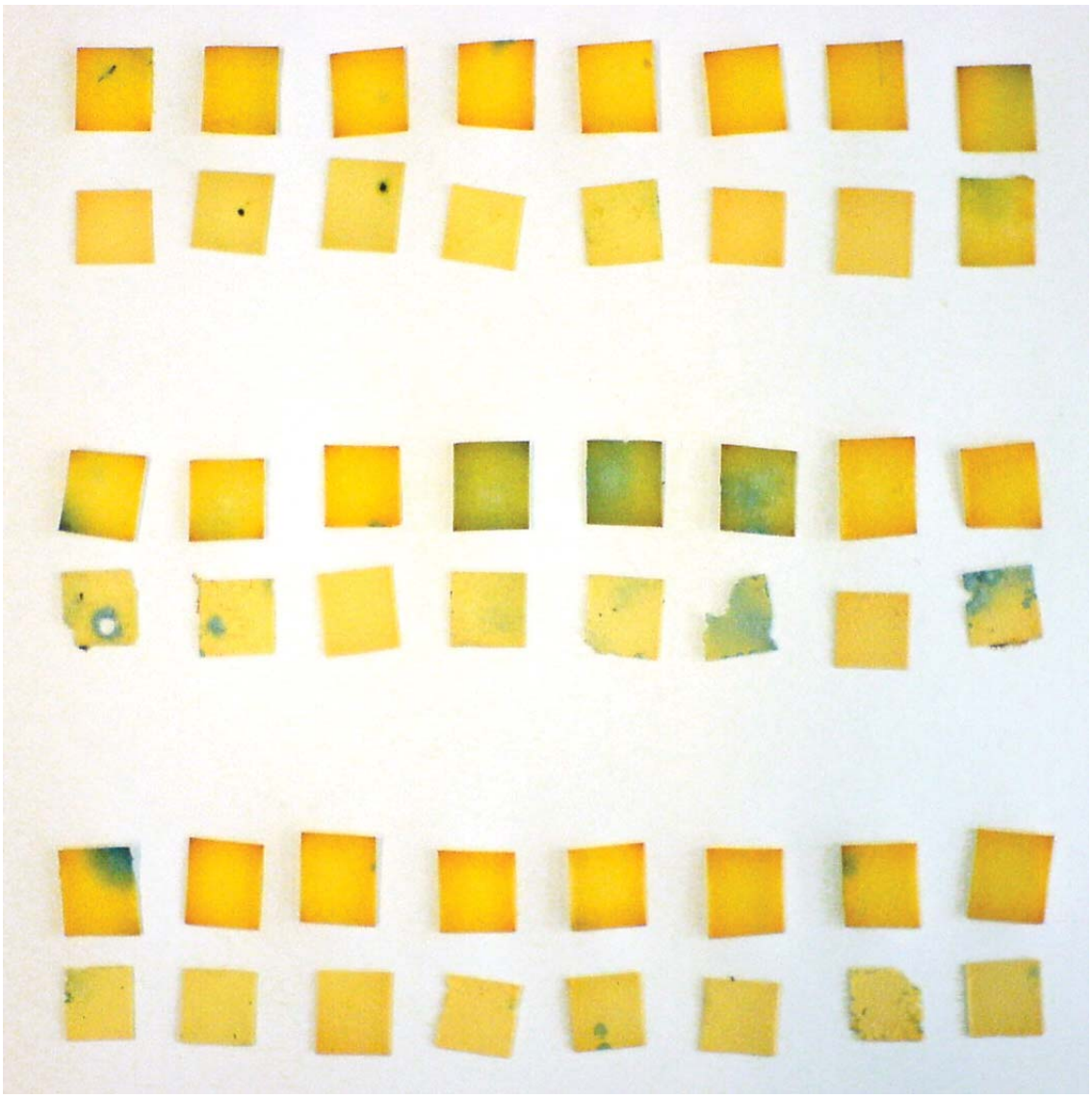

Figure 6.1: pH values of saliva residues of *Athalia rosae* (rows 1 and 2, previously fed *S. alba*) and *Spodoptera frugiperda* (rows 3 and 4, previously fed on artificial diet, rows 5 and 6, previously fed *S. alba*) as indicated by pH indicator paper moistened with glucose solution. Rows 1, 3, and 5 present pH paper fed upon for 1 hour. Rows 2, 4, and 6 present pH paper fed upon for 24 hours. The same individual insects were used in both tests and are aligned vertically.

What are the consequences of plant induction responses on the short-term behaviour of larvae and adults of *A. rosae*?

The defensive properties of induced plants were tested in feeding and oviposition biotests which were designed to represent subsequent attacks of larvae or of female adults occurring after the initial damage (Chapter 4). The feeding pattern of larvae was not influenced by the different plant induction states of either specialist or generalist feeding or mechanical damage. Female adults of *A. rosae* oviposited almost equally on induced and younger compared to older leaves. There was only a modest modification on mechanically damaged plants where more eggs were laid on older leaves. Larvae and adults were reported to respond to glucosinolates and isothiocyanates, but the intensity of the answers in electroantennogram trials were modulated by the side-chain structure of the metabolites (Barker *et al.*, 2006). Thus, the quantitatively different glucosinolate profiles of mechanically damaged plants with increased benzylglucosinolate levels could have shaped the behaviour of *A. rosae* females. In other systems the biological activity was different due to glucosinolate modifications, e.g. the resistance towards disease and specialist feeding (Giamoustaris and Mithen, 1995; Brader *et al.*, 2006; Burow *et al.*, 2006). Overall, the induction responses of *S. alba* in a controlled environment were not effective to produce protection against *A. rosae* larvae or adults within a period of 24 hours. However, damage was very evenly distributed within plant individuals. In *R. sativus* damage to four leaves did not reduce plant fitness compared to the same amount of damage concentrated on two leaves or on only one leaf (Mauricio and Bowers, 1990). In computational models these relationships were also found. Herbivore feeding patterns and host plant predictability were key determinants in the effectiveness of plant defences (Shelton, 2005; Tiffin *et al.*, 2006). Thus, increasing variability in general, e.g. by induction responses, decreases herbivore performance. In natural environments with a variety of herbivores and pathogens these reactions could well be protective, e.g. against generalist herbivores.

6.2 Variation between plant species and long-term effects

How is the glucosinolate-myrosinase system correlated to other plant defences and nutrition?

Although the glucosinolate-myrosinase system is characteristic for Brassicaceae, it is not the only defence mechanism employed by these plants (Hegnauer, 1964). The role of the glucosinolate-myrosinase system for the performance and preference of the sequestering specialist *A. rosae* was therefore determined in association with nutritional characteristics and mechanical defences (Chapter 5). The prediction of the univariate plant defence model

would be that strong negative correlations between defences exist (Agrawal, 2007). However, no such correlations were found in seven species of Brassicaceae. In contrast, strong positive correlations were found between trichome densities and proteinase inhibitors. Furthermore, nutritional characteristics were strongly correlated, e.g. specific leaf area, water, nitrogen, and protein content. In a multivariate analysis, three clusters were found which represented two plant defence syndromes. The clusters overlapped with those described by Agrawal and Fishbein (2006) for *Asclepias* species. Low nutritional quality was accompanied in both Asclepiadaceae and Brassicaceae by low defence levels. Higher nutritional quality of plant tissues coincided either with high mechanical or high chemical defences. The single plant traits of Brassicaceae were subsequently correlated multivariately by discriminant function analysis. Nutritional quality positively correlated with proteinase inhibitors and trichome densities, while chemical defences were more or less expressed independently. In contrast to the association of proteinase inhibitors with the "nutrition and defence" syndrome in Brassicaceae, high proteinase inhibitors were placed in the low nutritional quality syndrome within the plant defence syndrome triangle. From the plant's perspective, these correlations between proteinase inhibitors and nutritional properties might be adaptive because nutritional host quality is tightly linked to attraction for herbivores (Broadway and Colvin, 1992). Moreover, proteinase inhibitors reduce nitrogen acquisition of herbivores by interfering with their digestive system (Fan and Wu, 2005) and the ingestion of trichomes can cause gut lesions which require nitrogen-demanding repair (Raubenheimer and Simpson, 1999). These two plant defences are probably working synergistically. A second plant trait was found in a different association than that proposed in the hypothesis. In this subset of Brassicaceae, low water content was correlated to high nitrogen content and *vice versa*. Low water content is normally considered to create "hard to eat" tissues and was therefore associated with the low nutritional quality syndrome (Scriber and Slansky, 1981; Agrawal and Fishbein, 2006). In contrast, we argued that high water contents ($\sim$80 to 90 % of f.wt.) can dilute nutritional components of the plant tissue, e.g. nitrogen or proteins. Dilutions of artificial diets with water experimentally triggered compensatory feeding responses in caterpillars of *Anticarsia gemmatalis* HÜBNER (Lepidoptera: Noctuidae) (Slansky and Wheeler, 1992). Thus, a biphasic relationship of water content and insect resistance is suggested with an optimum below which mechanical feeding barriers are more important (Coley *et al.*, 2006) and above which the dilution of nutrients determines insect performance.

The expressions of plant traits were compared in this study across seven species and between two leaf ages, i.e. young, fully expanded and old, aging leaves. Although leaf age differences were considerable, both young and old leaves were always attributed to the same cluster. Thus, within a plant species the same defence strategy is employed in young and old leaves. In contrast to mobile nutritional and chemical molecules (Chen and

Andréasson, 2001), investment in trichomes is static and cannot be redistributed within the plant. Because most variability was found due to nutritional and mechanical properties, this might have restrained the power of detection of leaf age differences within the dataset. Furthermore, two of the three chemically defended plants are growth restricted (Tutin *et al.*, 1993). The two *Cardamine* species produce only three to five leaves per season. Age effects were less pronounced in these species. In contrast, differences between young and old leaves were highest in the low nutritional syndrome. Due to these objections, it is likely that in other species of Brassicaceae defence strategies, especially from "nutrition and (chemical) defence" to "low nutritional quality", might change with aging of leaves (Lambdon *et al.*, 2003).

Next to life-forms of plants, species phylogenetic history can also restrain the variability of syndromes (Rudgers *et al.*, 2004; Agrawal and Fishbein, 2006). Common ancestry can constrain the possible defence strategies a species can develop. In two clusters the species composition with respect to tribe association was very heterogeneous. Here phylogenetic constraints are unlikely. However, in the chemically defended plant cluster all three species belong to one tribe. One possible cause are phylogenetic constraints. Another explanation is that factors other than herbivory are responsible for this clustering. Species of the tribe Cardamineae typically occur in wet habitats and lack trichomes (Beilstein *et al.*, 2006). Trichomes are not only barriers against feeding but also function as light and transpiration protection which are not vital in wet habitats (Roy *et al.*, 1999).

How are long-term developmental parameters of *A. rosae* correlated?

In general, performance of larvae and preference of adults should be correlated to ensure the success of a species, which was termed "the mother-knows-best-principle" (Mayhew, 1997; Agosta, 2006). However, the complete picture is more complex. Several life-history parameters were measured for the turnip sawfly (Chapter 5). We were interested in the inter-correlations of these parameters and whether some parameters were more important than others for insect fitness. On the univariate level, adult weight and total developmental time were negatively correlated. Furthermore, developmental time correlated positively with total mortality. Male body composition was largely influenced by water content. As in plants, three clusters were aggregated in multivariate analysis. In one cluster, insects developed fast, were heavy as adults and mortality was low. The second cluster was similar to the first, but grouped insects with lower adult weights which had accumulated lower amounts of glucosinolates and nitrogen. The third cluster was characterized by very high mortality rates. Egg numbers of naïve females reflected these performance parameters and were highest in the first cluster, intermediate in the second and very low in the third. In subsequent discriminant function analysis, mortality rates were positively associated with male fat and glucosinolate content, but negatively with preference of naïve

females. Female adult weight was more or less independent from these parameters, but had low positive associations with male fat and glucosinolate content. For many species adult weight of females was often connected to (Awmack and Leather, 2002), but can also be independent of fecundity or fertility (Moreau *et al.*, 2006). For the turnip sawfly a connection between the number of eggs laid and host selectivity was confirmed. As observed for the pipevine swallowtail butterfly (Odendaal and Rausher, 1990), the more eggs a female produced the more host species were accepted also by the turnip sawfly. The egg load of a single *A. rosae* female could be modulated by the acquired fat content during the larval stage. In adult pine sawflies about half of the body fat is allocated to the eggs (Herz and Heitland, 2002). This connection should be pursued further because female fat content was estimated from male fat content, since other developmental parameters of male and females were very similar in this study. The preference of females, which were raised as larvae on *S. alba* and thus un-experienced in the seven test species, largely overlapped with the measured performance results with one exception. Larval performance was poor on Chinese cabbage, but this plant was readily accepted by naïve females for oviposition. On this plant species, egg mortality was exceptionally low compared to the other plants. Therefore, naïve females' choice correlated largely with the independently measured performance parameters, but egg performance also played an important role for this plant-insect interaction.

Which are the key components of Brassicaceae that influence the performance and preference of *A. rosae*?

The turnip sawfly is specialized to feed on glucosinolate-containing plants. However, it is not adapted to mechanical defences like trichomes. Therefore, changes in mechanical defences of the hosts should be more severe than changes in chemical defences. In comparison to generalist insects, specialists are more often restricted to a given host because a second suitable host is not easily found. Therefore, specialists need to display plasticity in development not only towards changing chemical defences, but also towards nutritional variations. Food specialists are forced to be nutrient generalists (Raubenheimer and Simpson, 1999). The multivariate analyses described in the sections above aggregated plant traits and insect parameters independently each in three clusters. The clusters largely overlapped with regard to host plant species. Insect development was as predicted in general better on chemically (plant cluster 1) than on mechanically defended plants (plant cluster 3). But for two species cluster affiliations were exchanged: *Alliaria petiolata* and *Cardamine pentaphyllos*. This exchange could be due to constraints in the insect's physiological capacities. *A. petiolata* displayed the "low nutritional quality" syndrome, but insect performance was very good on this plant. However, performance was poor on Chinese cabbage also displaying this syndrome, but with additional low levels of mechanical

defence. The insects should be nutrition generalists, but nutritional deficits might only be compensated for in the absence of mechanical defences. In contrast, *C. pentaphyllos* was chemically defended, but had very high myrosinase activity levels. The poor performance could thus be due to yet unknown costs for the larvae to inhibit myrosinases or due to negative effects of developing breakdown products (Müller and Wittstock, 2005). The variability of plant traits and of insect parameters was largely determined by one function in both discriminant function analyses. For plants, nutritional quality and mechanical defence were most important, while insect fitness was dominated by mortality rates, fat accumulation and glucosinolate sequestration of males, and oviposition of naïve females. Increased mortality rates were clustered together with longer developmental times (insect cluster 3) and both insect parameters were mostly increased by the predominant plant characteristics nutritional quality and mechanical defence. Severe deficits in protein could be lethal for early instars because of higher metabolic activities and growth rates (Raubenheimer and Simpson, 1999; Zalucki *et al.*, 2002). Later instars can compensate for these effects by prolonged growth which leads as a by-product to an over-ingestion of carbohydrates and also secondary defensive metabolites (Slansky and Wheeler, 1992). The turnip sawfly is a sequestering specialist and thus accumulates not only fat, but also glucosinolates in its body tissues. Within the same developmental time frame, fat and glucosinolate storage were also connected to adult weight (insect clusters 1 and 2). These correlations of insect parameters were also reflected in regression coefficients. The amount of sequestered glucosinolates was mostly determined by C:N ratio, as was found for adult weight, but also by mechanical defence. Fat accumulation largely decreased with increases of myrosinase activity, but also marginally increased with increases of mechanical defence. The explained variability of almost all insect parameters was very high, so that it is very likely that the chosen plant traits were accordingly highly important for the specialist *A. rosae*. In contrast, Agrawal and Fishbein (2006) did not find differences between clusters in caterpillar growth of *Danaus plexippus* L. (Lepidoptera: Danaidae) examined for periods of five days on different species of Asclepiadaceae. Furthermore, the multivariate linear models of their study only predicted less than a third of the overall variability for growth and survival. It might be important to follow the complete larval development as well as adult fitness parameters to be able to group larval parameters and match these with plant defence syndromes (Scheirs *et al.*, 2003).

6.3 Comparison between short-term and long-term effects

Although aging of tissues, herbivory and mechanical induction had considerable effects on plant chemistry and morphology, variations between species and genera were much

stronger. On both variation levels similar associations between the two components of the glucosinolate-myrosinase system and of defence and nutrition were found. Glucosinolates and myrosinases were weakly correlated within individual white mustard plants due to aging of tissues and upon induction at least in damaged leaves (Chapters 3 and 4). Across different taxa the same rather weak correlation was found (Chapter 5). Defence and nutritional properties were in contrast strongly correlated within plants and across taxa. The aging process of leaves and stems reduced not only protein and/or nitrogen contents, but also chemical defence levels in *S. alba*, *A. petiolata*, *Armoracia rusticana*, and *Brassica rapa*. However, induction responses in *S. alba* were expressed stronger in defences than in protein levels. Chemical and mechanical defences were negatively correlated across taxa, but also within individuals when considering the differences of defence patterns between leaves and stems. Mechanical defences pose feeding barriers towards generalists and specialists to the same degree. They cannot be circumvented by specialization of insect physiology. But as argued above they represent an investment for the plant which cannot be remobilized. These considerations might be important for the trade-off found between chemical and mechanical defences. Within plants of *S. alba*, predictions of the "optimal defence hypothesis" were mostly confirmed (Chapter 3). However, the vulnerability and the value for a plant need to be determined when comparing different organs. In contrast, the "plant defence syndrome hypothesis" explains defence differences only by vulnerability (= high edibility) of plants. In general, the association of high edibility and defence could be confirmed when comparing several species and genera (Chapter 5). Between plant species the associations of vulnerability and defence might be stronger than within individuals. Here, the value of a tissue may be a physiological one between differently aged leaves or a structural one between different organs, i.e. stems or flowers *versus* leaves. Stems were nutritionally poor but highly defended by chemical and mechanical defences. At this level the associations of the "plant defence syndrome hypothesis" as they are understood now may be different.

Despite similarities of defence variability, the effects of within plant and between taxa variations on the specialist *A. rosae* were very different. The performance of these specialists was mostly influenced by mechanical defences and deficits in nutrients. Furthermore, developmental times increased and fat accumulation of *A. rosae* decreased together with increases in myrosinase activity. Myrosinase activity correlated with preference, i.e. reduced egg numbers of naïve females (Chapter 5). On a *Brassica juncea* CZERN. homozygous line with high myrosinase activity adult weights of *A. rosae* were reduced, but fat accumulation was not tested (Müller and Sieling, 2006).

In a field trial, very high levels of isothiocyanates deterred female turnip sawflies from yellow traps (Barker *et al.*, 2006). Thus a very high pulse of released breakdown products

requiring very high amounts of the enzyme could repel or deter females from oviposition. In contrast, short-term changes within plants of the *S. alba* cv. Silenda were not efficient in modifying feeding or oviposition behaviour of the turnip sawfly. The strength of the plant responses might not be high enough to trigger changes in the behaviour of females. In the long-term, high levels of glucosinolates are necessary for an efficient poisoning of generalist larvae, but high levels of at least myrosinase activity are needed as protection against specialists (Siemens and Mitchell-Olds, 1996; Li *et al.*, 2000; Müller and Sieling, 2006; Chapter 5). Different combinations of glucosinolate and myrosinase levels could have different functions in performance and preference and against generalists and specialists. These could be reasons to explain the low correlation between glucosinolate concentrations and myrosinase activities.

The interactions of the sequestering specialist *A. rosae* with Brassicaceae host plants were shown to influence their interactions also with the next trophic level, i.e. predators. Glucosinolates play a major role because sequestered glucosinolates can protect larvae against ants and wasps (Müller *et al.*, 2002; Müller and Arand, 2007). These antagonists to the herbivores can exert top-down forces which could influence oviposition choice of *A. rosae* (Müller and Arand, 2007). However bottom-up effects on individual performance and preference were not influenced by the plant's glucosinolate content, but possibly by variation in myrosinase activity (Chapter 5). The mechanism of sequestration of glucosinolates and the circumvention of myrosinase activity in *A. rosae* needs to be determined. Thereby, costs and limits of the adaptation will be revealed. The role of the glucosinolate-myrosinase system is less important than that of mechanical defences and high nutritional deficits for long-term development of *A. rosae*. Furthermore, induction of the glucosinolate-myrosinase system did not protect the plant from subsequent attacks in the short-term. However, the selective increase of only myrosinase activity in systemic leaves of the cultivar Salvo and the overall short-lived responses could represent a solution for plants to the "lethal plant defence paradox". Similarly, asynchronies between defence and nitrogen distribution were found due to aging in *Brassica* species (Lambdon *et al.*, 2003). Nitrogen in older leaves was less well protected. The ratio of nutrition gain and detoxification expense would be more favourable for herbivores in older leaves. These defence distributions and induction responses could be used by plants to distribute damage evenly within the plant. The growth of an organ could be a further mechanism of the plant to regulate its fitness impacts due to herbivory. It can either outgrow lost tissue immediately (cultivar Salvo), or reduce organ growth to save nutrients for "better" times (cultivar Silenda). Whether these patterns can be reproduced in other plants or cultivars deserves to be investigated.

6.4 Perspectives and future prospects

Induction of defences is clearly a very important response mechanism in plants against changing environmental conditions. However, the impacts on herbivores are still not fully understood. No direct negative effects were found for the specialist *A. rosae*. In the future, the mechanism of induced resistance against single, as well as against a community of differently specialized herbivores should be investigated. In some cases these plant responses evoked by specialist induction eliminated generalist herbivores and thus shaped the composition of an herbivore community (Thaler *et al.*, 2001). In this context, the connection between direct and indirect defences should be evaluated. The emission of plant volatiles for the attraction of parasitoids might be more effective against specialist herbivores than the increase of direct defences. The effectiveness of the "mustard oil bomb" at different combinations of substrate and enzyme levels needs to be explored further. The interaction of organ growth and defence responses are clearly important. Disentangling the mechanistic basis for induction will also aid in understanding the evolution of specialist-plant-interactions. In this context, insect-derived elicitors and their chemical nature will be most interesting. The involved signalling cascades and expression of genes responsible for myrosinase and/or glucosinolate induction need to be investigated because in *S. alba* these two components were regulated independently. Influences on the performance of *A. rosae* using mutants of *Arabidopsis thaliana* might be useful to determine which nutritious needs and detoxification limits exist. Alternatively, an artificial diet could be developed which could represent the natural compartimentalization of glucosinolates and myrosinases. Preference behaviour should be further investigated for the role of breakdown products and nutritional components in host acceptance and oviposition. The complete rejection of *Bunias orientalis* as a host raises the question of possible allochemicals as deterrents. These could be exploited in the biological control of this insect for which pest warnings especially for autumn cultures of young oilseed rape plants increase (Abram, 2006; Rush, 2006).

Appendix A

Growth of organs after induction treatments

Plant defences are in general imposing costs in relation to growth and/or to fitness of individuals (Cipollini *et al.*, 2003b). Treatments with herbivores or mechanical wounding had short-term effects on photosynthesis (Tang *et al.*, 2006), and on seedling mortality (Buschmann *et al.*, 2006). Long-term effects were found on shoot and root biomasses (Birch *et al.*, 1996; Agrawal, 2000; Bossdorf *et al.*, 2004), and on seed production (Agrawal, 1999b; Lewis *et al.*, 2006). Competition with other plants for ressources can alter fitness effects exerted by defence (Siemens *et al.*, 2002; Rebek and O'Neil, 2005; Jones *et al.*, 2006). We measured organ weights within the framework of two studies on the induction of the myrosinase-glucosinolate-system in Brassicaceae (Chapters 3 and 4). Enhanced defences in the context of the glucosinolate-myrosinase-system were found in both studies.

Organ dry biomass was estimated from the water content determined of the organ halves, which were freeze-dried and analyzed for glucosinolate content, and from total organ fresh weight. The percentage of removed tissue area was determined by photo analysis of treated leaves only in one induction study (Chapter 4). Measured organ weight could be corrected for the damage within this data set. Effects on organ dry biomass evoked by herbivory or other treatments were statistically analyzed using univariate ANOVA and subsequent Tukey-HSD tests. Whether organ dry biomass was influenced by total leaf number was tested by Pearson product-moment correlation analysis within the single organs over the whole period or within single harvesting days. The development of total leaf numbers and organ dry biomassses over five days was followed with regression analysis for each treatment separately.

In the first induction study (Chapter 3) damage was only estimated by view and dry weights of *A. rosae* treated leaves could therefore not be corrected for lost tissue. In the following, differences in organ dry biomasses are therefore only considered for systemic organs. Bag treatment plants did not differ from untreated controls in leaf numbers or in dry biomasses of any organ on day 1. The total number of leaves varied between the tested individual plants. But significant differences were only found between plants treated with one large and three small larvae on day 2. Plants with higher leaf numbers were expected to have higher organ dry biomasses *per se*. Accordingly, over the whole period of five days total leaf number correlated significantly with dry biomasses of all four systemic organs (older leaves, $n = 87$, $R^2 = 0.39$, $P < 0.001$, younger leaves, $n = 87$, $R^2 = 0.55$, $P < 0.001$; older stems, $n = 85$, $R^2 = 0.40$, $P < 0.001$; younger stems, $n = 87$, $R^2 = 0.63$, $P < 0.001$). However significant correlations between total leaf number and organ dry biomass separated by harvesting day were found only in four out of sixteen correlations (day 2: older leaves, $n = 21$, $R^2 = 0.50$, $P = 0.020$, younger leaves, $n = 21$, $R^2 = 0.56$, $P = 0.008$; day 3: older stems, $n = 19$, $R^2 = 0.64$, $P = 0.003$; day 5: older stems, $n = 19$, $R^2 = 0.49$, $P = 0.033$). Organ dry biomass of older and younger systemic leaves in plants treated with one large larva were significantly increased compared to bag treated controls on all harvesting days. Treatment with three small larvae led to organ dry biomasses which did not differ statistically from bag treated plants. Growth rates of total leaf numbers within the five days were strongest in plants treated with three small larvae, followed by bag treated plants and weakest in plants treated with one large larva (Table A.1). Dry biomass growth rates of single organs could be determined with good reliability (R^2) only in younger leaves and both stems. These organs showed higher biomass increases within the five days than damaged and older leaves. In younger leaves and in older stems growth rates were strongly affected by larval feeding. Coefficients were about 1.5-times higher in plants when larvae irrespective of age fed on the plants. In younger systemic stems no differences in growth rates were found.

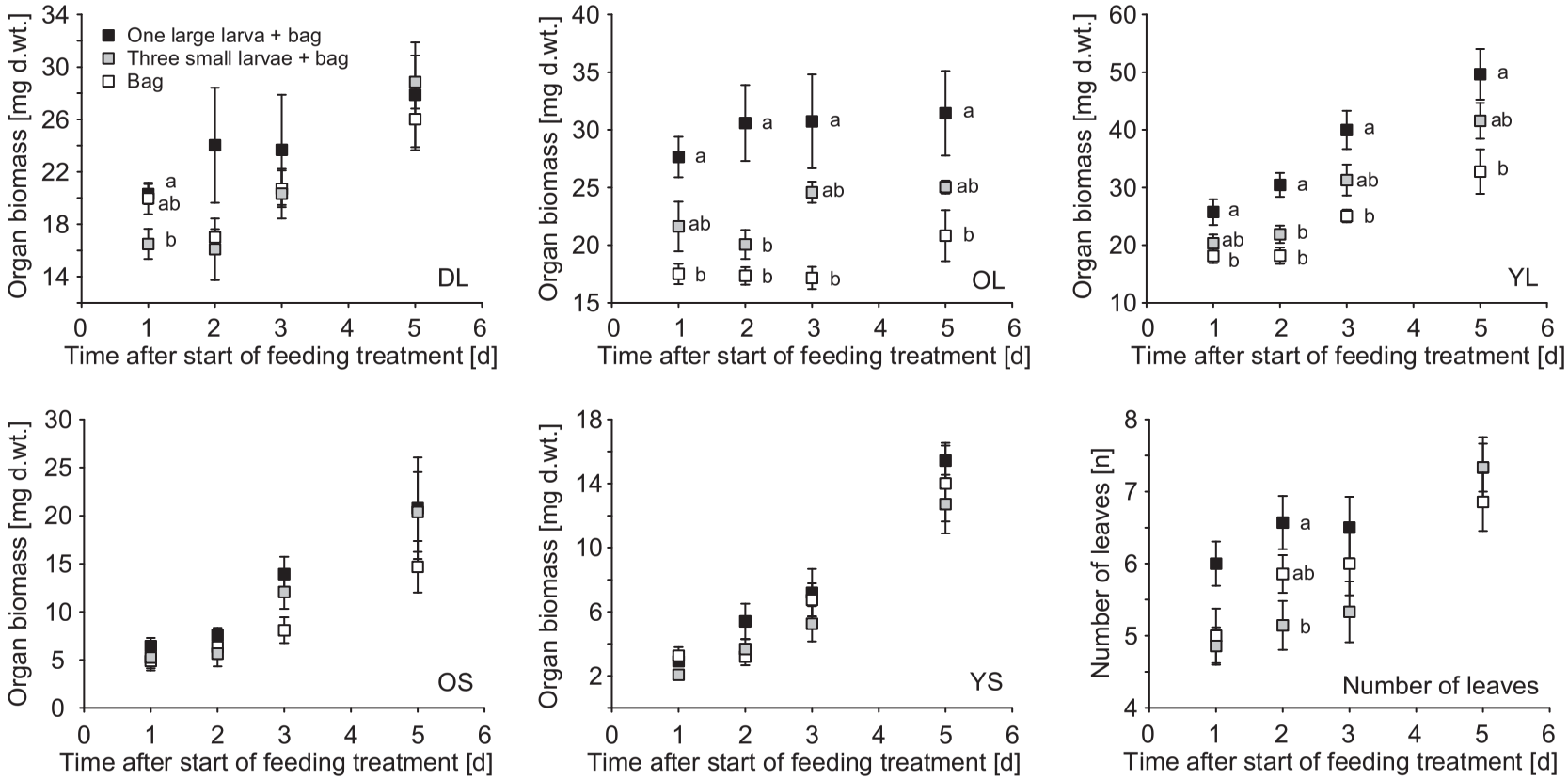

Figure A.1: Total numbers of leaves and organ dry biomasses of leaves and stems of *Sinapis alba* cv. Salvo. The second youngest leaf was treated with either one large larva of *Athalia rosae* or three small larvae, enclosed in a muslin bag for 24 hours (day 1). Organ biomasses of damaged leaves were not corrected for lost tissue. Bagged leaves without larvae served as controls (n = 7 plants per treatment). Treatment effects were tested by univariate ANOVA and subsequent Tukey-HSD tests (significant differences are marked with different letters, $P < 0.05$). DL: damaged leaf; OL: older leaf; YL: younger leaf; OS: older stem; YS: younger stem.

Table A.1: Regression analyses of total number of leaves or organ dry biomass depending on harvesting day.

Treatment	N	ANOVA R^2	F	df	P	Coefficient	SE
			Total number of leaves				
Bag	35	0.32	12.22	1	0.002	0.433 **	0.124
Three small larvae + bag	35	0.51	24.62	1	<0.001	0.618 ***	0.125
One large larva + bag	35	0.22	6.66	1	0.016	0.308 *	0.119
			Treated leaves				
Bag	35	0.30	10.99	1	0.003	1.831 **	0.552
Three small larvae + bag	35	0.51	25.18	1	<0.001	3.288 ***	0.655
One large larva + bag	35	0.08	2.14	1	0.156	1.727	1.179
			Older leaves				
Bag	35	0.12	3.65	1	0.067	0.859 †	0.450
Three small larvae + bag	35	0.17	4.79	1	0.039	1.100 *	0.503
One large larva + bag	35	0.03	0.61	1	0.441	0.829	1.058
			Younger leaves				
Bag	35	0.52	28.24	1	<0.001	3.960 ***	0.745
Three small larvae + bag	35	0.70	55.25	1	<0.001	5.633 ***	0.758
One large larva + bag	35	0.61	38.13	1	<0.001	6.164 ***	0.998
			Older stems				
Bag	35	0.43	18.42	1	<0.001	2.500 ***	0.582
Three small larvae + bag	35	0.54	27.83	1	<0.001	4.036 ***	0.765
One large larva + bag	35	0.44	18.85	1	<0.001	3.793 ***	0.874
			Younger stems				
Bag	35	0.60	38.20	1	<0.001	2.870 ***	0.464
Three small larvae + bag	35	0.70	54.81	1	<0.001	2.669 ***	0.361
One large larva + bag	35	0.76	75.50	1	<0.001	3.109 ***	0.358

Notes: Coefficients significantly different from zero are marked with asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † $P < 0.07$.

In the specificity experiment (Chapter 4), total number of leaves did not vary between the tested individual plants. Damage was determined by photo analysis and dry weights could therefore be corrected for lost tissue. Bag treatment plants did not differ from untreated control plants in total leaf numbers or in organ dry biomasses of any organ. Significant correlations between total leaf number and organ dry biomasses were found only for systemic older leaves ($n = 30$, $R^2 = 0.44$, $P = 0.016$). Organ dry biomasses were not influenced by mechanical wounding in any organ compared to bag treated plants. In contrast, feeding by both herbivore species reduced all organ dry biomasses. But significant differences from bag treated controls were only found for damaged and systemic younger leaves of *A. rosae* treated plants. Differences of organ dry biomasses between mechanically and herbivore treated plants were more pronounced than between bag and herbivore treated plants. In all three leaves significant reductions of 20 to 40 % in dry weights were found.

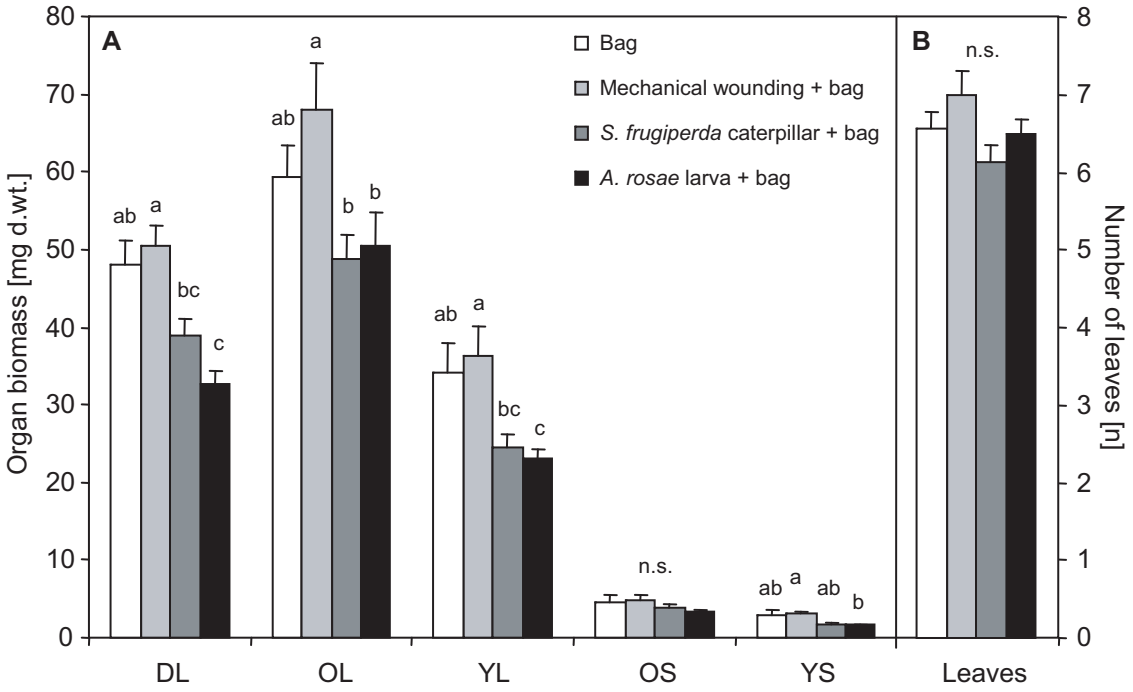

Figure A.2: Organ dry biomasses of leaves and stems (A) and total numbers of leaves (B) of *Sinapis alba* cv. Silenda. The second youngest leaf was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hours. Percentage removed tissue area was determined by photo analysis and organ weights of damaged leaves were corrected for lost tissue individually. Bagged leaves without any further treatment served as controls (n = 6-8 per treatment). Letters above bars indicate significant differences (ANOVA, Tukey-HSD tests: $P < 0.05$; n.s. - not significant). DL: damaged leaf; OL: older leaf; YL: younger leaf; OS: older stem; YS: younger stem.

Appendix B

Supplementary material for "Matching plant defence syndromes"

Table B.1: Plant leaf traits of seven Brassicaceae I. nutrition (mean ± SE, n = 4).

		Alliaria petiolata	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Water content	Y	81.93±0.42	79.44±1.98	88.90±1.21	81.17±0.60	84.99±0.49	85.04±0.34	83.68±0.71
[% of f.wt.]	O	86.98±1.37	80.64±2.21	91.51±1.32	81.39±0.67	85.01±0.44	84.60±0.42	86.63±0.72
Specific leaf area	Y	0.55±0.02	0.30±0.03	0.46±0.06	0.22±0.02	0.46±0.01	0.53±0.01	0.51±0.02
[cm^2 g^{-1}]	O	0.72±0.05	0.23±0.03	0.61±0.10	0.23±0.01	0.40±0.04	0.50±0.01	0.55±0.05
Soluble protein content	Y	3.00±0.44	6.10±0.76	2.19±0.34	6.47±1.19	3.01±0.69	3.55±0.65	4.65±0.68
[μg mg^{-1} f.wt.]	O	2.34±0.55	3.77±0.58	1.53±0.29	5.53±0.85	3.33±0.78	3.72±0.79	3.13±0.54
Nitrogen content	Y	6.44±1.08	9.23±0.51	3.90±0.31	9.53±0.42	5.61±0.60	6.97±0.43	7.55±0.89
[‰ of f.wt.]	O	4.71±0.94	8.42±2.50	3.37±0.27	9.26±0.68	5.90±0.65	6.64±0.45	6.33±0.74
C:N ratio	Y	13.15±2.43	10.20±1.06	12.04±1.57	8.66±0.41	11.80±0.85	9.66±0.31	9.43±0.61
	O	13.16±3.39	11.85±0.76	9.77±1.65	8.81±0.38	11.35±0.74	10.21±0.51	9.35±0.81

Notes: Y - young leaves, O - old leaves, f.wt. - fresh weight.

Table B.2: Plant leaf traits of seven Brassicaceae II. defence (mean ± SE, n = 4).

		Alliaria petiolata	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Glucosinolate concentration	Y	6.96±3.33	10.74±4.84	3.39±0.16	11.14±1.36	14.00±1.33	7.63±2.84	4.87±0.55
[μmol mg^{-1} f.wt.]	O	0.86±0.75	8.67±3.45	0.79±0.38	9.62±1.19	13.93±0.90	8.86±4.32	5.35±1.09
Myrosinase activity concentration	Y	11.71±3.12	14.44±5.21	0†	6.95±2.14	14.39±3.65	63.85±15.70	5.09±0.75
[nmol min^{-1} mg^{-1} f.wt.]	O	5.23±1.63	13.30±8.63	0†	7.73±2.90	14.05±3.45	100.86±10.29	4.51±0.66
Adaxial trichome density	Y	0	0	0.45±0.08	1.35±0.16	1.07±0.20	0	3.94±0.10
[no. hairs mm^{-2}]	O	0	0	0.11±0.03	4.65±0.24	1.05±0.10	0	3.17±0.42
Abaxial trichome density	Y	0	0	2.48±0.15	1.57±0.14	0.08±0.05	0	9.86±0.86
[no. hairs mm^{-2}]	O	0	0	0.24±0.01	4.85±0.29	0.03±0.03	0	11.91±0.54
Trypsin inhibitor activity	Y	0.25±0.25	0.25±0.25	3.00±0.41	3.75±1.11	2.50±0.50	1.25±0.63	5.50±0.29
[category per leaf disc]	O	0.25±0.25	0	0.50±0.29	3.75±1.44	2.50±0.50	1.00±0.71	5.75±0.63

Notes: Y - young leaves, O - old leaves, f.wt. - fresh weight; † no myrosinase activity could be detected within 24 hours.

Table B.3: Multifactorial ANOVA of plant leaf traits depending on species, leaf age, and the interaction term of species × leaf age using raw data (n = 56).

	Model		Species		Leaf age		Interaction		Variance	
	$df = 13$		$df = 6$		$df = 1$		$df = 6$		homogeneity	
Plant trait	F	P	F	P	F	P	F	P	F	P
Water content	9.631	**<0.001**	17.867	**<0.001**	8.126	**0.007**	1.645	0.159	2.114	0.034
Specific leaf area	13.396	**<0.001**	26.025	**<0.001**	1.433	0.238	2.760	**0.024**	2.312	0.020
Soluble protein content	4.542	**<0.001**	8.144	**<0.001**	4.749	**0.035**	0.905	0.500	1.009	0.460
Nitrogen content	4.490	**<0.001**	9.170	**<0.001**	1.785	0.189	0.260	0.952	3.183	0.002
C:N ratio	1.201	0.313	2.242	0.058	0.007	0.933	0.359	0.901	9.495	<0.001
Glucosinolate concentration	3.081	**0.003**	5.925	**<0.001**	1.399	0.243	0.518	0.791	8.072	<0.001
Myrosinase activity concentration	22.263	**<0.001**	44.973	**<0.001**	1.692	0.200	2.982	**0.016**	12.270	<0.001
Adaxial trichome density	111.383	**<0.001**	199.634	**<0.001**	14.356	**<0.001**	39.304	**<0.001**	5.815	<0.001
Abaxial trichome density	188.605	**<0.001**	388.477	**<0.001**	8.034	**0.007**	18.828	**<0.001**	6.178	<0.001
Trypsin inhibitor acitivity	9.719	**<0.001**	19.717	**<0.001**	1.349	0.252	1.115	0.370	3.772	0.001

Notes: For units of plant parameters see Tables B.1 and B.2.

Table B.4: Male insect parameters reared on seven Brassicaceae (mean±SE; performance parameters n = 30 at start).

		Alliaria petiolata	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Larval mortality	Y	3.33	34.48	10.00	100	13.33	6.90	100
[%]	O	6.67	10.00	3.33	100	6.67	10.34	37.93
Pupal mortality	Y	3.45	21.05	0	n.d. †	3.85	3.70	n.d.
[%]	O	0	0	17.24	n.d.	0	15.38	11.11
Total mortality	Y	6.67	48.28	10.00	100	16.67	10.34	100
[%]	O	6.67	10.00	20.00	100	6.67	24.14	44.83
Larval developmental time	Y	8.38±0.09	9.84±0.14	10.14±0.14	n.d.	10.43±0.09	10.37±0.12	n.d.
[d]	O	8.73±0.10	9.24±0.15	10.13±0.16	n.d.	10.36±0.12	10.93±0.12	12.99±0.24
Pupal developmental time	Y	12.43±0.08	12.51±0.12	11.99±0.08	n.d.	12.43±0.11	13.10±0.18	n.d.
[d]	O	12.38±0.06	12.08±0.13	12.45±0.14	n.d.	12.18±0.09	13.57±0.18	13.24±0.18
Total developmental time	Y	20.79±0.12	22.41±0.14	22.10±0.16	n.d.	22.86±0.16	23.40±0.24	n.d.
[d]	O	21.10±0.11	21.31±0.22	22.48±0.20	n.d.	22.50±0.16	24.52±0.24	26.04±0.26

Continued on following page.

Table B.4 continued.		*Alliaria petiolata*	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Eonymph weight	Y	32.44±0.43	27.12±0.68	26.15±0.63	n.d. †	28.07±0.43	27.98±0.54	n.d.
[mg]	O	31.71±0.50	30.92±0.69	24.22±0.75	n.d.	30.32±0.45	26.66±0.64	22.33±0.77
Adult weight	Y	11.71±0.18	9.72±0.26	8.54±0.26	n.d.	10.10±0.18	9.35±0.21	n.d.
[mg]	O	11.56±0.18	10.83±0.24	8.03±0.36	n.d.	10.94±0.19	8.60±0.18	8.64±0.27
Water content of adults	Y	76.93±0.33	77.22±0.43	78.88±0.51	n.d.	76.08±0.61	79.86±0.36	n.d.
[%]	O	76.74±0.39	74.83±0.86	80.80±0.98	n.d.	76.57±0.37	82.29±0.49	82.14±0.89
Non-fat content of adults	Y	14.82±0.33	14.55±0.50	12.46±0.80	n.d.	15.61±0.45	15.07±0.49	n.d.
[%]	O	14.00±0.55	16.36±0.70	10.43±1.21	n.d.	15.03±0.48	11.74±0.92	6.58±1.09
Fat content of adults	Y	8.23±0.25	8.06±0.45	8.34±0.40	n.d.	7.72±0.24	5.09±0.33	n.d.
[%]	O	8.81±0.39	8.29±0.28	9.36±0.39	n.d.	8.32±0.22	5.66±0.56	10.65±0.40
Glucosinolate concentration	Y	6.41±0.30	6.05±0.37	5.24±0.35	n.d.	7.28±0.22	4.44±0.24	n.d.
[μmol mg^{-1} f.wt.]	O	6.90±0.19	7.92±0.38	0.53±0.08	n.d.	7.31±0.26	4.20±0.21	5.79±0.26
Nitrogen content	Y	25.62±0.72	26.63±0.68	26.12±0.50	n.d.	26.89±0.56	23.21±1.10	n.d.
[‰ of adult f.wt.]	O	27.81±1.20	26.28±0.93	22.53±2.08	n.d.	26.80±0.99	22.30±0.90	22.53±1.67
C:N ratio	Y	4.60±0.08	4.59±0.08	4.22±0.07	n.d.	4.38±0.04	4.29±0.07	n.d.
	O	4.75±0.05	4.58±0.05	4.17±0.09	n.d.	4.50±0.05	4.31±0.07	4.11±0.06

Notes: Y - young leaves, O - old leaves, f.wt. - fresh weight. † n.d. - not determined due to high mortality.

Table B.5: Female insect parameters reared on seven Brassicaceae (mean±SE; performance parameters n = 50 at start; preference parameters n = 15 to 22).

		Alliaria petiolata	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Larval mortality ‡	Y	0	0	2.00	100	14.58	20.83	86.00
[%]	O	4.00	8.33	20.00	100	22.92	16.67	24.00
Pupal mortality	Y	6.00	16.33	12.24	n.d. †	0	7.89	28.57
[%]	O	2.08	4.55	22.50	n.d.	2.70	7.50	18.42
Total mortality	Y	6.00	16.33	14.00	100	14.58	27.08	90.00
[%]	O	6.00	12.50	38.00	100	25.00	22.92	38.00
Larval developmental time	Y	8.85±0.06	10.03±0.12	9.66±0.12	n.d.	10.23±0.30	10.89±0.28	14.30±0.53
[d]	O	9.25±0.22	9.89±0.12	10.75±0.15	n.d.	10.16±0.41	10.75±0.36	12.80±0.19
Pupal developmental time	Y	11.50±0.20	11.44±0.19	10.90±0.08	n.d.	11.39±0.14	12.56±0.47	11.70±0.18
[d]	O	11.58±0.15	11.14±0.09	11.20±0.18	n.d.	11.39±0.22	12.16±0.40	12.01±0.07
Total developmental time	Y	20.35±0.19	21.47±0.23	20.56±0.16	n.d.	21.61±0.25	23.44±0.67	26.00±0.45
[d]	O	20.83±0.25	21.02±0.14	21.95±0.25	n.d.	21.55±0.45	22.91±0.67	24.82±0.22

Continued on following page.

Table B.5 continued.		*Alliaria petiolata*	*Armoracia rusticana*	*Brassica rapa* ssp. *chinensis*	*Bunias orientalis*	*Cardamine heptaphylla*	*Cardamine pentaphyllos*	*Lunaria rediviva*
Eonymph weight	Y	48.97±0.81	42.64±1.21	34.95±0.80	n.d. †	40.62±1.49	33.33±0.76	26.86±2.69
[mg]	O	46.10±1.70	47.42±0.85	26.82±0.75	n.d.	43.61±2.03	35.28±2.23	30.58±0.99
Adult weight	Y	21.23±0.33	18.01±0.62	13.59±0.23	n.d.	17.47±0.43	12.81±0.53	11.62±0.71
[mg]	O	19.46±0.68	19.59±0.28	10.52±0.24	n.d.	18.95±0.91	13.77±0.67	13.21±0.41
Egg numbers of naïve females	Y	13.53±1.66	9.65±1.17	18.94±1.13	1.53±0.68	7.53±1.09	0.06±0.06	0.65±0.42
	O	7.24±1.00	2.94±0.73	12.59±1.53	0.12±0.08	13.06±1.31	0	0.88±0.65
Egg numbers of experienced females §	Y	21.41±1.84	23.73±3.09	22.83±1.49	n.d.	15.95±1.58	6.87±1.32	18.80±2.38
Host selectivity #	Y	2.91±0.17	3.27±0.22	2.13±0.13	n.d.	2.38±0.21	1.47±0.24	2.45±0.27

Notes: Y - young leaves, O - old leaves. † n.d. - not determined due to high mortality; ‡ Mortality rates could only be obtained from a mixture of both sexes; § Egg numbers of naïve females were obtained in leaf-age-choice tests, but times of bioassays were very short (3 hours) and egg numbers were highly different between plant species and leaf ages and thus allowed for approximation of data independence (overall minimum value: no eggs; maximum value: 29 eggs per female); # Host selectivity is expressed as mean numbers of accepted host plants of experienced females.

Table B.6: Multifactorial ANOVA for male and female insects separately depending on species, leaf age and the interaction of species × leaf age.

Insect parameter	n	Model $df = 10/11$ F	P	Species $df = 5$ F	P	Leaf age $df = 1$ F	P	Interaction $df = 4/5$ F	P	Variance homogeneity F	P
(a) male											
Larval developmental time	512	72.796	**<0.001**	138.350	**<0.001**	0.330	0.566	6.359	**<0.001**	2.424	0.008
Pupal developmental time	488	15.420	**<0.001**	25.925	**<0.001**	0.273	0.601	5.089	**0.001**	7.371	<0.001
Total developmental time	488	56.427	**<0.001**	102.414	**<0.001**	0.368	0.544	10.380	**<0.001**	5.666	<0.001
Eonymph weight	512	24.979	**<0.001**	43.347	**<0.001**	1.269	0.261	8.933	**<0.001**	2.380	0.009
Adult weight	457	33.787	**<0.001**	62.344	**<0.001**	0.585	0.445	7.134	**<0.001**	2.198	0.017
Water content	486	19.491	**0.003**	33.221	**<0.001**	1.667	0.197	5.680	**<0.001**	3.242	<0.001
Non-fat dry matter	374	11.795	**<0.001**	17.821	**<0.001**	5.765	**0.017**	4.379	**0.002**	5.865	<0.001
Fat content	375	14.951	**<0.001**	27.188	**<0.001**	6.581	**0.011**	0.244	0.913	10.263	<0.001
Glucosinolate concentration	375	44.287	**<0.001**	68.049	**<0.001**	8.358	**0.004**	30.903	**<0.001**	5.263	<0.001
C:N ratio	110	10.062	**<0.001**	19.455	**<0.001**	1.257	0.265	0.810	0.522	1.034	0.421
Nitrogen content	108	3.513	**0.001**	5.253	**<0.001**	0.615	0.435	1.726	0.150	3.353	0.001
(b) female											
Larval developmental time	196	50.490	**<0.001**	81.578	**<0.001**	0.218	0.641	8.060	**<0.001**	2.446	0.007
Pupal developmental time	196	5.779	**<0.001**	9.312	**<0.001**	0.000	0.984	1.155	0.333	3.390	<0.001
Total developmental time	196	35.119	**<0.001**	53.867	**<0.001**	0.114	0.736	5.357	**<0.001**	3.378	<0.001
Eonymph weight	196	48.625	**<0.001**	84.472	**<0.001**	0.281	0.597	10.611	**<0.001**	1.181	0.303
Adult weight	196	69.811	**<0.001**	126.652	**<0.001**	0.185	0.667	10.880	**<0.001**	2.057	0.026

Notes: For units of plant parameters see Tables B.4 and B.5.

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Summary

Brassicaceae and a few related plant families are characterized by possession of the glucosinolate-myrosinase system. Glucosinolates are amino-acid derived allelochemicals which are hydrolysed upon tissue damage by myrosinase enzymes to produce various degradation products which can be toxic for generalist insects. The larvae of the crucifer-specialist *Athalia rosae*, the turnip sawfly, sequester glucosinolates into their haemolymph. The role of the glucosinolate-myrosinase system for the interaction of the turnip sawfly with Brassicaceae was examined in this study from two different perspectives: variation within individual plants and between plant species. The plant responses to the feeding by herbivores and the short-term effects this induction had on insect behaviour were investigated in white mustard. Furthermore, plants can use multiple defences. Hence correlations of glucosinolates and myrosinase activities with other defences and nutritional quality and their long-term effects on the development of the insects were investigated in seven different plant species.

Within this context, reliable determinations of glucosinolate levels and myrosinase activities were very important. In contrast to the already well established methods for glucosinolate analysis, an enzyme assay was developed by which total activity of soluble and insoluble myrosinase fractions of myrosinases can now be measured under *in situ* conditions. According to this modified assay, myrosinase activity is measured as a glucose release from standardized concentrations of externally added substrate. A dynamic measurement of glucose *via* the GOD-PAP-method has a favorable signal to noise ratio in clear fractions containing soluble myrosinases and also in turbid suspensions containing not only insoluble myrosinase complexes, but also various cell fragments.

Induction of plant allelochemicals, e.g. glucosinolates, is of particular ecological importance for interactions with sequesterers which can exploit the increased investments of the plant in its defence. However, this plant dilemma, known as the "lethal plant defence paradox" has only been addressed once for Brassicaceae. In two cultivars of *Sinapis alba*, white mustard, glucosinolate and myrosinase activities levels increased after feeding by larvae of the turnip sawfly. The responses were stronger in local, damaged tissue than in systemic

tissue. Leaves were found to have higher defence levels than stems. The patterns of defence levels in leaves and stems of different age confirmed the predictions of the "optimal defence hypothesis". Older instars of *A. rosae* larvae evoked stronger increases in glucosinolates and myrosinase activities than younger larvae. The responses were transient and mostly declined within a day after the treatment. This rapid decline could represent one solution to the "lethal plant defence paradox" , because subsequent attackers would not profit from high glucosinolate levels. However, the recently proposed solution of plant induction responses being stronger towards susceptible, younger instars was not observed.

The responses of the glucosinolate-myrosinase system to the feeding by this specialist herbivore were clearly different from those evoked by a generalist herbivore which elicited only increases in glucosinolate, but not in myrosinase activity levels. However, mechanical wounding triggered responses similar to specialist feeding. Growth of single organs was reduced by both herbivores, but not by mechanical wounding. In biotests with larvae and female adults, neither larval feeding, nor the oviposition choices of specialist adult females were modified within 24 hours after the feeding treatment due to induction treatment. Therefore, specificity of plant responses, but not of their effects on the behaviour of an adapted herbivore was found in *S. alba*. Thus, in view of the arms race of plants and herbivores, the specialist herbivores could profit from increased glucosinolate levels *via* sequestration and protection against the next trophic level. The generalist herbivores could profit from a suppression of plant responses which possibly renders the food less toxic. The plant on the other hand is not protected by increased levels of allelochemicals against specialist *A. rosae*, but reduced organ growth could be a means to tolerate or escape the immediate attack of herbivores.

Multiple defence mechanisms of seven plant species and two leaf ages were investigated for their correlations. Univariate trade-offs between the ten investigated plant traits could not be found, however multivariate associations were found. The "plant defence syndromes hypothesis" was tested and as predicted the general plant traits "edibility" and "feeding barrier" characteristics were revealed as important trait associations for cluster formation. Three clusters were found in which plants with either low levels of nutrients and defences, with higher levels of nutrients and chemical defences, or with high levels of nutrients and mechanical defences were grouped. Variation between the two leaf ages was less pronounced than between different plant species and both leaf ages of one plant species were always found within the same cluster. Therefore, one species always employed the same defence strategy. To evaluate the fitness of the sawflies, which were raised on these seven host plants, several parameters were recorded. The complete larval development and adult fitness parameters were recorded to be able to group parameters and match these with plant defence syndromes. Mortality rates, fat and glucosinolate accumulation

were the most important performance parameters. Adult oviposition preference of the seven plant species was correlated to the registered performance. The insect parameters were also grouped in three clusters which coincided with those of the plants. As predicted for a specialist herbivore mechanical defences had stronger impacts on performance and preference than chemical defences. Nutritionally poor host quality was only compensated for in the absence of mechanical defences.

This study describes for the first time the responses of both components of the glucosinolate-myrosinase system after larval feeding of a leaf-chewing sequesterer within plant individuals. These reactions were shown to be specifically modified according to larval instar, to specialization of the insect and to plant cultivar. However, no short-term effects of increased defence levels were observed on the behaviour of the crucifer-specialist *A. rosae*. A wider range of variation in different plant species revealed multivariate associations of multiple defences. The long-term effects on performance and preference of *A. rosae* were dominated by mechanical defences, but nutritional traits and myrosinase activity also played key roles for host plant quality.

Zusammenfassung

Die Brassicaceen und einige nah verwandte Pflanzenfamilien zeichnen sich durch den Besitz des Glucosinolat-Myrosinase Systems aus. Glucosinolate sind von Aminosäuren abgeleitete Allelochemikalien, die nach Gewebezerstörung von Myrosinaseenzymen hydrolysiert werden. Die entstehenden Abbauprodukte wirken auf generalistische Insekten toxisch. Larven der auf Brassicaceen spezialisierten Rübsenblattwespe, *Athalia rosae*, sequestrieren Glucosinolate in ihre Hämolymphe. In der vorliegenden Studie wird die Rolle des Glucosinolat-Myrosinase Systems für die Interaktion von Brassicaceen mit der Rübsenblattwespe aus zwei unterschiedlichen Perspektiven untersucht: Variationen innerhalb einzelner Pflanzen und zwischen verschiedenen Pflanzenarten. Die pflanzliche Antwort innnerhalb einzelner Individuen auf Herbivorenfraß und deren kurzzeitige Auswirkungen auf das Insektenverhalten wurden am Weißen Senf untersucht. Des Weiteren nutzen Pflanzen multiple Abwehrmethoden. Daher wurden Korrelationen des Glucosinolat-Myrosinase Systems mit anderen Abwehrmethoden und mit dem Nährstoffgehalt der Pflanzen sowie deren langfristige Effekte auf die Entwicklung der Insekten an sieben verschiedenen Pflanzenarten untersucht.

Zuverlässige Bestimmungen von Glucosinolat- und Myrosinaseaktivitätsgehalten sind in diesem Zusammenhang sehr wichtig. Im Gegensatz zu den schon gut etablierten Methoden zur Glucosinolatanalyse wurde ein Enzymassay weiterentwickelt, mit dem die Gesamtaktivität von löslichen und unlöslichen Myrosinasefraktionen unter *in situ* Bedingungen bestimmt werden kann. Anhand dieses modifizierten Assays wird die Myrosinaseaktivität als eine Glucosefreisetzung aus standardisierten Konzentrationen eines extern hinzugefügten Substrats gemessen. Die dynamische Messung von Glucose mittels der GOD-PAP-Methode hat ein sehr gutes Verhältnis von Signal zu Hintergrund zum einen in klaren Fraktionen, die lösliche Myrosinasen enthalten, zum anderen aber auch in trüben Suspensionen, die nicht nur unlösliche Myrosinasekomplexe, sondern auch verschiedenste Zellfragmente enthalten.

Die Induktion von pflanzlichen Allelochemikalen, z. Bsp. Glucosinolate, ist von spezieller ökologischer Bedeutung für Interaktionen mit Sequestrierern, welche die erhöhten Investitionen der Pflanze in ihre Abwehr ausnutzen können. Dieses Dilemma aus der Sicht

der Pflanzen, „lethal plant defence paradox“ genannt, wurde bisher jedoch erst einmal bei einer Brassicaceenart untersucht. In der vorliegenden Studie konnte gezeigt werden, dass in zwei Kulturvarietäten des Weißen Senfs, *Sinapis alba*, Glucosinolat-, sowie auch Myrosinaseaktivitätsgehalte im Pflanzengewebe nach Fraß der Larven der Rübsenblattwespen erhöht waren. Die Reaktionen waren in lokal beschädigtem Gewebe ausgeprägter als in systemischem Gewebe. In Blättern wurden höhere Abwehrlevel gefunden als in Sprossachsenabschnitten. Die gefundenen Muster der Verteidigungslevel in Blättern und in Sprossachsenabschnitten bestätigten Vorhersagen der „optimal defence hypothesis“. Ältere Larvalstadien von *A. rosae* riefen stärkere Anstiege an Glucosinolat- und Myrosinaseaktivitätsgehalten hervor als jüngere Larven. Die Reaktionen waren nur kurzlebig und ließen in den meisten Fällen innerhalb eines Tages nach Behandlung nach. Dieser schnelle Rückgang könnte eine Lösung für das „lethal plant defence paradox“ darstellen, da nachfolgende Angreifer nicht weiterhin von erhöhten Glucosinolatgehalten profitieren könnten. Die kürzlich an anderer Stelle vorgeschlagene Lösung von stärkeren pflanzlichen Reaktionen auf jüngere, anfälligere Larven konnte jedoch nicht bestätigt werden.

Die Reaktionen des Glucosinolat-Myrosinase Systems auf Fraß dieses spezialisierten Herbivoren unterschieden sich deutlich von denen, die durch einen generalistischen Herbivoren hervorgerufen wurden. Letztere verursachten Anstiege im Glucosinolat-, aber NICHT im Myrosinaseaktivitätsgehalt der Pflanzen. Mechanische Verwundung löste jedoch ähnliche Reaktionen wie Spezialistenfraß aus. Das Wachstum einzelner Organe wurde durch Fraßaktivität beider Herbivorenarten, aber nicht durch mechanische Verwundung reduziert. Biotests mit Larven und adulten Weibchen ergaben, dass weder Larvalfraß noch Eiablagewahl der Weibchen innerhalb von 24 Stunden nach Behandlung der Pflanzen durch die unterschiedlichen Behandlungen beeinflusst wurden. So konnte zwar eine Spezifität der pflanzlichen Reaktionen von *S. alba*, aber nicht der Effekte auf das Verhalten dieses angepassten Herbivoren gefunden werden. Folglich kann, im Hinblick auf ein Wettrüsten zwischen Pflanzen und Herbivoren, der Spezialist von erhöhten Glucosinolatgehalten durch Sequestration und Schutz gegenüber der nächsten trophischen Ebene profitieren. Dem Generalisten würde die Unterdrückung von pflanzlichen Antworten nützen, da die Nahrungsquelle so möglicherweise weniger toxisch wirken kann. Die Pflanze auf der anderen Seite kann sich durch eine Erhöhung ihrer Allelochemikaliengehalte nicht gegen spezialisierte *A. rosae* schützen. Das reduzierte Organwachstum könnte einen Mechanismus darstellen, um die unmittelbare Attacke des Herbivoren und den damit verbundenen Schaden zu tolerieren.

In sieben verschiedenen Brassicaceenarten und zwei Blattaltern wurden die Korrelationen von multiplen Abwehrmechanismen untersucht. Univariate Zielkonflikte, sogenannte „trade-offs“, konnten zwischen den zehn untersuchten Pflanzenmerkmalen nicht gefunden

werden. Die Merkmale waren jedoch multivariat assoziiert. Die „plant defence syndrome hypothesis“ wurde hier getestet und die generellen Pflanzenmerkmale „Essbarkeit“ und „Fraßbarrieren“ wurden wie vorhergesagt als wichtige Merkmalsassoziationen zur Clusterbildung bestätigt. Drei Cluster wurden gefunden, in denen Pflanzen gruppiert wurden, die zum einen niedrige Gehalte an Nährstoffen und Verteidigung, zum anderen hohe Gehalte an Nährstoffen und chemische Verteidigung oder hohe Gehalte an Nährstoffen und mechanische Verteidigung aufwiesen. Die gefundene Variation zwischen den beiden Blattaltern war weniger intensiv als diejenige zwischen verschiedenen Pflanzenarten. Beide Blattalter einer Pflanzenart wurden in allen Fällen im gleichen Cluster gefunden. Daher scheint es, als ob innerhalb einer Pflanzenart immer die gleiche Abwehrstrategie Anwendung findet. Um die Fitness der Blattwespen beurteilen zu können, die auf diesen sieben verschiedenen Brassicaceenarten und zwei Blattaltern aufgezogen wurden, wurden verschiedenste Parameter aufgenommen. Die Parameter betrafen die komplette Larvalentwicklung, sowie das Adultstadium und wurden zu einer erneuten Clusteranalyse herangezogen. Das Ergebnis wurde dann mit dem der Pflanzenmerkmale verglichen. Sterberate, Fett- und Glucosinolatakkumulation waren die wichtigsten Entwicklungsparameter. Die Eiablagepräferenz der adulten Weibchen auf den sieben Pflanzenarten war mit der aufgenommenen Entwicklung korreliert. Die Insektenparameter waren ebenfalls in drei Cluster gruppiert, welche in der Pflanzenartenzusammensetzung mit denen der Pflanzencluster größtenteils übereinstimmten. Wie für einen spezialisierten Herbivoren vorausgesagt, hatten mechanische Abwehrmechanismen einen stärkeren Einfluss auf Entwicklung und Präferenz als chemische Abwehr. Nährstoffarmut der Pflanzen konnte nur in der Abwesenheit von mechanischen Abwehrmechanismen kompensiert werden.

In dieser Studie wurden zum ersten Mal die Antworten beider Komponenten des Glucosinolat-Myrosinase Systems auf Larvalfraß eines kauenden Sequestrierers innerhalb einzelner Pflanzen beschrieben. Es wurde gezeigt, dass diese Reaktionen spezifisch modifiziert werden je nach Larvalstadium, Spezialisierungsgrad des Herbivoren und je nach Kulturvarietät der Pflanze. Es wurden jedoch keine Kurzzeiteffekte der induzierten Abwehrlevel auf das Verhalten des Brassicaceen-Spezialisten *A. rosae* beobachtet. Ein breiteres Variationsspektrum in verschiedenen Pflanzenarten zeigte deutliche multivariate Assoziationen von multiplen Abwehrmechanismen auf. Die langfristigen Effekte auf Entwicklung und Präferenz von *A. rosae* wurden maßgeblich durch die mechanische Abwehr bestimmt. Nährstoffgehalt und Myrosinaseaktivität spielten daneben wichtige Rollen für die Gesamtqualität der Pflanzen als Wirte.

Publications and Conference contributions

Publications

- Travers-Martin, N. and Müller, C. (2007). Matching plant defense syndromes with performance and preference of a specialist herbivore. *Ecology*, submitted.

- Travers-Martin, N., Kuhlmann, F. and Müller, C. (2007). Revised determination of soluble and insoluble myrosinase activities in plant extracts. *Plant Physiology and Biochemistry*, submitted.

- Travers-Martin, N. and Müller, C. (2007). Specificity of plant responses in *Sinapis alba* L. and their effects on a specialist herbivore. *Journal of Chemical Ecology*, 33(8):1582-1597.

- Martin, N. and Müller, C. (2007). Induction of plant responses by a sequestering insect: Relationship of glucosinolate concentration and myrosinase activity. *Basic and Applied Ecology*, 8(1):13-25.

Conference contributions

- Müller, C. and Travers-Martin, N. (July, 2007). Matching plant defence syndromes with performance of a specialist herbivore. Poster presentation, 23^{rd} annual meeting of the International Society of Chemical Ecology in Jena, Germany.

- Martin, N. and Müller, C. (July, 2006). Specificity of plant responses: impacts of specialist vs. generalist feeding on the glucosinolate-myrosinase system in a Brassicaceae. Poster presentation, 22^{nd} annual meeting of the International Society of Chemical Ecology in Barcelona, Spain.

- Martin, N. and Müller, C. (October, 2005). Induction of plant defences by a sequestering insect. Oral presentation, 2^{nd} symposium of the Sonderforschungsbereich "Mechanismen der interspezifischen Interaktion von Organismen" in Würzburg, Germany.

- Martin, N. and Müller, C. (July, 2005). Induction patterns in a Brassicaceae evoked by herbivory of a sequestering insect: correlating glucosinolate concentration and myrosinase activity. Oral presentation, XVII International Botanical Congress in Vienna, Austria.

- Martin, N. and Müller, C. (February, 2005). Host range of a specialist herbivore: preference and performance of *Athalia rosae* (L.) (Hym.: Tenthredinidae) on native and alien Brassicaceae. Poster presentation, Graduate meeting "Evolutionary Chemical Ecology" of the "Deutsche Zoologische Gesellschaft" in Würzburg, Germany.

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Ehrenwörtliche Versicherung

Würzburg, den .....

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ferner erkläre ich, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat. Ich habe bisher noch keinen akademischen Grad erworben oder zu erwerben versucht.

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(Nora Travers-Martin)