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Evaluation of resistance mechanisms against *Delia radicum* L. and *Psylliodes chrysocephala* L. in brassicaceous accessions

Cuvillier Verlag Göttingen
Internationaler wissenschaftlicher Fachverlag

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Dissertation

zur Erlangung des Doktorgrades

der Fakultät für Agrarwissenschaften

der Georg-August-Universität Göttingen

vorgelegt von

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geboren in Hildesheim

Göttingen, März 2016

Bibliografische Information der Deutschen Nationalbibliothek
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.d-nb.de abrufbar.
1. Aufl. - Göttingen: Cuvillier, 2017
Zugl.: Göttingen, Univ., Diss., 2016

D 7

1. Referent:	Prof. Dr. Stefan Vidal
2. Korreferent:	Prof. Dr. Andreas von Tiedemann
Tag der mündlichen Prüfung:	22.04.2016

Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
Telefax: 0551-54724-21
www.cuvillier.de

1. Auflage, 2017
Gedruckt auf umweltfreundlichem, säurefreiem Papier aus nachhaltiger Forstwirtschaft.

ISBN 978-3-7369-9543-7
eISBN 978-3-7369-8543-8

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Chapter I

General Introduction

Oilseed rape production

Oilseed rape (*Brassica napus* (L.)) is the major oilseed crop in the European Union (EU); a total of 6.7 million hectares of oilseed rape were grown in the EU in 2014, 1.4 million hectares of which were grown in Germany (EUROSTAT 2015). Within the EU, winter oilseed rape is much more commonly grown (> 90% of total production) than spring oilseed rape (RAKOW 2011). Although oilseed rape has high yield potential, pests such as insects can cause significant yield losses and indeed, are the largest challenge in oilseed rape production worldwide (AHUJA *et al.* 2009; WILLIAMS 2010). Over the last decades, six insect pest species have been considered to be of major economic importance for winter oilseed rape: the cabbage stem flea beetle (*Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae)), the rape stem weevil (*Ceutorhynchus napi* (Gyll.) (Coleoptera: Curculionidae)), the cabbage stem weevil (*Ceutorhynchus pallidactylus* (Marsh.) (Coleoptera: Curculionidae)), the pollen beetle (*Brassicogethes aeneus*, syn. *Meligethes aeneus* (Fab.) (Coleoptera: Nitidulidae)), the cabbage seed weevil (*Ceutorhynchus obstrictus* (Marsh.) (Coleoptera: Curculionidae)) and the brassica pod midge (*Dasineura brassicae* (Winn.) (Diptera: Cecidomyiidae)) (WILLIAMS 2010). The cabbage root fly (*Delia radicum* (L.) (Diptera: Anthomyiidae)) has also increasingly become a significant pest in oilseed rape production within the last two decades (ALFORD *et al.* 2003; ERICHSEN & HUNMÖRDER 2005). Due to the damage that can potentially be caused by herbivores, chemical insecticides are commonly applied to protect plants. As a result of the intensive use of broad-spectrum insecticides, mainly pyrethroids, resistant populations of *M. aeneus* have been found in many European countries (SLATER *et al.* 2011); first populations of *P. chrysocephala* and *C. obstrictus* with a reduced sensitivity towards the pyrethroids have also been reported (HEIMBACH & MÜLLER 2013; HØJLAND *et al*. 2015).

Widespread, already in autumn damaging pest species, are *P. chrysocephala* and *D. radicum* (ALFORD *et al.* 2003; CAPINERA 2008). These species immigrate to rape fields shortly after the plants become established. In years with unfavourable growing conditions, the feeding activity of the *P. chrysocephala* and *D. radicum* larvae may considerably reduce plant density, which can lead to substantial economic losses (DOSDALL *et al.* 2000; ALFORD *et al.* 2003). From 2001 to 2013, neonicotinoid insecticides used for seed coating reduced the infestation of *P. chrysocephala* and *D. radicum* in oilseed rape plants in Germany. Moreover, pyrethroids are registered for spray applications that protect against *P. chrysocephala* adults and their larvae. For *D. radicum*, however, there is no chemical

control other than the seed coating effective to reduce the plant damage (ERICHSEN 2006). Since the use of neonicotinoids as a seed treatment in oilseed rape was strongly restricted by the EU in 2013 (BAROSO 2013), there is an urgent need to develop alternative control strategies for both *D. radicum* and *P. chrysocephala*.

Integrated pest management (IPM) may improve the efficiency, profitability and environmental acceptability of oilseed rape production and thereby, contribute to sustainable crop production systems (WILLIAMS 2010). Indeed, IPM "is an ecological approach to managing insect pests, by using different pest control methods, that are aimed at the entire pest complex of a crop ecosystem and finally ensures high quality agricultural production in a suitable, environmentally safe and economically sound manner" (BAJWA & KOGAN 2002). Especially cultivars that are resistant and tolerant to *D. radicum* and *P. chrysocephala* attacks may be an alternative to the problematic chemical approaches that promote the development of pest resistance and adversely affect the environment as well as non-target organisms like polyphagous predators and parasitoids (WILLIAMS 2004).

Cabbage stem flea beetle

The cabbage stem flea beetle (*Psylliodes chrysocephala* (L.)) (Coleoptera: Chrysomelidae) is located throughout the maritime regions of northern Europe (ALFORD *et al.* 2003). In late August to early September, shortly after seedling emergence the adult beetles invade into the oilseed rape fields (ALFORD *et al*. 2003) (Fig. 1).

The females feed for approximately two weeks (maturity feeding) before they begin to deposit their eggs in the soil (NUSS 2004). Egg-laying may continue throughout autumn and winter if weather conditions are favourable (high humidity and 4-16°C) (SCHULZ 1985). A developmental threshold of around 5°C has been determined for life stages (MATHIASEN 2015). The neonate larvae bore into the petioles of plants close to the nodality and burrow into the pith tissue (GODAN 1950). When the weather conditions are mild, the larvae continue to feed throughout the winter and during their development they move to younger leaves and later, into the stems (SCHULZ 1985). Mature thrid instar larvae leave the plant to pupate in the soil (DOBSON 1960). New generation adults emerge in late spring (May-July) and, before they begin to aestivate (summer diapause), they feed on the leaves of ripening oilseed rape plants, thereby causing no economic feeding damage. The cabbage stem flea beetle is univotile (ALFORD *et al.* 2003).

Feeding of post-aestivation adults on the emerging winter oilseed rape causes characteristic holes in cotyledons and young leaves. Severe feeding by the adults may lead to the death of seedlings (WILLIAMS 2010). The feeding of larvae, however, is considered to be more dangerous and may result in growth reduction and wilting; plant losses may occur if the vegetation point is damaged or if water penetrates the plants via injuries caused by the larvae and freezes during the winter, subsequently causing the plant tissue to burst (SCHULZ & DAEBLER 1984). Injuries may also act as entry points for fungal phytopathogens such as *Leptosphaeria maculans* (Desm.) (anamorph *Phoma lingam*) (BROSCHEWITZ *et al.* 1993). The host plants of the oliophagous cabbage stem flea beetle include glucosinolate-containing plants, like *B. napus*, *Bassica rapa* (L.) and *Brassica oleracea* (L.), as well as resedaceous (*Reseda alba* (L.)) and tropaeolaceous (*Tropaeolum majus* (L.)) plants (BARTLET & WILLIAMS 1991).

Figure 1: Adult *P. chrysocephala* beetle (upper left); damage caused by beetle feeding (upper right); damage pattern of larvae (lower left); *P. chrysocephala* larvae feeding in petiole (lower right).

Cabbage root fly

The cabbage root fly (*Delia radicum* (L.) syn. *D. brassicae*) (Diptera: Anthomyiidae) feeds on brassicaceous crops throughout the northern hemisphere; plant damage is caused by *D. radicum* larvae feeding on the roots (CAPINERA 2008) (Fig. 2). Three to four generations occur in Germany annually, with the third generation being the most damaging to winter oilseed rape crops, as it coincides with a highly vulnerable developmental stage of the plants. Commonly, oviposition of the third generation begins during the second week of September (ERICHSEN 2006). After eclosion, the flies feed nonspecifically on nectar and pollen. The gravid females deposit batches of eggs (up to 10 eggs per batch) beneath the soil surface, near the base of the brassicaceous host plants (CAPINERA 2008). The eggs may even stick to the plants' hypocotyls (ZOHREN 1968). Each female lays 300-400 eggs during its life span (CAPINERA 2008). First instar larvae feed on the root hairs, whereas second and third instar larvae (maggots) feed on the tissue of the taproot (MCDONALD & SEARS 1992). Extensive damage to the lateral root system may limit the plant's water and nutrient uptake and if the taproot it detached, the plants may die (MCDONALD & SEARS 1992). Aboveground symptoms of larvae feeding on the plant roots include purpling and wilting of leaves (CAPINERA 2008). Furthermore, injured roots are more vulnerable to fungal pathogens like *Verticillium longisporum* (KEUNECKE 2009) and *Fusarium spp*. (GRIFFITHS 1986). While winter oilseed rape can tolerate a moderate infestation level of the third and fourth *D. radicum* generation, a high larval infestation in autumn combined with suboptimal growth conditions (e.g. drought) may result in significant economic yield losses (ERICHSEN & HUNMÖRDER 2005). Thus far, economic thresholds for the cabbage root fly are not available and it is evident that more research is needed to quantify the economic impact of different levels of cabbage root fly infestation.

Larval development requires about four weeks and subsequent pupation in the soil lasts roughly two weeks, unless it is interrupted by cold temperatures during the winter or heat in the summer (COAKER & FINCH 1971). For the majority of life stages, a developmental threshold of approximately 6°C has been ascertained (CAPINERA 2008). The pupae of the third or fourth generation remain dormant during the winter and emerge the following spring. The feeding of the first and second generation larvae in spring and early summer does not damage the winter oilseed rape plants, as the root system is sufficiently developed at these times. The host plant range of *D. radicum* comprises vegetable *Brassicas*, brassicaceous weeds and *B. rapa* as well as *B. napus* (CAPINERA 2008).

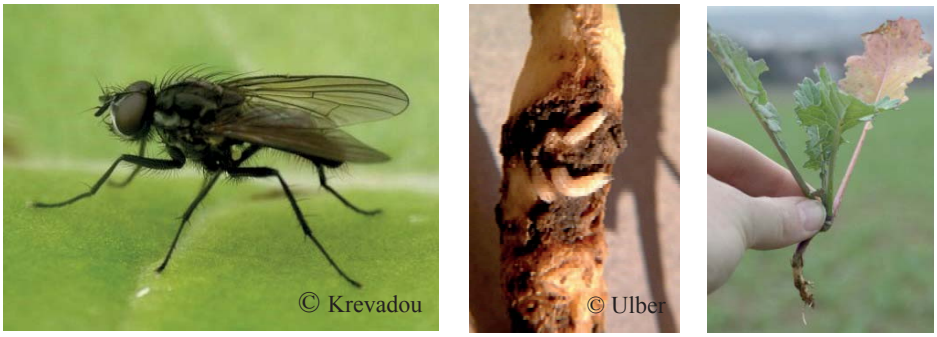

Figure 2: *D. radicum* adult (left); larvae feeding on roots (middle); oilseed rape plant with damage symptoms (right).

Plant resistance to insects

The aforementioned problems of pest control have shifted the focus to alternative measures for IPM and as a result, the use of cultivars that are resistant to pests and diseases should be a central part of any integrated crop management strategy (COOK *et al.* 2006), because they may reduce the current reliance on chemical insecticides (DOSDALL *et al.* 2000). Thus far, although cultivars of oilseed rape have been developed that are resistant to important fungal diseases such as *Plasmodiophora brassicae* (DIEDERICHSEN *et al.* 2009; FRAUEN 2011), few attempts have been made to breed cultivars that are resistant to insects (FRAUEN 2011). Moreover, these breeding programs often struggle to stay ahead of insect pest evolution (GOULD 1998). Host plant resistance to insect pests is generally based on reducing the host plant's attractiveness for colonization, feeding and egg deposition (antixenosis) and on the direct defence-responses and inhibitory effects of specific phytonutrients (antibiosis) (SCHOONHOVEN *et al.* 2005). Antixenosis is defined as "plant properties [that] evoke negative responses or total avoidance by insects" (SCHOONHOVEN *et al.* 2005), whereas antibiosis is characterized by reductions in the fecundity, longevity and development or by an increase in mortality of the pests and refers to plant properties that negatively affect the physiology of the herbivore (SCHOONHOVEN *et al.* 2005). Furthermore, a third defence mechanism is tolerance, which is described as the ability of a plant to support an insect infestation without loss of vigour and reduction of yield as compared to a susceptible plant (DENT 2000). Plants generally become resistant to insect pests through a combination of the defence-mechanism types (antixenosis, antibiosis and tolerance. Moreover, it is rare that the achieved degree of resistance to specific insect herbivores is complete; partial resistance is a more common phenomenon (DENT 2000;

SCHOONHOVEN *et al.* 2005). In contrast to complete resistance, partial resistance is expected to exert a lower selection pressure on insect populations and may therefore, more effectively counter the persistent adaptability of insects (SCHOONHOVEN *et al.* 2005).

Plant traits used for host selection and acceptance

The ability of an insect to detect a host plant relies on both biochemical and morphological plant traits (AHUJA *et al*. 2009). Concerning the biochemical plant traits in brassicaceous plants, volatiles and other secondary compounds such as glucosinolates, alkaloids and phenolics are discussed to influence the resistance of plants. Moreover, the effects of primary metabolites, like sugars are investigated. Additionally, morphological resistance traits like leaf colour, trichome density and epicuticular waxes may play a role in the insect-plant interaction (GATEHOUSE 2002; FÜRSTENBERG-HÄGG *et al*. 2013; HERVÉ 2014).

In addition to the direct defence of plants (plants produce physical barriers against herbivores or compounds that are repellent, antinutritive or toxic to herbivores (antixenosis and antibiosis menchanisms)), indirect defence mechanisms may protect plants against herbivory by attracting predators or parasitoids (FÜRSTENBERG-HÄGG *et al.* 2013). The defence traits employed by plants act as both constitutive "static" mechanisms through direct impairment (e.g. toxification or lower digestibility of plant tissue) and as inducible "active" mechanisms, which are accumulated in response to tissue damage by herbivores (GATEHOUSE 2002; AHUJA *et al.* 2009). In this study, we screen the direct antixenosis and antibiosis mechanisms of plants against the oilseed rape pests *P. chrysocephala* and *D. radicum.*

Biochemical plant traits: Numerous studies on insect-plant interactions have investigated the important role of the glucosinolates and their metabolites (AHUJA *et al.* 2009). Glucosinolates are a relevant group of secondary plant substances, which are only present in a limited group of plants. The most important family of plants containing glucosinolates are the *Brassicacea* (MITHEN *et al.* 2000). Approximately 30 different glucosinolates have been documented in brassicaceous species (FAHEY *et al.* 2001). All glucosinolates have a common chemical structure and consist of a beta-thioglucose moiety, a sulphonated oxime moiety and a variable side chain derived from an amino acid. Glucosinolates can be subdivided into three major classes according to their biosynthesis, i.e. aliphatic (derived from the amino acid methionine), indolic (derived from tryptophan)

and aromatic (derived from phenylalanin) (FEENY 1977). Different side chains result in differences in the biological activity of glucosinolates and their breakdown products, respectively (MITHEN 2001). Glucosinolates are stored in the cytoplasm of plant cells and damage to plant tissue (e.g. by herbivory) results in the enzymatic hydrolysis of glucosinolate molecules (via myrosinase) into glucose, aglucones and elementary sulphur. Depending on abiotic factors, like pH values and temperature, aglucones are further metabolized into isothiocyanates, nitriles and thiocyantes (MITHEN *et al.* 2000). The concentration and composition of glucosinolates within the plant tissue are controlled by various factors such as plant species and variety, plant organ, plant growth stage and environmental conditions (like sulphur and nitrogen availability) (MITHEN 2001; HOPKINS *et al.* 2009).

Glucosinolates may have a repellent effect on polyphagous pests (MOENS *et al.* 1992), but can act as cairomones for specialized insect herbivores and can trigger both feeding and oviposition (BARTLET *et al.* 1999a; BRUCE 2014). For specialised herbivores, glucosinolates are important host plant stimuli that, e.g. act as attractants to *P. chrysocephala* and *Phyllotreta spp.* (Coleoptera: Chrysomelidae) (LIBLIKAS *et al.* 2003; HENDERSON *et al.* 2004), as well as to *D. radicum* (ROESSINGH *et al.* 1992; FELKL *et al.* 2005), *C. obstrictus* (ULMER & DOSDALL 2006) and *D. brassicae* (BARTLET *et al.* 1999b). The role of glucosinolates as resistance factors has been documented numerous times (AHMAN 1993; GIAMOUSTARIS & MITHEN 1995; BARTLET 1996; UDDIN *et al.* 2009) and the negative effects of single glucosinolates have been demonstrated, e.g. on *C. obstrictus* (EICKERMANN *et al.* 2011), *Pieris rapae* (L.) (Lepidoptera: Pieridae) (AGRAWAL & KURASHIGE 2003) and *Phyllotreta spp*. (BODNARYK 1991). Nevertheless, the plant-insect interactions are evidently less influenced by the total amount of single glucosinolates than by their composition, e.g. the indolyl-aliphatic ratio. As the drastic reduction of the glucosinolate content in oilseed rape seeds of the 00-quality cultivars, however, did not change their attractiveness for specialized herbivores (WILLIAMS 1989; GIAMOUSTARIS & MITHEN 1995; BARTLET 1996).

In addition to glucosinolates, other secondary metabolites like several flavonoids have been found to act as feeding deterrents to insects (TREUTER 2006), for instance the flavonoid kampferol has been shown to reduce the larval performance of *C. obstrictus* (LEE *et al.* 2014). Moreover, phytoalexins (produced from flavonoids) have been documented to influence host plant selection of gravid *D. radicum* (BAUR *et al.* 1998).

Additionally, females have been shown to be strongly stimulated by a particular chemical surface compound (a complex tetracyclic carboxylic acid) (ROESSINGH *et al.* 1997) of *Brassica oleracea* leaves. Beside secondary metabolites, primary metabolites such as amino acids and sugars have been shown to affect host plant selection and utilization of insects (BERENBAUM 1995; HERVÉ *et al.* 2014). Sugars are especially phagostimulating to adults of *M. aeneus* (HERVÉ *et al.* 2014), *D. radicum* (HOPKINS *et al.* 1999) and *P. chrysocephala* (BARTLET *et al.* 1994). Moreover, the nutrient status of plants, particularly the nitrogen and sulphur content, may affect the host plant choice of insect pests (MARAZZI & STÄDLER 2005; RUSCH *et al*. 2013).

Morphological plant traits: Morphological plant traits such as leaf colour (SOUTHWOOD 1986; TANSEY *et al.* 2010a), wax layer (BODNARYK 1992; LAMBDON *et al.* 1998) or the pubescence of the leaves (SOROKA *et al*. 2011) have been shown to affect a plant's resistance to insects. The toughness of the plant tissue of below- and above ground parts may act as a physical barrier to chewing and sucking insects (FÜRSTENBERG-HÄGG *et al.* 2013). This morphological trait has been widely disregarded until recently. The compensatory regrowth of plant tissue of infested plants also influences the host plant-insect interaction (as reviewed in FÜRSTENBERG-HÄGG *et al.* 2013). Phenological factors, such as the growth stage of the plant, influences host plant selection (MCDONALD & SEARS 1992; SCHOONHOVEN *et al.* 2005), e.g. the infestation of *C. pallidactylus* on oilseed rape increases as the number of leaves per plant increases (EICKERMANN *et al.* 2011) and females of *D. radicum* were found to prefer larger plants for oviposition (MCDONALD & SEARS 1992;).

Current status of insect resistance breeding

In the continuous efforts to develop high-yielding oilseed rape varieties, the diversity related to defensive traits and resistance levels to herbivores might have been lost (SCHOONHOVEN *et al.* 2005). Rapeseed originated from the spontaneous hybridization of cabbage (*B. oleracea*) and turnip rape (*B. rapa*) and the breeding material currently in use might derive from only a small number of centuries-old interspecific hybrid plants (BECKER *et al.* 1995). While there is a relatively limited gene pool for *B. napus* (BROWN *et al*. 1997), the use of various resistance and quality traits from the progenitors and related plant species is a promising approach for breeding programs (GIRKE 2002). Differences in the susceptibility of brassicaceous species to insect attack are known and intensively studied for *D. radicum* (ELLIS *et al.* 1999; DOSDALL *et al.* 2000; JENSEN *et al.* 2002).

Whereas *B. napus*, *B. rapa* and *B. oleracea* are considered susceptible, a high level of resistance to insect attack is well documented for *S. alba* (RIPLEY & ARNISON 1990; BROWN *et al*. 1997; DOSDALL *et al.* 2000). For example, *S. alba* is reported to be widely resistant to attacks from of e.g. *C. obstrictus* (MCCAFFREY *et al.* 1999), *D. radicum* (DOSDALL *et al.* 2000) and exhibit a lower susceptibilty to flea beetles (*Phyllotreta spp*.) (BODNARYK 1991; GAVLOSKI *et al*. 2000) and *P. chrysocephala* infestation (DOERING 2012).

Concerning the development of pest-resistant cultivars in the last decade, progress has been made through the introgression of *S. alba DNA* into *B. napus* (DOSDALL & KOTT 2006; TANSEY *et al.* 2010b). In general, intergeneric hybridization is an effective means of broadening the genetic base and incorporating desired traits into cultivated *B. napus* (BROWN *et al.* 1997). To combine the seed quality and high yield of oilseed rape (*B. napus*) with the insect resistance of *S. alba*, progenies of intergeneric hybridization (*S. alba* x *B. napus*) were created (BROWN *et al.* 1997; DOSDALL & KOTT 2006). To overcome the reproductive barriers of *S. alba* and *B. napus*, "embryo rescue" was applied to the desired crosses (RIPLEY & ARNISON 1990). In this process, individual embryos were collected after crossing (before being aborted) and reared on artificial media. The process was followed by backcrosses with *B. napus* and the production of doubled haploid lines (RIPLEY & ARNISON 1990; SHARAMA *et al.* 1996).

In earlier investigations, introgressions of *S. alba* into *B. napus* carried genes for resistance to *C. obstrictus* from the *S. alba* parent into *B. napus* (DOSDALL & KOTT 2006). In field trials, 20 out of 230 introgression lines exhibited high levels of resistance to *C. obstrictus.* The females deposited less eggs (antixenotic resistance) and the larval development was prolonged (antibiotic resistance) (DOSDALL & KOTT 2006). In field experiments, several introgression lines were also less infested by root maggots (*Delia spp*.) (KOTT & DOSDALL 2004). *S. alba's* resistance to *D. radicum* attacks was also successfully transferred to vegetable rutabaga (*B. napus var. napobrassica* (L.)) (MALCHEV *et al.* 2010). A number of introgression lines (*S. alba* x *B. napus*) were moreover found to be resistant to *Phyllotreta spp*. attacks (DOSDALL *et al.* 2000), however no information about the resistance of introgression lines to infestation by *P. chrysocephala* is available.

Objectives

The aim of this study is to increase the knowledge on host plant selection and suitability as well as mechanisms of resistance of various brassicaceous accessions against two major insect pests of oilseed rape. We screened a total of 26 introgression lines (*S. alba* x *B. napus*), three *S. alba* cultivars and three *B. napus* cultivars for susceptibility towards cabbage stem flea beetle (*P. chrysocephala*) and cabbage root fly (*D. radicum*) infestations.

Specifically, we addressed:

- Host plant acceptance related to the oviposition of the cabbage root fly and feeding of the cabbage stem flea beetle.
- Host plant suitability for the larval performance of the cabbage root fly and the cabbage stem flea beetle.
- Mechanisms of host plant resistance (biochemical and morphological) that affect host plant-pest interactions.

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Chapter II

Screening of *Brassica napus*, *Sinapis alba* and introgression lines for antixenotic resistance to oviposition by cabbage root fly (*Delia radicum* L.)

Abstract

The cabbage root fly (*Delia radicum* (L.)) is a serious insect pest in European oilseed rape (*Brassica napus* (L.)) production. Plant damage occurs as a result of larvae feeding on the root tissue of young plants. The successful development of *D. radicum* larvae is dependent upon the host plant choice made by gravid females. As a component of an integrated management strategy, breeding for host plant resistance to *D. radicum* is a promising approach. The brassicaceous species *Sinapis alba* (L.) has been known to exhibit high levels of resistance to *D. radicum*. In a multi-choice oviposition screening under greenhouse conditions, two susceptible *B. napus* cultivars, two resistant *S. alba* cultivars and six introgression lines (*S. alba x B. napus*) were screened for their attractiveness to oviposition by gravid females of *D. radicum*. We found that a lower number of eggs was deposited on the introgression lines IL_140 and IL_165 in comparison to the standard *B. napus* cultivar Fenja. The *S. alba* cultivars, however, were as attractive for oviposition as the *B. napus* cultivars, indicating no antixenotic resistance of *S. alba* to females of *D. radicum.* Additionally, our results revealed that a plant's acceptability for oviposition of the cabbage root fly was highly mediated by their stem base diameter.

Therefore, antixenotic resistance mechanisms are not accountable for the low susceptibility of *S. alba* to *D. radicum* and antibiosis appears to be the stronger mechanism of resistance to *D. radicum* attacks.

Introduction

The cabbage root fly (*Delia radicum* (L.)) (Diptera*: Anthomyiidae*) (CRF) is an important pest of brassicaceous vegetables (CAPINERA 2001) and is increasingly damaging crops of winter oilseed rape (*Brassica napus* (L.)) (ALFORD *et al.* 2003). The damage is caused by larvae feeding on the root tissue of young plants (ALFORD *et al*. 2003; CAPINERA 2008). Three to four generations of the CRF occur annually in Germany, with the third generation being the most harmful to winter oilseed rape crops. Commonly, oviposition of the third generation begins during the second week of September (ERICHSEN 2006). Adult females deposit their eggs in batches near the hypocotyl and hatching larvae develop over the course of three stages while feeding on the root tissue, before pupating in the soil (MCDONALD & SEARS 1992; CAPINERA 2008).

Recent control strategies for the CRF have focused on the use of insecticides via seed coating with neonicotinoids to avoid severe larval damage of young oilseed rape

plants in autumn (ERICHSEN 2006). Since 2013, the use of neonicotinoids as seed treatments in oilseed rape has been suspended in the EU (BAROSO 2013). Consequently, alternative strategies for controlling the CRF are urgently needed. Although host plant resistance to insects may be an important component of integrated pest management (IPM) systems, no cultivars have yet been produced that are resistant to insect attack (COOK *et al.* 2006; FRAUEN 2011). Breeding oilseed rape cultivars that exhibit high levels of antixenotic resistance to the CRF may be an alternative to chemical plant protection. Antixenotic plant defence is generally based on plant traits that deter insects from colonization, feeding and oviposition (SCHOONHOVEN *et al.* 2005).

A theory widely used to explain the host plant choice of insect herbivores is the preference-performance hypothesis, also known as the "mother knows best" principle (VALLADARES & LAWTON 1991; JOHNSON *et al.* 2006). This theory is based on the assumption that larvae of insect herbivores are limited in their mobility and therefore, females have to choose the optimal host plant for survival and development of their offspring. By promoting antixenotic resistance traits in plants that deter females from oviposition, or by eliminating "token stimuli" for oviposition, infestation and the subsequent damage to plants might be reduced.

The search for an oviposition site by the female flies can be subdivided into two phases: pre-alighting and post-alighting behaviour (HOPKINS *et al.* 1999). In the pre-alighting phase, the females find a host using olfactory (KERGUNTEUIL *et al.* 2014) and visual stimuli, since the adult female is able to detect plants due to colour, size and pattern of leaves (ROESSINGH & STÄDLER 1990). In this context, it has been shown that the number of eggs laid by the CRF is strongly dependent on the growth stage of plants, as females prefer to oviposit on host plants that are in the fourth or fifth leaf stage (MAACK 1977; MCDONALD & SEARS 1992). In the subsequent post-alighting behaviour, further sensory information, especially olfactory and mechano-sensory cues, lead to host acceptance or rejection (HOPKINS *et al.* 1999). Chemical compounds present in the leaf surface, such as glucosinolates and their hydrolysis products, also provide important stimuli to *Delia* flies after landing (ROESSINGH *et al.* 1992). In addition to chemical cues, physical stimuli via morphological plant characteristics (such as leaf structures) have been reported to affect host plant acceptance by adult females (ROESSINGH & STÄDLER 1990; FÜRSTENBERG-HÄGG *et al.* 2013). Insects that prefer brassicaceous crops, such as the CRF, rely on their attraction to glucosinolates and their volatile breakdown products during host plant

selection and host plant acceptance (HOPKINS *et al.* 1999). After the decision to accept or reject a plant for oviposition, CRF females perform a fixed sequence of behavioural patterns, as described by ZOHREN (1968). In addition to host plant quality, the soil substrate itself should fulfil females' requirements for oviposition, e.g. with regard to particle size and conditions of humidity (KOSTAL *et al.* 2000). During the described process of host plant selection, antixenosis is the operating mechanism of resistance if the fly is deterred by the biochemical and morphological plant characteristics (SCHOONHOVEN *et al.* 2005).

In former investigations, *S. alba* was found to be highly resistant to CRF attacks (MCDONALD & SEARS 1992; KOTT & DOSDALL 2004) and researchers attempted to transfer resistance traits from *S. alba* into *B. napus* via embryo rescue and backcrossing into *B. napus* (RIPLEY & ARNISON 1990; BROWN *et al.* 1997). For a number of the resulting intergeneric hybrids (*S. alba* x *B. napus*), the root damage by CRF larvae was reduced to the level of the resistant *S. alba* parent and thus, KOTT & DOSDALL (2004) assumed that resistance genes were successfully transferred from the *S. alba* parent to these hybrids. To verify the genetic inheritance of resistance in intergeneric hybrids, two quantitative trait loci (QTLs) were identified that explain approximately 50% of resistance (EUKERE *et al.* 2005). The ideas concerning the resistance mechanisms employed by *S. alba* plants are inconsistent: DOSDALL *et al.* (1994) have concluded that antixenosis is the major mechanism of resistance in *S. alba* to infestation by CRFs, whereas JYOTI *et al.* (2001) have emphasized the importance of antibiosis for the reduced susceptibility of *S. alba* to CRF attacks. In Canada, the evaluation of intergeneric hybrids and introgression lines, respectively, for resistance to the CRF has mainly been performed under field conditions by scoring the larval damage on roots (DOSDALL *et al.* 2000; EUKERE *et al.* 2005). However, evaluating plant resistance based on the damage done by herbivores under field conditions is not sufficient to completely identify the plant traits that affect insects or any effects on the insect itself (DENT 2000; HERVÉ 2014). Until recently, there has only been limited information about plant traits that deter female CRFs from oviposition.

In this study, a multi-choice experiment was performed under laboratory conditions to evaluate differences in the suitability of several brassicaceous accessions for oviposition by female CRFs. The following major questions were addressed:

1) Are there differences in the host plant preference of the CRF between *S. alba*, *B. napus* and the introgression lines?

2) Is the resistance of *S. alba* based on a lower attractiveness for CRF oviposition?

3) Which plant traits are associated with the number of deposited eggs?

Potential antixenotic effects of brassicaceous accessions were assessed using the number of eggs deposited per plant, focusing especially on the relationship between the number of eggs deposited by female flies and morphological plant traits, i.e. stem base diameter and leaf size.

Material and Methods

Insects: A *D. radicum* (CRF) population from a laboratory colony maintained in the Department of Agroentomology (Goettingen) and mass reared on roots of swede (*Brassica napus* (L.) var. *napobrassica*) was used for the experiments. Adults were reared at 20°C, with 65-80% relative humidity and under natural light conditions in insect rearing cages (30 cm x 30 cm x 30 cm). Adult flies were fed a diet of dry food consisting of dextrose, skim milk powder, soy flour and brewer's yeast (10:10:1:1 ratio) and wet food consisting of honey, soy flour and brewer's yeast (5:1:1 ratio) [ratios in g:g]. Water was additionally supplied.

Experimental setup: A total of 10 brassicaceous accessions were tested: 2 cultivars of oilseed rape (*B. napus*) (Fenja, Visby), 2 cultivars of white mustard (*S. alba*) (Base, Martigena) and 6 introgression lines (*S. alba* x *B. napus*). The *B. napus* cultivars were chosen because of their importance in commercial winter oilseed rape production (Visby) and their growing type (spring oilseed rape: Fenja). The introgression lines used in this experiment were selected due to their varying attractiveness for the oviposition of female cabbage root flies in comparison to the standard oilseed rape cultivar Fenja. The data were obtained by screening of a total of 26 introgression lines in preceding multi-choice oviposition experiments.

The test plants were sown and raised in a soil substrate consisting of potting soil (Fruhsdorfer Erde, Typ 25), loamy soil and sand in a 2:1:1 ratio [vol:vol]. Based on previous investigations, the seeding dates of single brassicaceous accessions were shifted (max. 5 days) to standardize the phenology of the test plants. One week following germination, the plants were transplanted individually into 11 cm wide plastic pots. All plants were grown in a greenhouse with a photoperiod of 16:8 h (L:D), using artificial light (7000 lux). Beginning at the four leaf stage, plants were fertilized weekly with 75 ml of 0.2% HakaPhos Blau® (15% N, 10% P_20_5, 15% K_20). Host plant suitability of accessions

for oviposition and parameters of plant characteristics were assessed on six replicate plants per accession, respectively. Plants were moreover grown to determine the trichome density on the leaf lamina of five replicate plants per accession.

Oviposition screening: The multi-choice oviposition experiment began once the brassicaceous accessions reached the four leaf stage, because this growth stage is known to be highly attractive to the CRF (MAACK 1977). For each plant, the potting substrate was covered by a polyethylene plastic film tightly surrounding the plant stem (Fig. 1). Sieved, washed and dried sand with a grain size ≤ 2 mm was used as a top layer (2 cm) to supply a standardized substrate for oviposition.

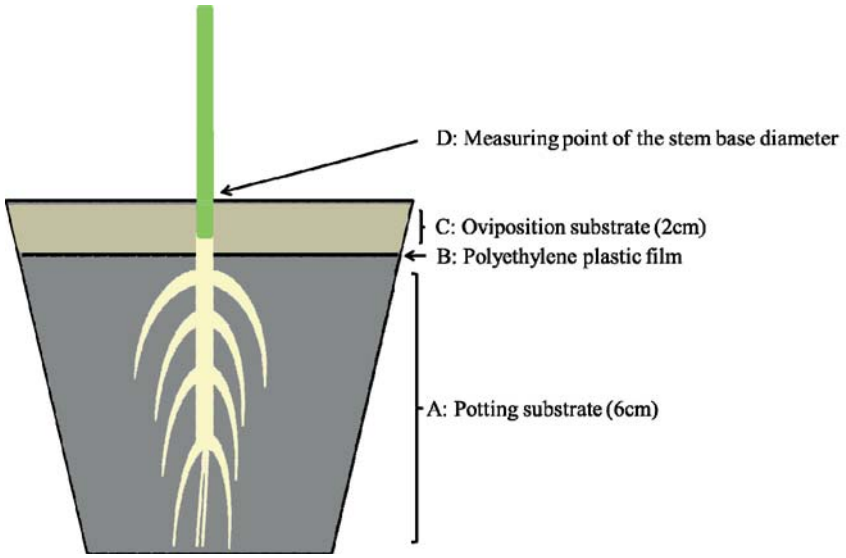

Figure 1: Side view of an experimental plant for the cabbage root fly (*Delia radicum* (L.)) oviposition screening.

A total of 60 plants (10 accessions, 6 replicated plants per accession) were arranged in a randomized complete block design within a gauze cage (SeranPVDC, mesh width 425 µm) measuring 4.0 m × 2.0 m × 1.8 m. The distance between the plants was 20 cm. At the start of the experiment, 160 flies (80♀: 80♂, six to seven days old) were released into the gauze cage and were allowed to mate and oviposit. All flies had no previous experience with plants. Every 48 h the top layer of sand of each plant that contained eggs was poured into labelled dishes; eggs were subsequently extracted from the sand by flotation and counted. The sand layer of each plant was continuously replaced every 48 h until the end of the experiment and plants were repeatedly exposed to the same adult flies. After 10 days, the experiment was terminated.

During the experiment adult flies were additionally fed a diet of dry food and wet food as previously described. Water was additionally supplied.

Plant characteristics: To characterise the development of the test plants, measurements were recorded for the stem base diameter, the leaf size and the trichome density. The stem base diameter (mm) of each plant was measured at the start of the experiment as well as at the end of the experiment (Fig. 1) and the mean hypocotyl diameter was calculated for the measurements from both dates. To measure the leaf lamina area (cm²) of the third and fourth leaf of each plant at the end of the experimental period, an area meter (LI-3100C, LI-COR Inc., USA) was used. Moreover, the length (cm) of these leaves' petioles were recorded. The trichome density (number of trichomes/cm²) was assessed for five leaf discs per lamina of 1 cm², which were excised from the third and fourth leaf of each plant using a cork borer. The number of trichomes was determined from the upper surface of the leaf discs using a stereo microscope (Stemi 2000-C, Zeiss, Germany).

Statistical analysis: The effect of accession on the total number of CRF eggs deposited, basal stem diameter, size of the leaf lamina, petiole length and trichome density (number of trichomes/cm²) was tested using a mixed model analysis of variance (ANOVA) (PROC Mixed) (SAS 9.4, SAS Institute, USA). Normality of data and homogeneity of variance were checked by inspecting residuals (QQ-plots). All data were square root transformed to meet the assumption of the statistical models. The level of significance was set at a confidence interval of 95%. To identify differences between accessions, an a-posteriori Tukey test was used and p-values of multiple comparisons were adjusted, in accordance with Tukey. Spearman's rank-order correlations were used to analyse the relationship between individual plant traits (i.e. basal stem diameter, area of the leaf lamina, petiole length and trichome density) and the total number of eggs deposited. A stepwise multiple regression analysis was performed to determine the impact of the plant traits on the total number of eggs deposited. As just one plant trait was included in the regression, the best-fitting factor is presented in a linear regression with the dependent variable.

Results

Oviposition: Due to the huge variation in the number of eggs deposited per plant for single periods of 48 h, egg numbers of the experimental period of ten days were pooled for statistical analyses. The total number of eggs deposited by *D. radicum* (CRF) females differed significantly between accessions (F 4.14, $p < 0.001$). Female flies laid significantly more eggs on plants of the introgression lines IL_114 and IL_175 than on plants of the introgression line IL_165 and the oilseed rape cultivar Visby, respectively (Fig. 2). Two of the introgressions received clearly more eggs than the standard oilseed rape cultivar Fenja (31.50 ± 2.71), whereas the number of eggs laid by females was lower on plants of two introgression lines, the oilseed rape cultivar Visby and the *S. alba* cultivars (Martigena and Base) than on plants of the standard cultivar Fenja (all results are not significant). Furthermore, no significant differences were found between the number of eggs laid in the *S. alba* cultivars and the number of eggs laid in the introgression lines. Moreover, the number of eggs did not significantly differ between plants of the *S. alba* cultivars and the standard oilseed rape cultivar Fenja.

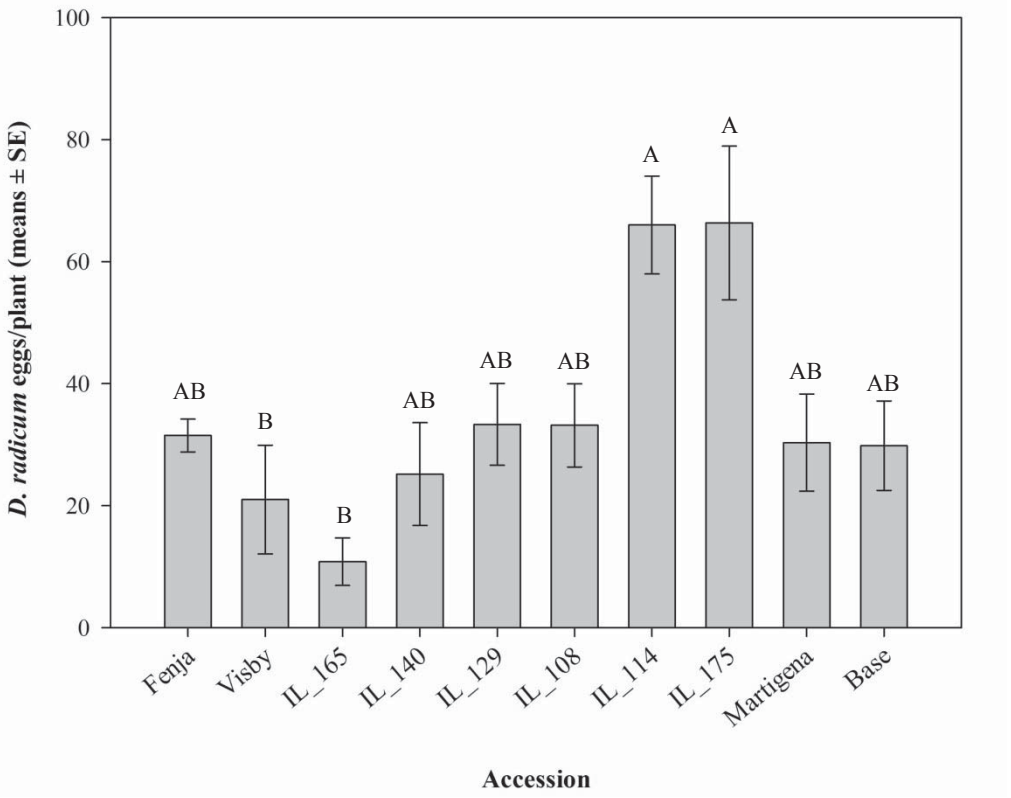

Figure 2: Number of *D. radicum* eggs laid on plants of test accessions in the multi-choice oviposition experiment. Different letters indicate significant differences between accessions (ANOVA, Tukey test, $p \leq 0.05$). Data are presented as arithmetic means ± SE.

Plant characteristics: The stem base diameter varied significantly between accessions (F 8.46, $p < 0.001$) (Tab. 1) and was significantly larger in introgression lines IL_175, IL_108 and IL_114 than in IL_140 and IL_165. Furthermore, the stem base diameter of the introgression line IL_165 was significantly smaller than that of the standard oilseed rape cultivar Fenja. The largest stem base diameter was recorded for plants of the introgression line IL_175 (4.88 mm ± 0.21 mm). There were no differences in stem base diameter between accessions and the two *S. alba* cultivars.

The leaves of *S. alba* were pinnate, whereas the leaves of the *B. napus* cultivars were planar undivided. The leaf shapes for all of the introgression lines were similar to *B. napus* leaves. The area of the lamina of the third and fourth leaf differed significantly between accessions (F 35.50, $p < 0.001$) (Tab. 1), as values of the introgression line IL_140 and the *S. alba* cultivars were significantly smaller compared to the standard cultivar Fenja. Furthermore, the lamina of the introgression line IL_140 was significantly smaller than the other accessions. The petiole length also differed significantly between accessions (F 26.98, $p < 0.001$) (Tab. 1), with significantly shorter petioles found on the introgression lines IL_140 and IL_108 and both *S. alba* cultivars than on the standard oilseed rape cultivar Fenja.

Table 1: Plant characteristics of the test accessions screened in the multi-choice oviposition experiment. Different letters within columns indicate significant differences (ANOVA, Tukey test, $p \leq 0.05$). Data presented as arithmetic means ± SE.

Accession	Basal stem diameter (mm) (means ± SE)	Lamina area (cm²) (means ± SE)	Petiole length (cm) (means ± SE)	Trichomes/cm² (means ± SE)
Fenja	3.92 (± 0.14) BC	66.13 (± 3.43) AB	8.12 (± 0.63) AB	0.16 (± 0.04) CDE
Visby	3.54 (± 0.16) BCD	61.86 (± 2.80) AB	7.85 (± 0.20) AB	1.44 (± 0.43) B
IL_165	3.04 (± 0.14) D	54.95 (± 1.76) BC	7.41 (± 0.30) BC	0.50 (± 0.01) BCDE
IL_140	3.29 (± 0.12) CD	43.65 (± 4.04) CD	6.56 (± 0.24) CD	0.32 (± 0.22) CDE
IL_129	3.75 (± 0.18) BCD	62.03 (± 2.41) AB	7.87 (± 0.19) AB	0.04 (± 0.04) E
IL_108	4.17 (± 0.08) AB	75.89 (± 2.31) A	8.71 (± 0.15) A	0.70 (± 0.19) BC
IL_114	4.25 (± 0.14) AB	68.66 (± 1.21) AB	8.28 (± 0.21) AB	0.60 (± 0.18) BCD
IL_175	4.88 (± 0.23) AB	73.44 (± 3.89) A	8.55 (± 0.26) A	0.02 (± 0.01) DE
Martigena	3.54 (± 0.21) BCD	23.86 (± 1.27) E	4.87 (± 0.21) E	10.40 (± 0.95) A
Base	3.67 (± 0.10) BCD	36.90 (± 2.80) D	6.05 (± 0.24) D	9.10 (± 0.78) A

The trichome density on the lamina surface differed significantly among accessions (F 74.39, $p < 0.001$) (Tab. 1) and was significantly less for the standard cultivar Fenja than for the *S. alba* cultivars and Visby. We found only low numbers of trichomes for 5 out of 10 accessions (≤ 0.5 trichomes/cm²). The introgression lines IL_108, IL_165 and the oilseed rape cultivar Visby had an intermediate trichome density (from 0.6 to 1.5

trichomes/cm²), whereas the *S. alba* cultivar Martigena and Base exhibited a distinct hairiness (> 9 trichomes/cm²).

Correlation and multiple regression analysis: The correlation analysis revealed a significant, positive interaction between the stem base diameter and the number of eggs deposited within the experimental period of 10 days (r = 0.88, p = < 0.001). Furthermore, the number of eggs was positively correlated with petiole length (r = 0.62, p = 0.054) and lamina area (r = 0.62, p = 0.054). No significant correlation was found between trichome density and the number of eggs deposited by females (r = -0.42, p = 0.229). Stepwise multiple regression analyses demonstrated that the number of eggs laid by *D. radicum* females was significantly affected by the stem base diameter of plants (adj. R^2 = 0.76); neither the petiole length nor the lamina area were included as significant factors in the regression model. The number of *D. radicum* eggs deposited per plant was significantly positive depending on the basal stem diameter (Fig. 3).

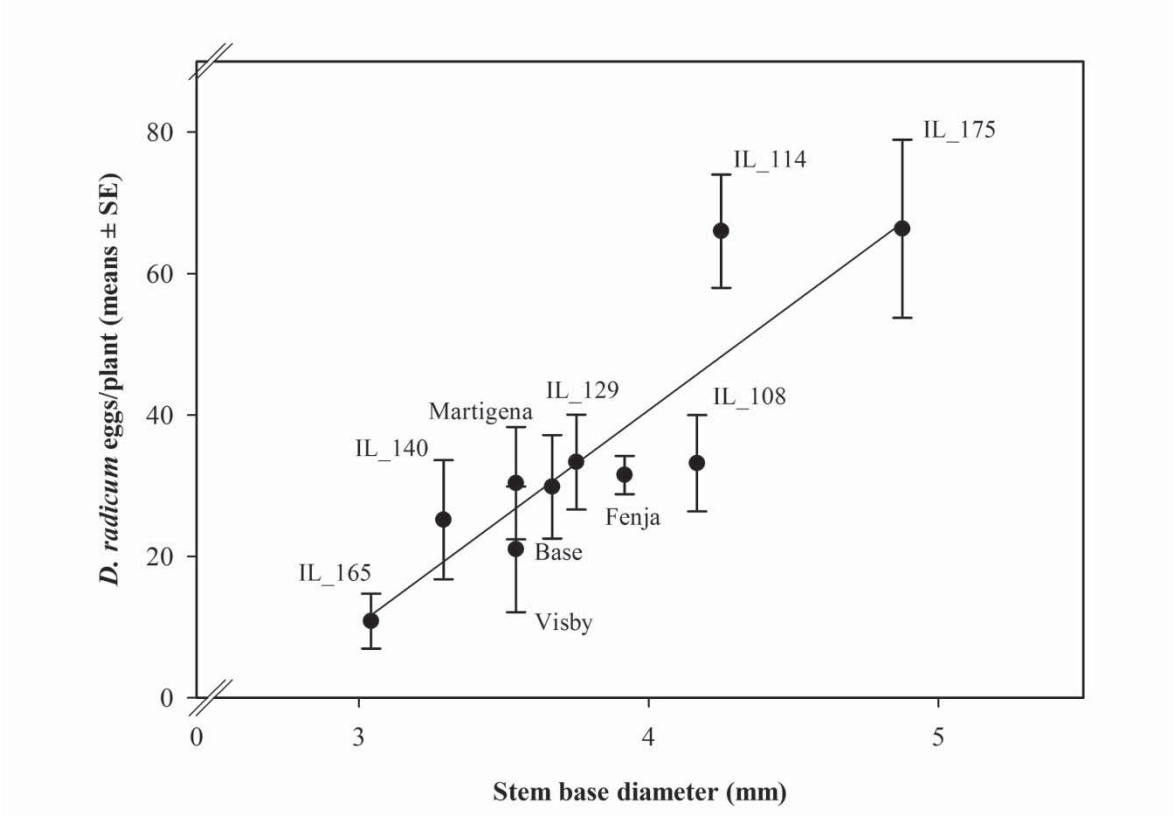

Figure 3: Relationship between the number of *D. radicum* eggs/plant of the 10 brassicaceous accessions tested in the multi-choice experiment and the basal stem diameter. Linear regression model: (y = - 80.421+ 30.275x, F 30. 45, p < 0.001, adj. R^2 = 0.76).

Discussion

The results of our multi-choice experiment reveal that the *S. alba* cultivars were as attractive as the *B. napus* standard cultivar Fenja to CRF females. The experiment further indicates that the stem base diameter strongly influences the selection and acceptance of plants for oviposition by CRFs. Less eggs were deposited on the introgression lines IL_140 and IL_165 than on the *B. napus* standard cultivar Fenja.

Due to the fact that several plant types can be simultaneously offered, multi-choice experiments are a useful tool to assess plants' suitability for herbivorous insects (e.g. host selection for oviposition) (BARTLET & WILLIAMS 1991; DEGEN 1999). Females are able to examine alternatives for oviposition and may select a host plant according to their preferences, with the number of eggs deposited on one accession depending on the attractiveness of other accessions. Multi-choice bioassays, however, do not reflect field situations in which only one cultivar is available as a potential host plant (SCHOONHOVEN *et al.* 2005). Therefore, no-choice experiments, where just one accession is available, are more closely related to field conditions, but would exclusively demonstrate distinct differences in suitability between host plants (FARELL 1977). Consequently, no-choice or even dual-choice experiments provide information about differences in the attractiveness if an exceptionally susceptible plant genotype is incorporated into the screening (DEGEN 1999). Multi-choice tests may also reveal minor differences in the attractiveness of different host plants.

The highest number of eggs was recorded for plants of the introgression lines IL_175 and IL_114, with twice as many eggs deposited on these introgression lines than on the standard oilseed rape cultivar Fenja (all results were not significant). Conversely, the lowest numbers of eggs were found on the introgression lines IL_165 and IL_140 as well as on the oilseed rape cultivar Visby. These results are in accordance with previous dual-choice experiments, as CRF females deposited significantly more eggs on plants of the standard cultivar Fenja (255.00 ± 15.02 eggs) than on plants of the introgression lines IL_140 (135.75 ± 32.59 eggs) ($n = 5$, Wilcoxon-Test; $p = 0.043$) within 72 h (HENNIES 2015, unpublished data). Unexpectedly, the number of eggs deposited on plants of both "resistant" *S. alba* cultivars was almost comparable to the standard Fenja and two of the introgression lines. On the one hand, this observation is in accordance with ELLIS *et al.* (1999), who reported that several *Brassica* species were equally attractive to oviposition by CRFs (no differences in antixenotic resistance), but varied in host quality for the larvae

(antibiotic resistance). On the other hand, our findings that *S. alba* is suitable for the oviposition of CRFs contradicts the observation of former studies that have documented antixenotic resistance as the main resistance factor to CRF attacks (DOSDALL *et al.* 1994; JYOTI *et al.* 2001). If we assume that antibiosis to larvae will be the operating resistance mechanism in brassicaceous species (also in *S. alba*), our results are inconsistent with the preference-performance hypothesis, as the females oviposited on plants that will not be a suitable host for the development of their offspring.

A plant's development stage has been shown to be an important factor in determining the host plant choice of CRF females for oviposition, as plants in the fourth and fifth leaf stage are highly attractive (MAACK 1977; MCDONALD & SEARS 1992). According to ROESSINGH & STÄDLER (1990), females prefer larger leaves over smaller leaves and we also found a positive relationship between leaf size and number of eggs deposited. The shape of leaves may also influence host selection by *D. radicum*. DEGEN & STÄDLER (1996) reported that models of pinnate leaves were more attractive for oviposition by the carrot fly (*Psila rosae* (F.) (Psilidae: Chamaepsila)) than simple leaves, but found the leaf shape to be of minor importance for the CRF. Leaves of *S. alba* were pinnate, whereas the leaves of the *B. napus* cultivars were planar undivided. The leaf shape of all introgression lines were similar to that of the *B. napus* leaves. We found differences in the number of eggs per plant among these introgression lines, but not principally in comparison to the *S. alba* cultivars. Consequently, we assume that the leaf shape does not affect the suitability of plants for oviposition by *D. radicum*.

Morphological structures, such as trichomes, may also influence the antixenotic resistance level of plants (DALIN *et al.* 2008). It has been demonstrated that a higher density of trichomes impedes the feeding activity of adult *Phyllotreta spp*. (Coleoptera: Chrysomelidae) on brassicaceous crops (SOROKA *et al.* 2011). JOYTI *et al.* (2001) have found that the trichomes on *S. alba* plants deter CRF oviposition. In our study, however, we could not detect a negative influence of pubescence on host plant choice by female flies, as there was no interaction between this morphological plant trait and the number of eggs deposited. Our results demonstrate that the stem base diameter is the most important factor for host plant choice and indeed, explains 76% of the variation in the number of eggs deposited per plant. The potential impact of the stem base may be evident during the process of host plant evaluation when females move down the stem and start circling

around the stem base before depositing the eggs in the soil, as described by ZOHREN (1968).

Our multiple-choice experiment did not detect any strong antixenotic resistance source within the set of introgression lines tested. In contrast to our expectations, the cultivars of *S. alba* exhibited no antixenotic resistance to oviposition by *D. radicum* females. We further found that the stem base diameter strongly affects the host plant choice of the CRF. Further investigations of potential mechanisms involved in antixenotic resistance should notably concentrate on biochemical plant traits as oviposition stimulants or deterrents, e.g. the content and composition of volatile organic substances in the leaf surface, which were beyond the scope of our study.

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Chapter III

Screening of *Brassica napus*, *Sinapis alba* and introgression lines for antibiotic resistance to cabbage root fly larvae (*Delia radicum* L.)

Abstract

The cabbage root fly (*Delia radicum* (L.)) is considered one of the most important autumn pests in European oilseed rape production (*Brassica napus* (L.)). Plant damage occurs as a result of larvae feeding on the root tissue of young plants. In recent years, larval damage has been controlled by insecticide seed coating via neonicotinoids. However, since December 2013 the use of this chemical class as a seed treatment has been strongly restricted within the EU. Hence, there is an urgent need for alternative strategies to control the cabbage root fly population; cultivars of oilseed rape that are resistant to this insect pest may be an important component of an integrated pest management system. No-choice feeding experiments were conducted under controlled laboratory conditions to evaluate the level of resistance of 31 brassicaceous accessions (2 *Sinapis alba* (L.) cultivars, 3 *B. napus* cultivars and 26 introgression lines of *S. alba* x *B. napus*) to infestation by cabbage root fly larvae. Plants were artificially infested with eight eggs per plant at either the five or six leaf stage. Neonate larvae were allowed to feed on plants for a period of 4 weeks. Roots were scored according to the extent of feeding damage by larvae and different parameters of larval performance were recorded. From the set of accessions tested, only the *S. alba* cultivars demonstrated antibiotic effects through the presence of less root damage, a reduction in the survival rates of pupae and larvae, reduced weight of the larvae and pupae and reduced size of the pupae. One introgression line significantly reduced the larval performance (lower survival rates of larvae and pupae) compared to the *B. napus* standard cultivar Fenja.

Introduction

The cabbage root fly (*Delia radicum* (L.)) (Diptera: *Anthomyiidae*) (CRF) is a widespread pest of winter oilseed rape (*Brassica napus* (L.)) across Europe (ALFORD *et al.* 2003; SCHÖNBERGER 2012). Three to four generations of the CRF occur annually in Germany, with the third generation being the most damaging to winter oilseed rape crops (ERICHSEN 2006). Adult females deposit their eggs in batches near the hypocotyl and hatching larvae (maggots) develop over the course of three larval stages, before they pupate in the soil (CAPINERA 2008). First instar larvae feed on the root hairs, whereas second and third instar larvae feed on the taproot's tissue (MCDONALD & SEARS 1992). Extensive damage to the lateral root system as a result of larval feeding may limit the water and nutrient uptake (MCDONALD & SEARS 1992) and taproot detachment can cause plants

to die. Furthermore, injured roots are more vulnerable to infection by soil-borne fungal pathogens like *Verticillium longisporum* (KEUNECKE 2009) and *Fusarium spp*. (GRIFFITHS 1986). Recent control strategies for CRFs in oilseed rape production have focused on chemical control using insecticidal seed coatings. Especially compounds of the chemical class of neonicotinoids were widely used throughout the previous decade (ERICHSEN 2006). Since December 2013, however, the use of neonicotinoids as a seed treatment in oilseed rape has been suspended within the EU (BAROSO 2013). Hence, there is an urgent need for alternative strategies to control the CRF.

One of the major goals of a sustainable oilseed rape production is the establishment of an integrated pest management (IPM) system and the reduction of negative side effects of pesticides on the environment, such as any negative impacts on non-target organisms. In IPM systems, one of the basic approaches is the use of varieties that exhibit resistance to pest species (COOK *et al.* 2006). Thus, breeding oilseed rape cultivars that are resistant to CRF attacks is a promising approach for the sustainable and long-term control of this pest (DOSDALL *et al.* 2000).

Wild / undomesticated relatives of *B. napus* have been found to carry genes for resistance to CRF attacks (ELLIS *et al.* 1999; DOSDALL *et al.* 2000). Differences in the susceptibility to CRFs have been well documented for several brassicaceous plant species (ELLIS *et al.* 1999; DOSDALL *et al.* 2000; JENSEN *et al.* 2002). In Canada, different brassicaceous species were evaluated for their susceptibility to *Delia spp*. in field experiments. Variation in susceptibility occurred between and within single plant species. According to DOSDALL *et al.* (2000), the susceptibility of the studied brassicaceous plants to CRFs can be ranked as follows: *Brassica carinata* (L.) > *Brassica rapa* (L.) > *Brassica oleracea* (L.) > *Brassica juncea* (L.) > *Brassica tournefortii* (Gouan.) > *B. napus* > *Crambe abyssinica* (Hochst.) > *Brassica nigra* (L.) > *Eruca sativa* (Mill.) > *Sinapis alba* (L.). Furthermore, *Brassica fructilosa* (Cirillo) and *Brassica spinescence* (Pomel) are highly resistant to maggot feeding (ELLIS *et al*. 1999; FELKL *et al.* 2005). Consequently, *S. alba* is considered to be a promising source of resistance. Host plant resistance to insect pests is generally based on a reduced attractiveness of the host plant for colonization, feeding and egg deposition (antixenosis) as well as on the direct defense-responses and inhibitory effects of specific phytonutrients (antibiosis) (SCHOONHOVEN *et al.* 2005). DOSDALL *et al.* (1994) have concluded that antixenosis is the major mechanism of resistance to infestation by CRFs in *S. alba*, whereas JYOTI *et al.* (2001) have emphasized

the importance of antibiosis for the reduced susceptibility of *S. alba* to CRF infestation. Efforts were made to transfer resistance traits of *S. alba* into *B. napus* via hybridization, embryo rescue and backcrossing (e.g. KOTT & DOSDALL 2004). A number of the resulting intergeneric hybrids (*S. alba* x *B. napus*) exhibited reduced CRF larval root damage (damage on the level of *S. alba* parent) and thus, KOTT & DOSDALL (2004) assumed that resistance genes were transferred from the *S. alba* parent to these hybrids. To prove the genetic inheritance of resistance in intergeneric hybrids, two quantitative trait loci (QTLs) were identified that explain approximately 50% of resistance (EUKERE *et al.* 2005). The objective of this experiment is to evaluate the susceptibility of introgression lines (*S. alba* x *B.napus*) to infestation by CRF larvae compared to *B. napus* and *S. alba* and to test for antibiotic effects based on the development of larvae. In the no-choice experiments, 26 introgression lines, three *B. napus* and two *S. alba* cultivars were screened under controlled laboratory conditions using a standardized inoculum of laboratory propagated *D. radicum* eggs. We further investigated the glucosinolate (GSL) content of the roots and root toughness on selected accessions to identify potential resistance traits.

Material and Methods

Insects: *D. radicum* (CRF) eggs were taken from a laboratory where they were bred and reared on swede (*Brassica napus* L. *var. napobrassica*) in insect rearing cages (30 cm x 30 cm x 30 cm) at 20°C, with 65-80% relative humidity and natural light conditions. Adult flies were fed a diet of dry food consisting of dextrose, skim milk powder, soy flour and brewer's yeast (in a 10:10:1:1 ratio) and wet food consisting of honey, soy flour and brewer's yeast (in a ratio 5:1:1) [ratios in g:g].

Six days after flies eclosed from pupae, a Petri dish containing washed, sieved (≤ 2 mm) and dried sand and a piece of swede (2 cm²) was placed in the cages for oviposition. The Petri dish was replaced every 24 h and eggs were extracted via flotation (MAACK 1977). Eggs deposited during the first 24 h were discarded to ensure a high percentage of fertilized eggs. Eggs from the second day of oviposition onward were stored on filterpaper for 24 h at 6˚C until they were used in the experiments.

Experimental setup: A total of 31 brassicaceous accessions was tested: 3 oilseed rape cultivars (*B. napus*: Fenja, Campino, Visby), 2 white mustard cultivars (*S. alba*: Sirte, Martigena) and 26 introgression lines (*S. alba* x *B. napus*). The summer oilseed rape cultivar Fenja was used as the standard.

Seeds of all brassicaceous accessions were sown in multipot-plates filled with a soil substrate (Loam, Fruhstorf T2, sand 2:1:1) [vol:vol]. Four weeks following germination, plants were transplanted individually into 13 cm wide plastic pots containing the aforementioned soil substrate. The plants were grown under controlled conditions at approximately 19°C for a photoperiod of 16:8 h (L:D) and with artificial light (7000 lux). From the second leaf stage onward, plants were fertilized weekly with 75 ml of 0.2% HakaPhos Blau® (15% N, 10% P_20_5, 15% K_20). The experiment consisted of three subsequent runs with an identical experimental setup for the assessment of larval damage, different parameters of CRF performance and stem base diameter of plants (31 accessions; 3 runs x 5 infested replicate plants per accession). Within each run, plants were arranged in a randomized complete block design.

Furthermore, plants were grown to investigate the GSL content of roots and root toughness. We therefore selected accessions with varying susceptibility to larval damage from the main experiment to investigate the GSL content (4 accessions; 5 infested replicate plants per accession, 5 non-infested replicate plants per accession) and root toughness (14 accessions; 2 runs x 5 non-infested replicate plants per accession).

Root damage: All 31 accessions with five replicate plants per accession were artificially infested with eight eggs per plant between the fith and the sixth leaf stage. Eggs were placed at the base of each plant, 1 cm beneath the soil surface using a fine camel's hairbrush (Fig. 1). Hatching larvae were allowed to feed on the roots. To ensure that the most larvae were able to pupate, while avoiding eclosion of adults, the experiment was terminated 30 days post-inoculation.

Soil from the pots was then floated and examined for larvae and pupae. Taproots were washed and the degree of root injury was determinated as the percentage of root surface destroyed by larval feeding. We established a scale ranging from 0% (no damage) to 100% (complete damage), subdivided into 5% intervals of destroyed root surface.

Figure 1: Inoculation of plants in five leaf stage. Eight *D. radicum* eggs per plant (within the red border) were positioned at the taproot 1 cm beneath the soil surface.

Larval performance: Larvae and pupae were counted and weighed using a microbalance (MC 5, Satorius, Germany). The length of the pupae was measured using a stereo microscope (M3Z, Wild, Switzerland). Based on the number of larvae and pupae per plant, the recovery rate (%) (defined as the ratio of pooled number of larvae and pupae successfully developed on roots to inoculated eggs*100) was calculated to express differences in the developmental success of larvae.

Glucosinolate analysis: The glucosinolate (GSL) content of the roots of four selected accessions were analysed. To account for differences in GSL profiles due to larval damage, we analysed the GSL content of non-infested plants and infested plants (30 days post egg inoculation) with five replicate plants per accession, respectively. Root samples were immediately shock frozen at -80 ˚C and then freeze dried. Samples were homogenized using a mill (Krups KM 75, Germany). Bulk samples per accession and treatment were prepared for analyses. The extraction of desulphoglucosinolates followed the protocol of CLEEMPUT & BECKER (2011). Individual desulphoglucosinolates were analysed using a high performance liquid chromatography (HPLC) (Shimadzu, Germany) equipped with a Nucleodur 100-3 C18 column (Macherey-Nagel, Germany) and separated using a water-acetonitrile gradient (solvent A water, solvent B acetonitrile; phase 1: 0-20 min 1-20% B; phase 2: 20-25 min 20% B; phase 3: 25-27 min 20% B; phase 4: 27-34 min 1% B) at a flow rate of 0.6 ml/min. Desulfoglucosinolates were identified by retention times of known standards. The GSL content is expressed as µmol/g dry root weight.

Morphological characteristics: The stem base diameter of the 31 accessions (five replicate plants per accession) was measured 30 days post-inoculation. To detect differences in the strength of root tissue between accessions, the root toughness of 14

selected accessions with five replicate non-infested plants per accession was measured between the fith and the sixth leaf stage using a texture analyser (TA.XT2, Stable Micro Systems Ltd., UK). Roots were cut at the stem base and positioned in the centre of the texture analyser in alignment with the height of the first lateral roots. A commercial sewing needle (size no. 9, Ø 0.6 mm) was used for penetration (see Appendix Fig. 1). The penetration depth was standardized to 1 mm. Results are expressed as the maximum force needed (N/cm²) to fracture the root surface. The data were analysed using the texture expert program (version 1.10, Stable Micro Systems Ltd., UK).

Statistical analysis: The effects of plant genotype on root damage, different parameters of CRF performance (see Tab. 2), stem base diameter and toughness of root tissue were tested using general linear models (GLM) (ANOVA) (SAS 9.4, SAS Institute, USA). Normality and homogeneity of variance were verified by inspecting the residuals. For all parameters, a Dunnett post hoc test was performed to test for differences between the standard oilseed rape cultivar Fenja and the other accessions. The level of significance was set at a confidence interval of 95% and p-values were adjusted according to Dunnett. Linear regression models were used to describe the relationship between larval performance and root damage. Bulk samples from five replicate plants per accession were taken for GSL analyses and the results are presented in a descriptive form.

Results

Root damage: The accessions exhibited considerable differences in the susceptibility to feeding by *D. radicum* larvae (F 4.67, $p < 0.001$). Compared to the standard *B. napus* cultivar Fenja; the root surface damage ranged between 50% less than and 30% more than Fenja (Fig. 2). The root surface damage from larval feeding for Fenja was, on average, 58.57% ± 5.58% (Tab. 2). We detected no significant differences in larval damage between the 26 introgression lines and the standard cultivar Fenja. Root damage was significantly less for the *S. alba* cultivars Sirte (> 37% reduced; $p = 0.019$) and Martigena (> 47% reduced; $p = 0.002$), but did not differ between the *B. napus* cultivars Visby and Campino and the standard cultivar Fenja, respectively. Nevertheless, root damage was reduced in 16 out of the 30 accessions, compared to the standard Fenja (Fig. 2). From these, the introgression lines IL_138, IL_140, IL_175 and IL_101 were less affected by *D. radicum* larvae, with damage reduced by 6% to 10% compared to the standard cultivar Fenja. The introgression line IL_165 had the lowest feeding damage, with a damage reduction of 26% compared to the standard cultivar Fenja.

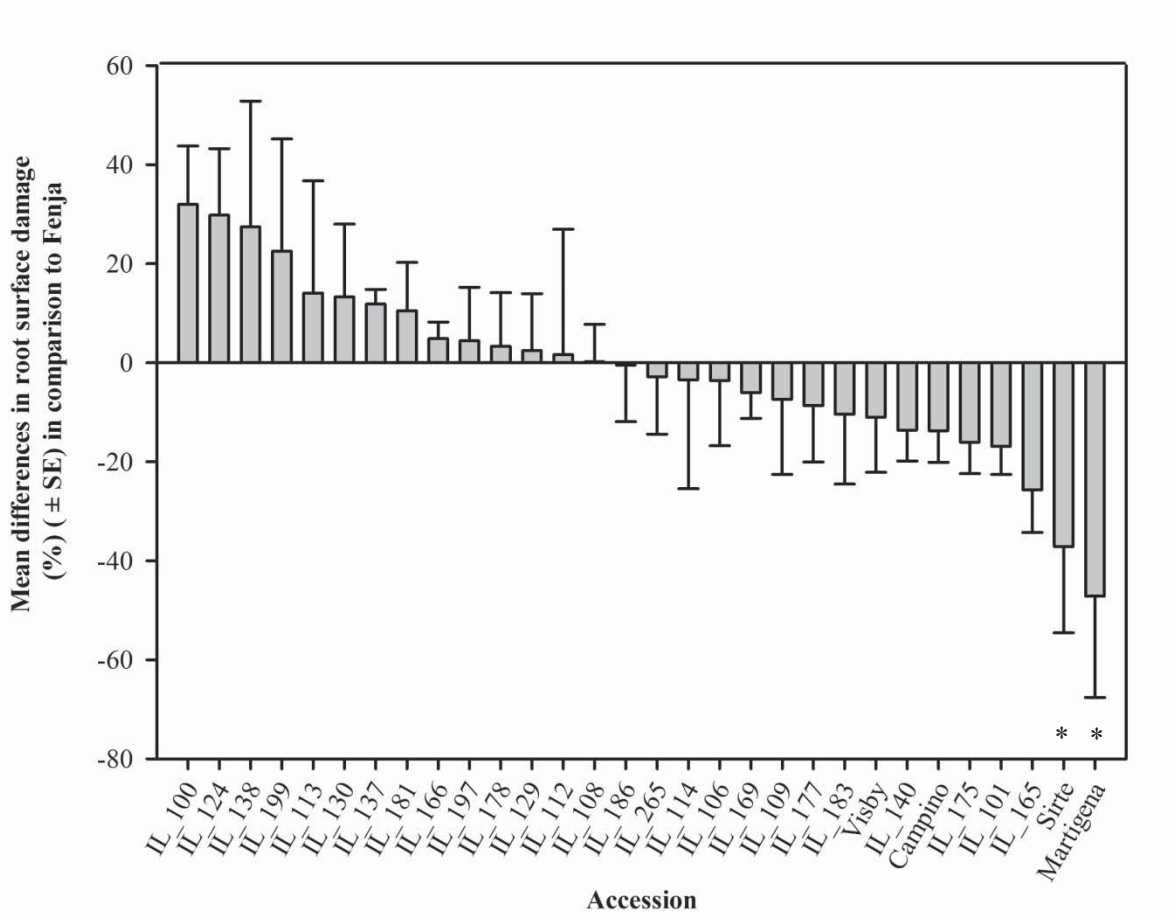

Figure 2: Differences in the damaged root surface by *D. radicum* larvae on tested accessions in relation to damaged area on standard cultivar Fenja. Data presented as arithmetic means ± SE. Significant differences to Fenja are marked with an asterisk (ANOVA, Dunnett test, *$p \leq 0.05$).

Larval performance: The totalled number of developed larvae and pupae per plant was less (for all 30 accessions) than for the standard *B. napu*s cultivar Fenja (Tab. 1). The recovery rate of larvae and pupae on plants for the standard cultivar Fenja was, on average, 73.44% ± 4.82%. Significantly lower numbers of *D. radicum* larvae and pupae were found for the *S. alba* cultivars Sirte ($p < 0.001$) and Martigena ($p < 0.001$) and the introgression line IL_183 ($p = 0.036$), compared to the standard cultivar Fenja. The number of larvae ranged between 2.44 ± 0.5 larvae for Fenja and zero larvae (Sirte); the number of larvae was significantly lower in 14 of the introgression lines and the *S. alba* cultivars compared to the standard cultivar Fenja.

Table 1: Root surface damage due to larval feeding, parameters of larval performance and stem base diameter 4 weeks after inoculation of eight eggs per plant (no-choice test). Significant differences to the standard cultivar Fenja are marked with asterisks (ANOVA, Dunnett-Test, *$p \leq 0.05$). Data are presented as arithmetic means ± SE.

Accession	Larval feeding damage % (means ± SE)	Recovery rate % (larvae + pupae) (means ± SE)	No. larvae (means ± SE)	Larval weight (mg) (means ± SE)	No. pupae (means ± SE)	Pupal weight (mg) (means ± SE)	Pupal size (mm) (means ± SE)	Basal stem diameter (mm) (means ± SE)
Fenja	58.75 (± 5.58)	73.44 (± 4.82)	2.44 (± 0.50)	12.16 (± 0.52)	3.44 (± 0.48)	14.10 (± 0.36)	5.84 (± 0.05)	9.34 (± 0.23)
Martigena	28.67 (± 4.40)*	4.17 (± 2.64)*	0.20 (± 0.20)*	8.34 (± 0.00)	0.13 (± 0.09)*	6.36 (± 0.27)*	4.52 (± 0.00)*	6.43 (± 0.22)*
Sirte	35.00 (± 3.99)*	4.17 (± 2.64)*	0.00 (± 0.00)*		0.33 (± 0.21)*	9.55 (± 0.41)*	5.09 (± 0.09)*	7.33 (± 0.27)*
IL_165	44.00 (± 5.05)	60.00 (± 8.38)	1.07 (± 0.32)	12.06 (± 1.52)	3.73 (± 0.74)	14.89 (± 0.43)	5.86 (± 0.05)	9.03 (± 0.24)
IL_101	48.50 (± 7.71)	71.25 (± 8.75)	0.70 (± 0.33)*	12.93 (± 0.54)	5.00 (± 0.63)	13.32 (± 0.46)	5.72 (± 0.05)	8.10 (± 0.29)
IL_175	50.00 (± 5.47)	54.46 (± 7.48)	0.79 (± 0.26)*	13.51 (± 0.91)	3.57 (± 0.49)	13.89 (± 0.59)	5.75 (± 0.06)	8.07 (± 0.26)*
Campino	50.28 (± 5.46)	50.69 (± 6.74)	1.50 (± 0.33)	12.48 (± 1.01)	2.56 (± 0.53)	13.37 (± 0.53)	5.67 (± 0.07)	8.56 (± 0.29)
IL_140	51.25 (± 5.41)	65.63 (± 7.26)	2.06 (± 0.55)	10.85 (± 0.87)	3.19 (± 0.59)	12.29 (± 0.68)	5.56 (± 0.10)	7.53 (± 0.35)*
IL_183	51.33 (± 4.48)	36.67 (± 6.84)*	1.60 (± 0.45)	10.52 (± 1.29)	1.33 (± 0.49)	12.80 (± 0.29)	5.63 (± 0.05)	8.50 (± 0.25)
Visby	51.33 (± 3.79)	66.67 (± 8.15)	0.27 (± 0.15)*	16.94 (± 1.68)	5.07 (± 0.69)	16.43 (± 0.26)	6.14 (± 0.03)	6.97 (± 0.22)*
IL_177	52.67 (± 3.74)	55.00 (± 7.50)	1.27 (± 0.38)	13.32 (± 1.07)	3.13 (± 0.58)	13.22 (± 0.54)	5.72 (± 0.07)	9.07 (± 0.28)
IL_109	52.86 (± 2.50)	66.96 (± 5.34)	1.57 (± 0.53)	12.10 (± 1.37)	3.79 (± 0.45)	13.96 (± 0.67)	5.73 (± 0.08)	10.00 (± 0.28)
IL_169	55.67 (± 6.63)	59.17 (± 9.25)	0.87 (± 0.35)*	9.31 (± 1.17)	3.87 (± 0.78)	12.02 (± 0.68)	5.50 (± 0.10)	7.07 (± 0.22)*
IL_106	56.00 (± 3.63)	54.17 (± 5.13)	2.00 (± 0.37)	10.01 (± 0.75)	2.33 (± 0.57)	12.88 (± 0.54)	5.62 (± 0.08)	8.43 (± 0.25)
IL_114	56.07 (± 7.00)	47.32 (± 7.88)	1.00 (± 0.46)	12.00 (± 0.76)	2.79 (± 0.62)	12.74 (± 0.57)	5.62 (± 0.09)	7.46 (± 0.21)
IL_265	56.33 (± 5.29)	45.00 (± 7.70)	1.60 (± 0.48)	13.38 (± 1.00)	2.00 (± 0.51)	14.02 (± 0.63)	5.90 (± 0.07)	9.13 (± 0.18)
IL_112	56.79 (± 4.56)	53.57 (± 8.12)	0.79 (± 0.26)*	8.56 (± 1.41)	3.50 (± 0.66)	12.04 (± 0.54)	5.61 (± 0.08)	8.43 (± 0.25)
IL_108	56.79 (± 5.83)	56.25 (± 5.52)	1.93 (± 0.59)	12.65 (± 0.39)	2.57 (± 0.60)	14.35 (± 0.40)	5.84 (± 0.09)	8.43 (± 0.22)
IL_186	57.67 (± 4.17)	69.17 (± 4.20)	0.87 (± 0.29)*	11.12 (± 0.80)	4.67 (± 0.48)	15.54 (± 2.33)	5.67 (± 0.11)	8.20 (± 0.30)
IL_129	59.93 (± 5.86)	60.83 (± 8.61)	1.40 (± 0.38)	11.21 (± 1.18)	3.47 (± 0.57)	13.30 (± 0.54)	5.66 (± 0.06)	7.67 (± 0.22)*
IL_178	60.00 (± 5.52)	62.50 (± 8.95)	0.38 (± 0.14)*	9.07 (± 0.56)	4.62 (± 0.81)	13.51 (± 0.49)	5.72 (± 0.07)	9.50 (± 0.19)
IL_197	60.36 (± 3.83)	68.75 (± 8.26)	0.36 (± 0.17)*	15.55 (± 1.62)	5.14 (± 0.64)	14.13 (± 0.75)	5.78 (± 0.10)	8.27 (± 0.21)
IL_166	61.25 (± 4.88)	67.97 (± 4.84)	0.94 (± 0.35)*	10.67 (± 0.87)	4.50 (± 0.46)	12.20 (± 0.67)	5.50 (± 0.09)	8.75 (± 0.21)
IL_181	64.69 (± 3.37)	67.19 (± 6.02)	0.50 (± 0.18)*	15.29 (± 1.17)	4.88 (± 0.49)	15.18 (± 0.36)	5.98 (± 0.06)	10.03 (± 0.22)
IL_113	65.31 (± 4.78)	62.50 (± 6.04)	0.81 (± 0.26)*	12.05 (± 1.04)	4.19 (± 0.56)	13.96 (± 0.51)	5.77 (± 0.07)	8.44 (± 0.21)
IL_130	65.33 (± 6.31)	71.67 (± 7.26)	0.53 (± 0.22)*	13.91 (± 1.56)	5.20 (± 0.60)	15.10 (± 0.64)	5.96 (± 0.08)	7.20 (± 0.26)*
IL_137	66.33 (± 3.89)	70.00 (± 5.15)	0.67 (± 0.25)*	16.39 (± 0.78)	5.00 (± 0.44)	15.73 (± 0.34)	5.97 (± 0.04)	7.50 (± 0.31)*
IL_138	69.25 (± 5.28)	51.88 (± 7.44)	0.75 (± 0.22)*	10.13 (± 1.21)	3.40 (± 0.61)	13.48 (± 0.52)	5.69 (± 0.07)	7.78 (± 0.19)*
IL_199	69.33 (± 3.45)	71.67 (± 5.10)	2.33 (± 0.51)	12.16 (± 0.97)	3.40 (± 0.62)	12.37 (± 0.70)	5.63 (± 0.10)	8.47 (± 0.27)
IL_124	75.33 (± 5.31)	60.83 (± 6.78)	0.73 (± 0.25)*	13.08 (± 1.79)	4.13 (± 0.57)	12.96 (± 0.64)	5.72 (± 0.10)	7.73 (± 0.39)*
IL_100	76.43 (± 6.80)	58.04 (± 4.06)	0.93 (± 0.35)	12.36 (± 1.30)	3.71 (± 0.54)	14.39 (± 0.49)	5.80 (± 0.07)	7.21 (± 0.41)*

The larval weight differed significantly between accessions (F 5.97, $p < 0.001$), but we detected no significant difference to the standard cultivar Fenja. With an average of 5.07 ± 0.69 pupae Visby had the highest number of pupae, whereas a low number of pupae was found on the *S. alba* cultivars (< 0.5 pupae per plant). Significantly less larvae developed to pupae on plants of Sirte ($p < 0.001$) and Martigena ($p < 0.001$) in comparison to the standard Fenja. The pupal weight was significantly reduced on Martigena ($p = 0.002$), as well as Sirte ($p = 0.003$) and also the size of the pupae was significantly lower for the *S. alba* cultivars (Martigena $p = 0.002$, Sirte $p = 0.003$), than those of the standard cultivar Fenja (Tab. 1).

The larval feeding damage scores of root surface were significantly influenced by the recovery rate ($R^2 = 0.39$, $p < 0.001$), the number of pupae developed per plant ($R^2 = 0.33$, $p < 0.001$), the weight of larvae plus pupae ($R^2 = 0.27$, $p = 0.002$) as well as the pupal weight ($R^2 = 0.25$, $p = 0.002$) (Tab. 2). Furthermore, the recovery rate was positively related to the weight of larvae plus pupae ($R^2 = 0.85$, $p < 0.001$).

Table 2: Parameter estimates for significant linear relationships between infestation parameters assessed in the no-choice experiment, a is the intercept of the linear regression equation and b is the slope of the linear regression equation, *($p \leq 0.05$).

Independent variable	Dependent variable	a	b	Coefficient of determination (R^2)	df	F
Recovery rate	Larval feeding damage	0.40	34.12	0.39	31	20.55*
Number of pupae	Larval feeding damage	4.69	40.49	0.33	31	15.60*
Weight (larvae + pupae)	Larval feeding damage	0.29	37.83	0.27	31	12.07*
Weight pupae	Larval feeding damage	0.27	41.67	0.25	31	11.05*
Recovery rate	Weight (larvae + pupae)	1.07	4.65	0.85	31	167.14*

Glucosinolate analysis: In all accessions, the total GSL content of infested plants was substantially more than non-infested plants (Fig. 3). While indolyl GSLs was the dominant group in non-infested plants of Fenja and IL_183, aliphatic GSLs prevailed in the non-infested introgression line IL_114 (67%). In infested roots of the *B. napus* cultivar Fenja and the two introgression lines, the content of indolyl GSLs, however, increased to > 80% of the total GSL content (in comparison to the non-infested plants) with especially higher contents of glucobrassicin (GBC) and neoglucobrassicin (NEO) (Tab. 3). The content of aliphatic GSLs was reduced in infested roots of Fenja and IL_114, whereas it was slightly higher in infested roots of the introgression line IL_183. Overall, the content of the aliphatic GSLs glucoraphanin (RAA) and glucoraphenin (RAE) increased compared to non-infested plants.

Both the non-infested and infested roots of the *S. alba* cultivar Martigena had the highest content of aromatic GSLs of the accessions investigated. The predominant aromatic GSL in Martigena was sinalbin (SIB). Root infestation of the *S. alba* cultivar Martigena resulted in a slight increase in total GSL content compared to non-infested plants, due to higher contents of 4-hydroxyglucobrassicin (4OH), GBC and SIB. The content of the aliphatic GSL sinigrin (SIN) decreased due to infestion in the roots of Martigena.

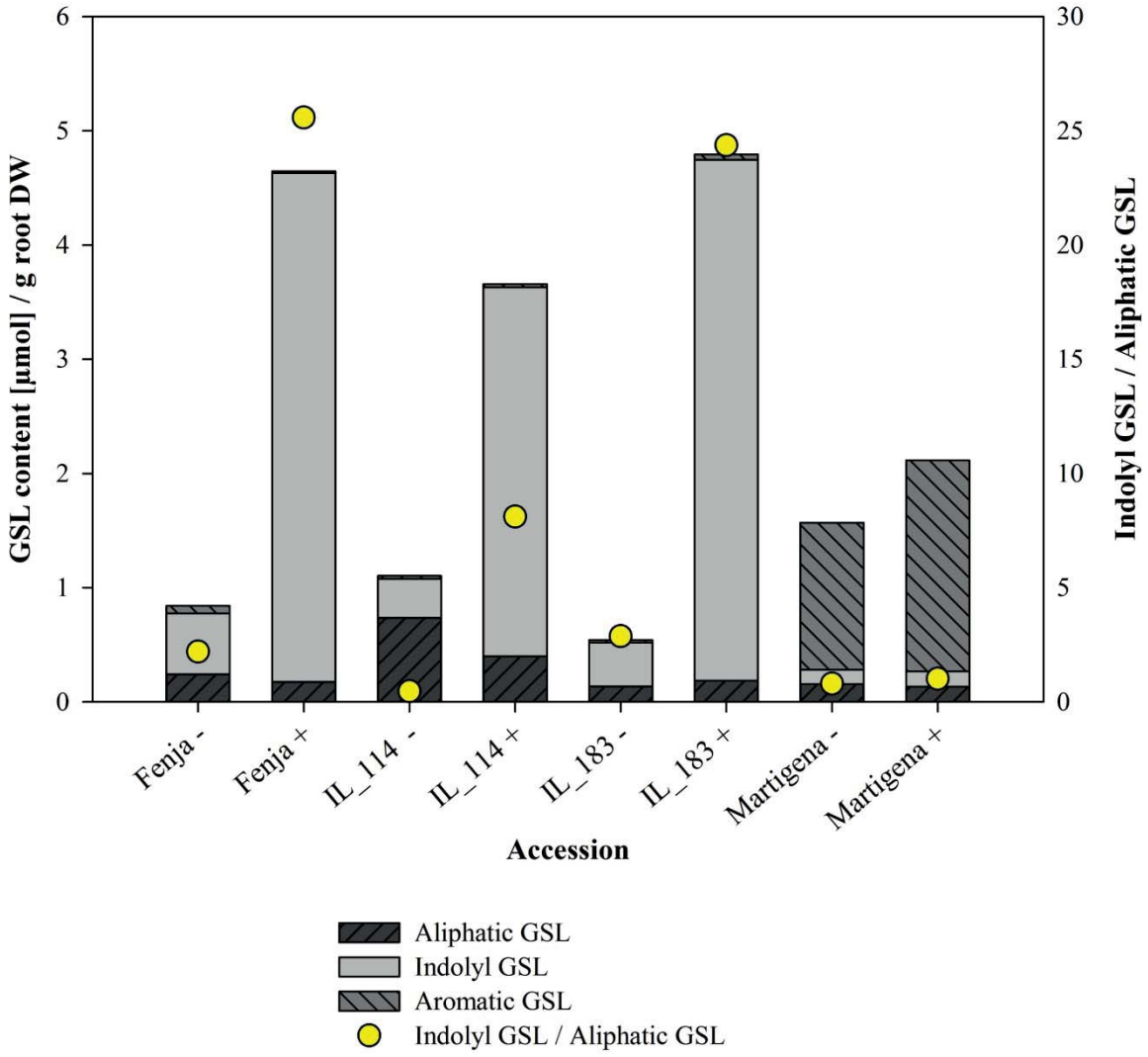

Figure 3: Contents of glucosinolates (GSL) (total groups and ratio) (µmol/g DW) in roots of control plants (-) and inoculated plant (+). Data presented as results of bulk samples.

Table 3: Glucosinolate (GSL) contents (total, groups and single substances) (µmol/g DW) in roots of infested (+) and non-infested (-) test accessions 30 days post inoculation. Data of bulk sample analyses are presented.

Accession and Treatment	Total GSL	Aliphatic GSL	Indolyl GSL	Aromatic GSL	I/A	Aliphatic GSL									Indolyl GSL				Aromatic GSL	
						IBE	PRO	SIN	GNL	RAA	RAE	GNA	GBN	ERU	4OH	GBC	4ME	NEO	SIB	NAS
Fenja -	0.84	0.24	0.53	0.07	2.20	0.04	0.00	0.01	0.00	0.03	0.05	0.01	0.10	0.00	0.11	0.32	0.00	0.11	0.07	0.00
Fenja +	4.65	0.17	4.45	0.02	25.58	0.01	0.05	0.00	0.00	0.04	0.00	0.02	0.06	0.00	0.06	1.02	0.00	3.38	0.02	0.00
IL_114 -	1.10	0.73	0.34	0.03	0.47	0.01	0.57	0.01	0.00	0.09	0.00	0.02	0.04	0.00	0.04	0.15	0.00	0.16	0.03	0.00
IL_114 +	3.66	0.40	3.23	0.03	8.11	0.03	0.20	0.01	0.00	0.07	0.00	0.01	0.07	0.00	0.06	1.02	0.00	2.15	0.03	0.00
IL_183 -	0.54	0.13	0.39	0.02	2.88	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.08	0.00	0.08	0.13	0.00	0.17	0.02	0.00
IL_183 +	4.79	0.19	4.56	0.05	24.37	0.00	0.00	0.00	0.00	0.07	0.06	0.01	0.05	0.00	0.05	1.00	0.00	3.52	0.05	0.00
Martigena -	1.57	0.16	0.12	1.29	0.80	0.00	0.03	0.06	0.00	0.05	0.00	0.02	0.00	0.00	0.01	0.01	0.00	0.10	1.27	0.02
Martigena +	2.11	0.13	0.13	1.85	1.01	0.02	0.00	0.04	0.00	0.03	0.02	0.02	0.00	0.00	0.02	0.02	0.00	0.10	1.85	0.00

I/A = Ratio of Indolyl GSL/Aliphatic GSL; IBE = glucoiberin; PRO = progoitrin; SIN = sinigrin; GNL = gluconapoleiferin; RAA = glucoraphanin; RAE = glucoraphenin; GNA = gluconapin; GBN = glucobrassicanapin; ERU = glucoerucin; 4OH = 4-hydroxyglucobrassicin; GBC = glucobrassicin; 4ME = 4-methoxy-glucobrassicin; NEO = neoglucobrassicin; SIB = sinalbin; NAS = gluconasturtiin.

Morphological plant characteristics: The stem base diameter differed significantly among accessions (F 8.61, $p < 0.001$); it was significantly lower for 11 introgression lines and the *S. alba* cultivars (Martigena and Sirte) compared to the standard *B. napus* cultivar Fenja (Tab. 4). No significant correlation was found between the stem base diameter and feeding damage by CRF larvae. The maximum force needed to fracture the root surface differed significantly among accessions (F 2.09, $p = 0.034$), but there were no differences in root toughness between accessions and the standard cultivar Fenja. Root toughness was neither correlated with larval feeding damage nor with various parameters of larval performance (see Tab. 2).

Table 4: Root toughness of non-infested plants in the six leaf stage. Significant differences to the standard *B. napus* cultivar Fenja are marked with asterisks (ANOVA, Dunnett test, $*p \leq 0.05$). Data are presented as arithmetic means ± SE.

Accession	Root toughness (N/cm^2) (means ± SE)
Fenja	61.05 (± 3.28)
Martigena	61.28 (± 5.66)
IL_165	49.24 (± 8.24)
IL_140	42.24 (± 5.44)
IL_183	46.34 (± 8.59)
IL_114	32.98 (± 7.22)
IL_165	35.62 (± 8.83)
IL_108	37.72 (± 5.49)
IL_186	36.32 (± 7.92)
IL_129	37.61 (± 8.19)
IL_181	63.39 (± 5.31)
IL_130	44.37 (± 6.76)
IL_100	35.04 (± 1.75)

Discussion

The results of our study reveal differences in the feeding damage to the roots and the larval performance of CRFs. The *S. alba* cultivars demonstrated reduced damage to plants, reduced rates of recovery and weights of larvae and pupae, as well as reduced pupal size in comparison to the standard oilseed rape cultivar Fenja. Moreover, introgression line IL_183 significantly reduced the larval performance (lower recovery rate and smaller larvae and pupae) in comparison to the standard cultivar Fenja. The GSL content in root tissues was altered by CRF infestation: the total GSL content was higher in infested roots, mainly due to an increase in indol GSLs. While aliphatic and indol GSLs were dominant in infested and non-infested plants of Fenja and the introgression lines, the aromatic GSL SIB was prevalent in infested and non-infested roots of the *S. alba* cultivar. It was determined that root toughness neither influenced the extent of root damage nor larval performance.

The precise knowledge of potential sources and mechanisms of host plant resistance is a prerequisite for developing insect resistant cultivars. It is especially important to have profound information about the plant traits that help protect an accession from damage in order to design an appropriate experimental setup that can identify accessions which may carry resistance (STONER & SHELTON 1988; JYOTI *et al.* 2001). In general, plant resistance to insect pests may be caused by antibiosis, antixenosis, tolerance or a combination of these (PAINTER 1951; KOGAN & ORTMAN 1978). Constitutive host plant resistance can be defined as the consequence of heritable plant characteristics that result in a plant being relatively less damaged than a plant without these characteristics (TEETES 2013). The level of resistance can be measured by comparing test accessions to susceptible cultivars of the same plant species (SINGH & SINGH 2005).

The major aim of our study is to evaluate the level of resistance of 26 introgression lines (*S. alba* x *B. napus*) to CRF infestations. Therefore, we compared the antibiotic effects of these 26 accessions to the susceptible standard *B. napus* cultivar Fenja, two additional susceptible oilseed rape cultivars and two resistant *S. alba* cultivars. The host plant quality of different test accessions was determined by scoring the root injury caused by larval feeding and the different parameters of larval performance. Antibiotic properties of the host plant may have a direct lethal effect on neonate larvae or may lead to mortality during larval development. Individuals that survive antibiosis may suffer from prolonged developmental times, reduced body size and lower body weight (JYOTI *et al.* 2001). To test accessions for antibiotic resistance to the CRF, a no-choice bioassay was ideal, since under

natural conditions gravid CRF females select host plants (as described and investigated in Chapter II) and the larvae must live with this choice since they are immobile and cannot search for alternative hosts (ZOHREN 1968; KERGUNTEUIL *et al*. 2014).

We documented differences in the feeding damage of the roots and in larval performance of *D. radicum* on different test accessions. Consequently, the observed differences in larval performance suggest antibiotic resistance. Within the literature, *S. alba* has been repeatedly described to be highly resistant to CRF attacks (MCDONALD & SEARS 1992; KOTT & DOSDALL 2004). In our study, the two *S. alba* cultivars exhibited significantly reduced root damage compared to the standard *B. napus* cultivar Fenja and negatively influenced the performance of larvae. The introgression lines, deriving from progenies of the interspecific hybridization between *S. alba* and *B. napus,* exhibited considerable variation in the levels of susceptibility to CRF attacks; the introgression line IL_183 exhibited a low development of larvae, although feeding damage to the roots was pronounced (> 50% root surface damage). The absence of a complete resistance to CRF infestation, i.e. feeding inhibition of larvae on resistant plants to a certain degree, is in accordance with the general acceptance that plant resistance to insects is often only partial (DENT 2000). The development of a complete resistance (absence of any feeding damage on the resistant plants) cannot be expected within the approach of transferring resistance traits from *S. alba* into *B. napus,* as even plants of *S. alb*a have root damage caused by larval feeding and a small number of larvae successfully develop into flies (DOSDALL *et al.* 2000; JYOTI *et al.* 2001). Nevertheless, the extent to which the plants are damaged due to larval feeding in *S. alba* is low in comparison to *B. napus*. A partial resistance further possesses the advantage that the selective pressure on the pest populations is lower compared to a complete resistance (DENT 2000).

The larval development of insect pests on host plant roots is influenced by biochemical and morphological characteristics (BIRCH 1988). Morphological plant characteristics may have a significant impact, especially on the success of establishment and infestation of insect pests (SCHOONHOVEN *et al*. 2005; PRICE *et al*. 2011). Root toughness is determined by macro-molecules such as lignin, cellulose and callose (JOHNSON *et al.* 2010). In our study, we measured the toughness of the root surface as a possible resistance factor affecting the feeding and performance of CRF larvae for the set of selected accessions, but did not find this morphological characteristic to be associated with decreased feeding damage. Similar results were obtained by BIRCH (1988), who did

not detect any effect of root toughness on the damage caused by larval feeding of *Delia floralis* (L.) (Diptera: Anthomyiidae) on swedes in field trials. However, future investigations are needed to determine how the accessions' root toughness (especially *S. alba*) changes during plant development and whether an increase in toughness is induced by larval feeding.

In our study, the total GSL content of roots increased due to the feeding of *D. radicum* larvae. The content of indolic GSLs was higher in the infested than in the non-infested roots of Fenja and the introgression lines IL_114 and IL_183. Especially the content of the indole GSL GBC increased compared to non-infested plants. An increase in indole GSLs and a reduction in the content of aromatic GSLs in roots due to larval feeding of the CRF have also been observed in *B. oleracea* by VAN DAM & RAAIJMAKERS (2006). Moreover, our results are in accordance with former studies that have revealed that the feeding of *D. floralis* larvae on roots of several *Brassica* genotypes results in an increase in the total GSL content due to an increase in indole-based compounds, especially GBC (BIRCH *et al.* 1992; GRIFFITHS *et al.* 1994).

The non-infested roots of the *S. alba* cultivar possessed the highest total GSL content and the predominant GSL was SIB in both infested and non-infested roots, which accounted for 87% of the total GSL content in the infested roots. The GSL SIB is known to negatively influence a wide range of both generalist and specialist insect herbivores (BODNARYK 1991) and may also explain the lower root damage and lower level of larval performance of CRF larvae for *S. alba* in our study. Furthermore, the roots of the *S. alba* cultivar Martigena contained the aliphatic GSL SIN, which is known to be hydrolysed by myrosinase into the volatile allyl-isothiocyanate, which is known to have an antibiotic effect on the larval performance of *Pieris rapae* (L.) (Lepidoptera: Pieridae) (AGRAWAL & KURASHIGE 2003). Induced changes to the content and composition of GSLs in plants (local and systemic) may affect the primary pest species itself and may alter the degree of acceptance of the plant to other herbivores (both generalists and specialists) that subsequently colonize the induced plant. Moreover, volatile compounds, such as volatile isothiocyanates (hydrolyses products of aliphatic and aromatic GSLs), which are released in higher amounts in response to herbivory, are known to attract parasitoids and predators (AHUJA *et al.* 2009).

It might be beneficial to use the available partial antibiotic resistance of introgression lines to CRF attacks in a trap cropping strategy: accessions that combine a

high attractiveness (no antixenotic resistance) to oviposition by *D. radicum* with pronounced antibiotic traits (e.g introgression line IL_183) may lead to a "dead-end" for the insect pest, since only a few individuals will successfully develop on the roots. These accessions may be used as a trap crop in combination with the susceptible main crop (which is less attractive for oviposition) to reduce the number of infestations in the commercial crop to below the level of economic damage (KERGUNTEUIL *et al.* 2014). Furthermore, the population growth of the CRF may be hampered.

Screening the introgression lines has not yet revealed any strong source of resistance to the CRF, but the experimental set-up provides useful information on the genetic potential of tested accessions towards CRF maggots for further testing. Future investigations, on a larger set of intergeneric hybrids and introgression lines, should search for accessions with a more pronounced resistance. The larval performance and root damage of these accessions should be low to protect plants from economically relevant damage. While the root toughness in our study did not affect the performance of *D. radicum* larvae, the GSL content of roots was altered by larval feeding and there is a high probability that the larval performance was affected by the GSL substances, however this interaction needs to be investigated in greater detail.

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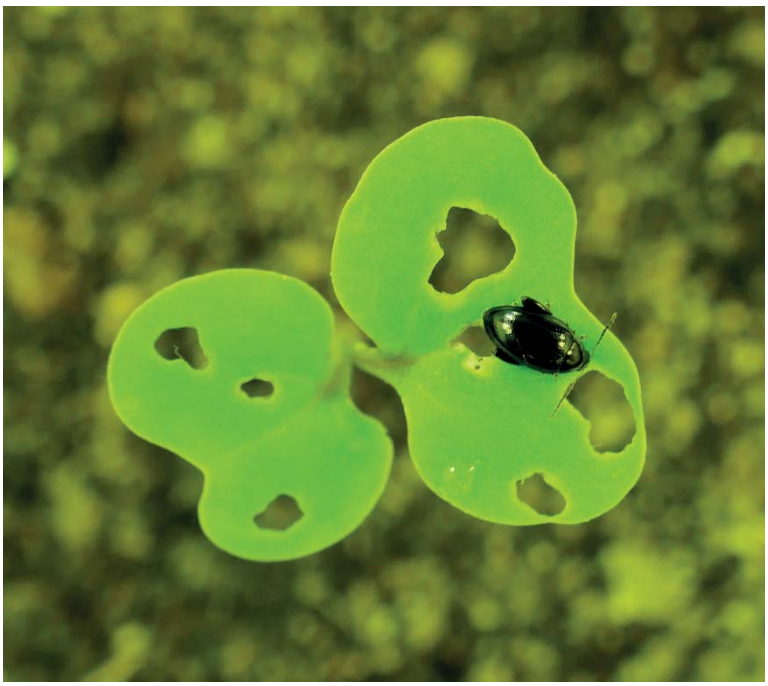
Chapter IV

Screening of *Brassica napus*, *Sinapis alba* and introgression lines for antixenotic seedling resistance to feeding by cabbage stem flea beetle (*Psylliodes chrysocephala* L.)



Abstract

The cabbage stem flea beetle (*Psylliodes chrysocephala* (L.)) is an important pest in winter oilseed rape production (*Brassica napus* (L.)) in Europe. In recent decades, *P. chrysocephala* has been controlled by neonicotinoid seed coating and the foliar application of pyrethroids. Since December 2013, however, the usage of neonicotinoids for a seed treatment has been suspended within the EU. Moreover, resistance of *P. chrysocephala* to pyrethroids was detected in northern Germany in 2008. Hence, there is an urgent need for alternative strategies to control the cabbage stem flea beetle population and cultivars of oilseed rape that are resistant to this insect pest may be an important component of an integrated pest management system. Especially the brassicaceous species *Sinapis alba* (L.) is known to be an inappropriate host for several insect pest species.

In this study, we screened the seedlings of 11 introgression lines (*S. alba* x *B. napus*) for antixenotic resistance to the feeding of *P. chrysocephala* adults. No-choice experiments exmained the seedling damage caused by adult feeding in comparison to a susceptible oilseed rape cultivar and two cultivars of *S. alba*. We further assessed several morphological plant traits (i.e. trichome density, cotyledone size and seedling dry weight) and the plants' glucosinolate profiles as potential factors of host plant resistance. Compared to the *S. alba* cultivars, seedling damage was higher in all introgression lines and the oilseed rape cultivar. We found a significantly positive correlation between the total content of aliphatic glucosinolates in seeds and the damage caused by adult feeding, whereas the content of the single glucosinolate substances sinalbin and gluconapoleiferin were significantly, negatively related to the feeding of adults on cotyledons of the test accessions. Our results also indicate that the trichome density of cotyledons as well as the dry matter weight of seedlings influence the feeding of *P. chrysocephala.*

Introduction

The cabbage stem flea beetle (CSFB) (*Psylliodes chrysocephala* (L.)) (Coleoptera: Chrysomelidae) is a major pest of oilseed rape (*Brassica napus* (L.)) in humid regions throughout Europe (ALFORD *et al.* 2003). Between late August and early September adults colonize newly emerged crops of winter oilseed rape (WILLIAMS 2004). Following a maturity feeding period of approximately two weeks, females start to deposit their eggs into the soil near plants (NUSS 2004). Females have a high fecundity and may oviposit more than 600 eggs during their life-span (MATHIASEN *et al.* 2015). Neonate larvae bore

into the petioles near the nodality and mine within the pith tissue of the petioles (GODAN 1950). Damage is primarily caused by larval feeding and may result in growth reduction and wilting. Moreover, plant losses may occur if the vegetation point is damaged or if water penetrates the plants via injuries caused by larval feeding and freezes during the winter (SCHULZ & DAEBLER 1984; NILSSON 2002). However, the initial feeding of adult CSFBs on oilseed rape plants can also be severe. Especially attacks by adult CSFBs on young seedlings are known to negatively affect *Brassica* crops; such attacks reduce plant density and delay the growth and development of the plants, resulting in lower yields (WILLIAMS 2010).

Insecticides are the primary means of controlling the CSFB through the foliar spray application of pyrethroids and insecticidal seed coatings. Over the last decade, the chemical class of neonicotinoids was primarily used as a seed treatment (ERICHSEN 2006), but these insecticides have, since December 2013, been strongly restricted within the EU due to their potential harmful effects on bees (BAROSO 2013). Furthermore, populations of CSFB that exhibit a genetically manifested "knock down resistance" (kdr) to pyrethroids are increasing (HØJLAND *et al*. 2015). Consequently, it is assumed that it will become increasingly difficult to control the CSFB through the use of insecticides. In addition to agronomical strategies like reduced tillage regimes (ULBER & SCHIERBAUM - SCHLICKER 2003), adapted plant densities (NUSS 2004) and the promotion of natural enemies as biological control agents (ULBER 2003), breeding oilseed rape cultivars that are resistant to CSFB attacks may be a potential component of integrated pest management (IPM) systems for oilseed rape (WILLIAMS 2004).

Detailed information on host plant traits that affect the behaviour and the physiological processes of a pest species can markedly improve progress towards developing resistant varieties (AHUJA *et al.* 2009). Host plant location and acceptance by herbivorous insects generally rely on biochemical and morphological plant traits (SCHOONHOVEN *et al.* 2005; RUSCH *et al.* 2010). According to KOGAN & ORTMAN (1978), antixenosis is defined as plant traits that interfere with insects' behaviour of host plant choice and impact on subsequent plant-insect interactions, whereas antibiotic resistance traits may negatively affect pest survival and development (SCHOONHOVEN *et al.* 2005). The enforcement of antixenotic resistance of young oilseed rape plants to flea beetle attacks is of major importance, since young plants are highly vulnerable to feeding damage (LAMB 1984) and antixenosis may prevent damage by deterring adults from feeding and

ovipositing. The feeding of the CSFB is induced by olfactory, mechanosensory and gustatory cues of the host plant (BARTLET 1996). Numerous studies on insect-plant interactions have investigated the impact of glucosinolates (GSLs) and their metabolites on herbivores of brassicaceous plants. While GSLs are known to have a repellent effect on generalist pest species (MOENS *et al.* 1992), they can serve as cairomones, mediating the feeding and oviposition of highly specialized insect herbivores (BARTLET *et al*. 1999a; RASK *et al.* 2000; MÜLLER 2009). Furthermore, morphological plant factors like pubescence (DUFFEY 1986; SOROKA *et al.* 2011), colour (SOUTHWOOD 1986; BÜCHI 1990; TANSEY *et al.* 2010a) or wax layer (BODNARYK 1992; LAMBDON *et al.* 1998) have been found to influence host plant selection and infestation by insect pests.

In an effort to develop higher yielding cultivars, defensive diversity and the resistance level of the progenitors have simultaneously vanished (SCHOONHOVEN *et al.* 2005). The genepool of *B. napus* currently used in breeding programs is limited (BROWN *et al.* 1997), whereas a high level of resistance to insect attacks is exhibited by *Sinapis alba* (L.) (white mustard) (RIPLEY & ARNISON 1990; BROWN *et al.* 1997). White mustard has been reported to be widely resistant to infestation by *Ceutorhynchus obstrictus* (Marsh.) (Coleoptera: Curculionidae) (MCCAFFREY *et al.* 1999 and 2004) and less vulnerable to flea beetle (*Phyllotreta spp*.) (Coleoptera: Chrysomelidae) attacks (BRANDT & LAMB 1993; GAVLOSKI *et al*. 2000). Reduced feeding damage by adults and reduced larval performance for the CSFB on S. *alba* plants, in comparison to *B. napus,* have been reported by DOERING (2012). With regard to the development of pest-resistant oilseed rape cultivars in the last decade, progress has been made in Canada by introgressing *S. alba* DNA into *B. napus*; several of the resulting introgression lines carry genes for resistance to *C. obstrictus* and *Phyllotreta spp.* from the *S. alba* parent (DOSDALL & KOTT 2006; TANSEY *et al.* 2010b).

In this experiment, we tested a total of 14 brassicaceous accessions: 1 susceptible *B. napus* cultivar (Fenja), 2 resistant *S. alba* cultivars (Base and Martigena) and 11 introgression lines (*S. alba* x *B. napus*) for differences in host plant suitability for the CSFB. Antixenotic effects of all accessions were determined in a laboratory no-choice test. Special emphasis was placed on the relationship between the feeding activity of beetles and the GSL content of seeds. Moreover, we assessed trichome density, size and dry weight of cotyledons as potential plant characteristics of resistance.

Material and Methods

Insects: Third instar larvae of *P. chrysocephala* (CSFB) were collected from a non-insecticide-treated oilseed rape field (cultivar Visby) in southern Lower Saxony around the city of Goettingen (Germany) in March 2015. Larvae that had burrowed within the petioles were transferred to small plastic vessels filled with 6 cm soil substrate consisting of loamy soil and sand mixed in a 2:1 ratio [vol:vol]. Cups were closed and stored at room temperature and the larvae were fed with petioles of the oilseed rape cultivar Mozart until they were ready to pupate in the soil. Adult CSFBs emerged about three weeks after pupation. They were fed for 48 h with leaves of the oilseed rape cultivar Mozart. In this experiment, we only used beetles that emerged within a time span of 48 h. Before the experiment began the beetles were starved for 48 h and their sex was determined according to morphological characteristics, as described by COOK *et al.* (2006).

Experimental setup: A total of 14 brassicaceous accessions were tested: 1 oilseed rape cultivars (*B. napus*: Fenja), 2 white mustard cultivars (*S. alba*: Base, Martigena) and 11 introgression lines (*S. alba* x *B. napus*). Single seeds of all accessions were sown in transparent vessels (500 ml, Polypropylen). The soil substrate consisted of potting soil (Fruhsdorfer Erde, Typ 25), loamy soil and sand in a 2:1:1 ratio [vol:vol]. Plants were grown under greenhouse conditions. For the feeding experiment we used 15 replicate plants per accession and to determine seedling characteristics we additionally grew 35 replicate plants per accession.

Adult feeding: The experiment was conducted under controlled conditions at 18°C ± 2°C, with 65-80% relative humidity and a 16:8 h (L:D) photoperiod. Test vessels were arranged in a randomized complete block design with 15 replicate plants per accession. The experiment started 5 days after the seeds had been sown, when seedlings had fully developed cotyledons. Two adult CSFB (1♀:1♂) were released into each test vessel. Vessels were closed with a plastic gauze cover and the feeding damage of cotyledons by adults was scored 24 h, 48 h and 72 h after beetle release using a semi-quantitative scale, ranging from 0% = no feeding to 100% = cotyledons completely consumed (death of plant), subdivided into 5% intervals of consumed cotyledon area. Furthermore, the survival rate of CSFBs was documented. Plants were sprinkled with H_2O water daily to supply sufficient humidity to beetles.

Plant characteristics: The trichome density of all accessions was measured for 15 replicate non-infested seedlings per accession, 6 days after sowing, by cutting a leaf disc of

0.25 cm² (one leaf disc per cotyledon) with a cork borer. The number of trichomes was counted from the upper surface of leaf discs under a microscope (Zeiss, Stemi 2000-C, Germany). To measure the size of the cotyledons (cm²) of 20 non-infested seedlings per accession, an area meter (LI-COR Inc., LI-3100C, USA) was used. Subsequently, the dry weight of these seedlings (cotyledons and stems) was documented. Therefore, seedlings were cut at soil surface; the cotyledone surface (cm²) was measured; and the plant material was dried at 60˚C for 48 h. Seedling dry weight (mg DW/seedling) was recorded using a microbalance (Satorius, MC 5, Germany).

Glucosinolate analysis: Glucosinolate (GSL) contents were extracted from milled samples (Krups, KM 75, Germany) of 1.5 g seeds. The extraction of desulphoglucosinolates followed the protocol of CLEEMPUT & BECKER (2011). Individual desulphoglucosinolates were analysed using a high-performance liquid chromatography (HPLC) (Shimadzu, Germany) equipped with a Nucleodur 100-3 C18 column (Macherey-Nagel, Germany) and separated using a water-acetonitrile gradient (solvent A water, solvent B acetonitrile; phase 1: 0-20 min 1-20% B; phase 2: 20-25 min 20% B; phase 3: 25-27 min 20% B; phase 4: 27-34 min 1% B) at a flow rate of 0.6 ml/min. Desulfoglucosinolates were identified by the retention times of known standards. Glucosinolate contents are expressed as µmol/g seed.

Statistical analysis: The variations in cotyledon area consumed by adults and dry matter weight of seedlings between accessions were tested using a mixed model analysis of variance (ANOVA) (PROC Mixed) (SAS 9.4, SAS Institute, USA). The effect of genotype on variation in trichome density was also tested using a mixed model analysis (ANOVA); only accessions with trichomes were included in this analysis. Normality and homogeneity of variance were verified by inspecting the residuals (QQ-plots). Data of adult feeding damage were arcsine transformed and trichome density and dry matter weight of plants were square root transformed to meet the assumption of the statistical models. Because all plants were completely destroyed by adult feeding in two accessions 72 h after beetle release, these were excluded from the final analysis. The level of significance was set at a confidence interval of 95%. To identify differences between accessions, an a-posteriori Tukey test was used and p-values of multiple comparisons were adjusted, in accordance with Tukey. Since bulk samples of seeds were used for the GSL analyses, results are presented in a descriptive form. Spearman's rank-order correlations were used to analyse the relationship between cotyledon area consumed by adults and trichome density, dry

matter weight, cotyledon area and GSL content, respectively. A multiple regression analysis was used to determine the impact of morphological plant characteristics (trichome density, dry matter weight, cotyledon area) on feeding damage by CSFBs. Factors that have been proven to be significant explanatory variables were included in the model via a stepwise selection technique. The survival rate of test plants was calculated based on the scoring results of adult feeding. The Kruskal-Wallis test and the Holm-Procedure (Makro OWL.sas; BRUNNER & LANGNER 1999), with a t-approximation for non-parametric data, were used to identify significant differences between plant survival of accessions 24 h, 48 h and 72 h after the release of the beetles.

Results

Susceptibility to feeding by *P. chrysocephala*: The cotyledon area consumed by adult beetles was significantly higher for the introgression line IL_186 line IL_1330 after 24 h, for the introgression lines IL_265, IL_165 and IL_186 after 48 h and for the introgression line IL_165 after 72 h compared to the *S. alba* cultivars Base and Martigena. Furthermore, 72 h after beetle release, the cotyledon area of all seedlings of the introgression line IL_165 and IL_186 were completely consumed by the beetles. At the end of the experimental period (72 h), the feeding damage of eight introgression lines (IL_100, IL_114, IL_183, IL_130, IL_140, IL_108, IL_129 and IL_181) and Fenja was not significantly different from the *S. alba* cultivar Base. The mean proportion of cotyledon area consumed by adults ranged from 3.57% (± 0.48%) to 37.80% (± 10.56%) after 24 h, from 18.53% (± 7.69%) to 71.67% (± 10.17%) after 48 h and from 42.3% (± 9.57%) to 100% (± 0.00%) by the end of the experiment (72 h) (Tab. 1). Moreover, 24 h after beetle release, the feeding damage of the introgression lines IL_100, IL_114 and IL_183 did not significantly differ from the *S. alba* cultivar Base, but the values were clearly below the feeding damage of the *B. napus* cultivar Fenja (Tab. 1).

Table 1: Consumed cotelydon area (%) of different brassicaceous accessions by *P. chrysocephala* 24 h, 48 h and 72 h after beetle release (no-choice test). Different letters within columns indicate significant differences (ANOVA, Tukey test, $p \leq 0.05$) between accessions after 24 h; 48 h and 72 h. Data are presented as arithmetic means ± SE.

Accession	Consumed cotyledon area (%) after 24 hours (means ± SE)	Consumed cotyledon area (%) after 48 hours (means ± SE)	Consumed cotyledon area (%) after 72 hours (means ± SE)
Fenja	18.63 (± 6.28) AB	58.00 (± 10.03) ABC	72.33 (± 9.11) ABC
Base	3.57 (± 0.48) B	18.53 (± 7.69) C	42.30 (± 9.57) C
Martigena	3.77 (± 0.80) B	38.70 (± 10.47) BC	55.83 (± 11.63) BC
IL_100	8.83 (± 2.39) AB	58.17 (± 11.15) ABC	73.00 (± 10.24) ABC
IL_114	10.13 (± 2.49) AB	60.15 (± 11.01) ABC	78.67 (± 9.07) ABC
IL_183	10.33 (± 4.40) AB	67.40 (± 11.32) ABC	83.67 (± 8.80) ABC
IL_130	15.00 (± 2.88) AB	56.13 (± 10.71) ABC	84.33 (± 8.51) ABC
IL_140	19.17 (± 6.30) AB	38.00 (± 10.31) ABC	91.00 (± 6.14) AB
IL_108	20.13 (± 6.93) AB	61.82 (± 11.28) AB	88.50 (± 7.86) AB
IL_265	25.33 (± 8.08) AB	67.00 (± 10.31) A	96.00 (± 2.77) A
IL_165	30.47 (± 9.73) AB	62.40 (± 11.44) A	100.00 (± 0.00) -
IL_129	30.77 (± 9.65) AB	65.50 (± 10.97) AB	88.83 (± 7.66) AB
IL_181	32.43 (± 11.09) AB	58.07 (± 11.85) ABC	72.80 (± 10.47) ABC
IL_186	37.80 (± 10.56) A	71.67 (± 10.17) A	100.00 (± 0.00) -
DF	13.00	13.00	11.00
F	6.25	4.02	4.31
p	<0.001	<0.001	<0.001

Plant characteristics: Due to the absence of trichomes on the cotyledons of the majority of the accessions, this factor could only be analysed for the standard cultivar Fenja, the *S. alba* cultivars Martigena and Base and the introgression line IL_183. The trichome density significantly differed between the four accessions (F 30.11, $p < 0.001$). The *S. alba* cultivars Martigena and Base had significantly higher trichome densities on the cotyledons (on average > 3.80 trichomes per cm²) than the standard cultivar Fenja (2.68 trichomes per cm²) and the introgression line IL_183 (2.66 trichomes per cm²), respectively. By grouping the accessions into two sub sets: with trichomes (Fenja Martigena, Base and IL_183) and without trichomes (IL_100, IL_114, IL_130, IL_140, IL_108, IL_265, IL_165, IL_129, IL_181, IL_186) and considering the accessions as replicates, a significantly higher mean cotyledon damage was found for accessions without trichomes (F 8.55, $p = 0.013$).

The dry weight of seedlings (mg DW/seedling) varied significantly between accessions (F 31.82, $p < 0.001$). The highest dry weight was found for the *S. alba* cultivars Martigena and Base, with an average of > 5.96 mg DW/seedling, while the lowest weight

was measured for the introgression line IL_186 (2.68 mg DW/seedling) (Fig. 1). The cotyledon area varied significantly between the accessions (F 17.33, $p < 0.001$). The cotyledon area of the standard oilseed rape cultivar Fenja (1.30 cm² ± 0.05 cm²) was significantly smaller than the cotyledon area of the *S. alba* cultivars Base and Martigena and those of the introgression lines IL_130, IL_140 and IL_129 (Fig. 1). The smallest mean cotyledon area was recorded for IL_186. The cotyledons of the *S. alba* cultivar Base were significantly larger than the cotyledons of all of the introgression lines and Fenja.

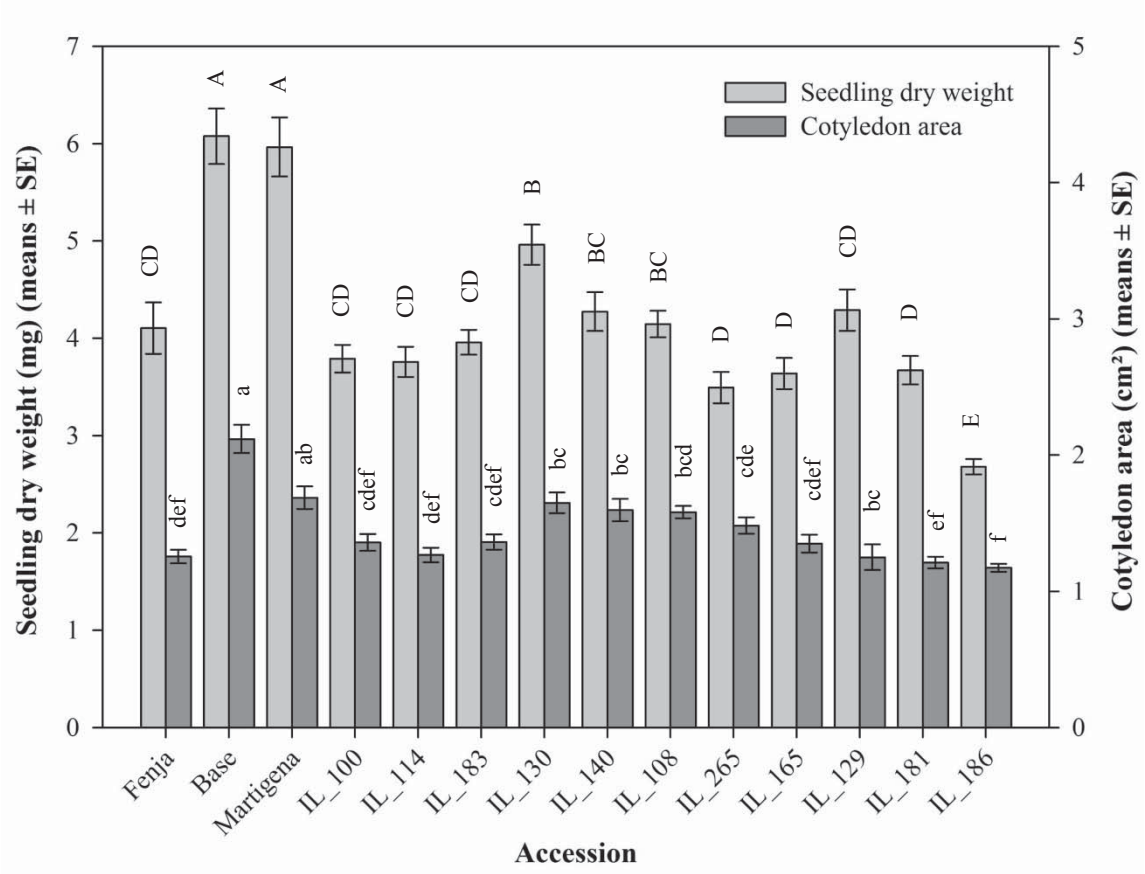


Figure 1: Seedling dry weight (mg) and cotyledon area (cm²) of non-infested seedlings of the accessions tested in the no-choice feeding experiment. Different letters indicate significant differences in the seedling dry weight (upper case letters) and cotyledon area (lower case letters) (ANOVA, Tukey test, $p \leq 0.05$). Data are presented as arithmetic means ± SE.

The seedling dry weight was positively correlated with the cotyledon area ($r = 0.83$, $p = 0.003$). We found no significant interaction between the cotyledon area and feeding by adults, whereas the dry matter of seedlings was negatively correlated to feeding damage from CSFBs ($r = -0.59$, $p = 0.027$). Furthermore, trichome density had a significant, negative correlation with feeding damage from CSFBs ($r = -0.66$, $p = 0.010$). The seedling dry weight and trichome density were included in the multiple regression model as

explanatory variables and together, explain 78% ($R^2 = 0.78$, $p < 0.001$) of the variation of cotyledon feeding damage achieved by CSFBs (Tab. 2).

Table 2: Results of stepwise multiple regression analysis of seedling dry weight (DW), trichome number and cotyledon area on feeding damage by adult *P. chrysocephala*. Standardised regression coefficients are given for the variables.

	df	SS	MS	F	P > F	Regression-coefficent (standardised)	adj. R^2
Regression model	3	1361.38014	680.69007	24.10	< 0.001		0.78
Intercept					< 0.001	0	
Seedling DW					0.008	-0.576	
Trichome no.					0.047	-0.403	
Residuals	11	310.73565	28.24870				

The HPLC analysis of seeds from all 14 accessions revealed a total of 14 different GSL compounds (Tab. 3). The highest total GSL content were found for the *S. alba* cultivars Base (63.05 µmol/g seeds) and Martigena (63.05 µmol/g seeds), with sinalbin (SIB) accounting for 93.55% and 94.98% of the total GSL content in seeds, whereas the cultivar Fenja exhibited the second lowest total GSL content (4.67 µmol/g seeds). For all introgression lines and the oilseed rape cultivar Fenja, aliphatic GSL was the predominant group, followed by indolic compounds; meanwhile, the content of the aromatic GSL gluconasturtiin (NAS) and SIB were below 10% of the total GSL content in all introgression lines and the standard cultivar Fenja. Aromatic GSLs could not be detected in the introgression lines IL_183 and IL_165 and were extremely low in the introgression line IL_100. The cultivar Fenja and the introgression line IL_114 had high indol/alkenyl ratios (> 0.95), reflecting a balanced proportion of indolic and aliphatic GSL in seeds. Conversely, we determined a lower indol/alkenyl ratio in seeds for the introgression line IL_100 (0.45), indicating the higher proportion of aliphatic GSL.

The GSL glucobrassicanapin (GBN) was the predominant aliphatic GSL in the majority of introgression lines and Fenja, with the lowest values found in the introgression line IL_129 and the highest values found in IL_100 (Tab. 3). Seeds of Fenja and of all introgression lines also varied widely with regard to their concentration of gluconapin (GNA), with the highest values found for introgression lines IL_181, IL_186 and IL_130 and the lowest values found for the introgression line IL_129. The predominant indolic GSL in seeds of all introgression lines and Fenja was 4-hydroxy-glucobrassicin (4OH), followed by glucobrassicin (GBC). Moreover, 8 out of 11 introgression lines contained small amounts of SIB (0.03 to 0.28 µmol/g), which was the dominant GSL of *S. alba*. In

addition to the predominant aromatic GSL SIB, seeds of the *S. alba* cultivars contained the highest amounts of the aliphatic GSLs sinigrin (SIN) and glucoraphanin (RAA) and for the indolic GSLs, especially high amounts of 4OH and GBC (Tab. 3).

The content of aliphatic GSLs was positively correlated with mean feeding damage (24 h - 72 h) scores ($r = 0.66$, $p = 0.011$) as well as with damage scores 48 h ($r = 0.72$, $p = 0.004$) and 72 h after beetle release ($r = 0.64$, $p = 0.013$). The content of the single aliphatic GSL gluconapoleiferin (GNL) exhibited a negative correlation with feeding scores 24 h after insertion of beetles ($r = -0.55$, $p = 0.040$). Likewise, the aromatic GSL SIB was negatively correlated with feeding scores 24 h after beetle release ($r = -0.61$, $p = 0.002$) and overall damage scores (24 h - 72 h) ($r = -0.56$, $p = 0.037$). The total amount of aromatic GSLs was negatively correlated with adult feeding scores 24 h ($r = -0.54$, $p = 0.048$) and 48 h ($r = -0.57$, $p = 0.034$) after beetle release.

The survival rate of plants as an indicator of their tolerance to feeding by CSFBs differed significantly among accessions 24 h (χ^2 26.74, $p = 0.014$), 48 h (χ^2 21.33, $p = 0.05$) and 72 h (χ^2 45.46, $p < 0.001$) after beetle release. Feeding on the *S. alba* cultivars Martigena and Base was both delayed (see Tab. 1) and less severe, resulting in a lower proportion of dead plants (Fig. 2). In contrast, adult feeding on the introgression lines IL_186 and IL_165 led to a higher number of dead plants 48 h after release of beetles and to 100% mortality of plants by the end of the experiment (72 h). The survival of plants of the introgression lines IL_186 was significantly lower than those of the *S. alba* cultivars Base and Martigena both 48 h and 72 h after beetle release. Furthermore, survival of the introgression lines IL_130, IL_129, IL_108, IL_183, IL_265, IL_165 and IL_140 was significantly reduced compared to the *S. alba* cultivar Base 72 h after beetle release. At this time, the survival of plants of the introgression lines IL_186 and IL_165 was also significantly lower than the survival of the *S. alba* cultivar Martigena.

Accessions did not affect the survival of beetles, since all beetles survived independent of the accession.

Table 3: Contents of total glucosinolates (GSL) and single GSL in seeds of different brassicaceous accessions (µmol/g seeds). Data are presented as results of bulk sample analyses.

Cultivar or Accession	Total GSL	Aliphatic GSL	Indolyl GSL	Aromatic GSL	I/A	Aliphatic GSL									Indolyl GSL				Aromatic GSL	
						IBE	PRO	SIN	GNL	RAA	RAE	GNA	GBN	ERU	4OH	GBC	4ME	NEO	SIB	NAS
Fenja	4.67	2.35	2.23	0.09	0.95	0.15	0.00	0.00	0.00	0.04	0.00	0.35	1.80	0.00	1.96	0.20	0.04	0.03	0.00	0.09
Base	61.96	3.18	0.82	57.97	0.26	0.09	0.12	2.65	0.03	0.28	0.00	0.00	0.00	0.00	0.44	0.32	0.03	0.03	57.97	0.00
Martigena	63.05	2.31	0.85	59.88	0.37	0.20	0.12	1.68	0.00	0.31	0.00	0.00	0.00	0.00	0.44	0.36	0.03	0.02	59.88	0.00
IL_100	8.79	6.03	2.68	0.07	0.45	0.13	1.52	0.25	0.07	0.01	0.07	1.51	2.47	0.00	2.52	0.17	0.00	0.00	0.07	0.00
IL_114	4.93	2.34	2.29	0.30	0.98	0.17	0.00	0.00	0.00	0.06	0.00	0.67	1.44	0.00	2.12	0.12	0.03	0.01	0.28	0.03
IL_183	9.04	5.81	3.22	0.00	0.55	0.04	2.47	0.00	0.30	0.27	0.00	2.29	0.45	0.00	2.91	0.30	0.00	0.01	0.00	0.00
IL_130	8.42	4.74	3.47	0.20	0.73	0.14	0.00	0.10	0.00	0.05	0.00	2.54	1.92	0.00	2.88	0.58	0.00	0.01	0.12	0.08
IL_140	5.32	2.96	2.28	0.08	0.77	0.28	0.00	0.00	0.00	0.07	0.00	0.75	1.86	0.00	2.14	0.10	0.03	0.01	0.05	0.03
IL_108	8.35	5.06	2.98	0.30	0.59	0.18	1.81	0.00	0.00	0.06	0.00	1.39	1.62	0.00	2.76	0.22	0.00	0.00	0.23	0.07
IL_265	6.56	4.41	2.07	0.08	0.47	0.14	1.12	0.00	0.00	0.05	0.01	1.14	1.95	0.00	1.91	0.13	0.02	0.01	0.08	0.00
IL_165	6.54	4.49	2.05	0.00	0.46	0.00	0.00	1.54	0.00	0.00	0.00	1.17	1.78	0.00	1.94	0.11	0.00	0.00	0.00	0.00
IL_129	0.70	0.44	0.20	0.06	0.47	0.01	0.15	0.00	0.00	0.00	0.01	0.08	0.19	0.00	0.20	0.01	0.00	0.00	0.05	0.01
IL_181	7.60	4.50	2.51	0.59	0.56	0.26	0.00	0.09	0.00	0.06	0.00	2.51	1.58	0.00	2.25	0.22	0.03	0.01	0.03	0.56
IL_186	8.45	5.22	3.02	0.21	0.58	0.14	0.00	0.18	0.00	0.06	0.00	2.51	2.33	0.00	2.61	0.35	0.04	0.01	0.00	0.21

I/A = Ratio of Indolyl GSL/Aliphatic GSL; IBE = glucoiberin; PRO = progoitrin; SIN = sinigrin; GNL = gluconapoleiferin; RAA = glucoraphanin; RAE = glucoraphenin; GNA = gluconapin; GBN = glucobrassicanapin; ERU = glucoerucin; 4OH = 4-hydroxyglucobrassicin; GBC = glucobrassicin; 4ME = 4-methoxy-glucobrassicin; NEO = neoglucobrassicin; SIB = sinalbin; NAS = gluconasturtiin.

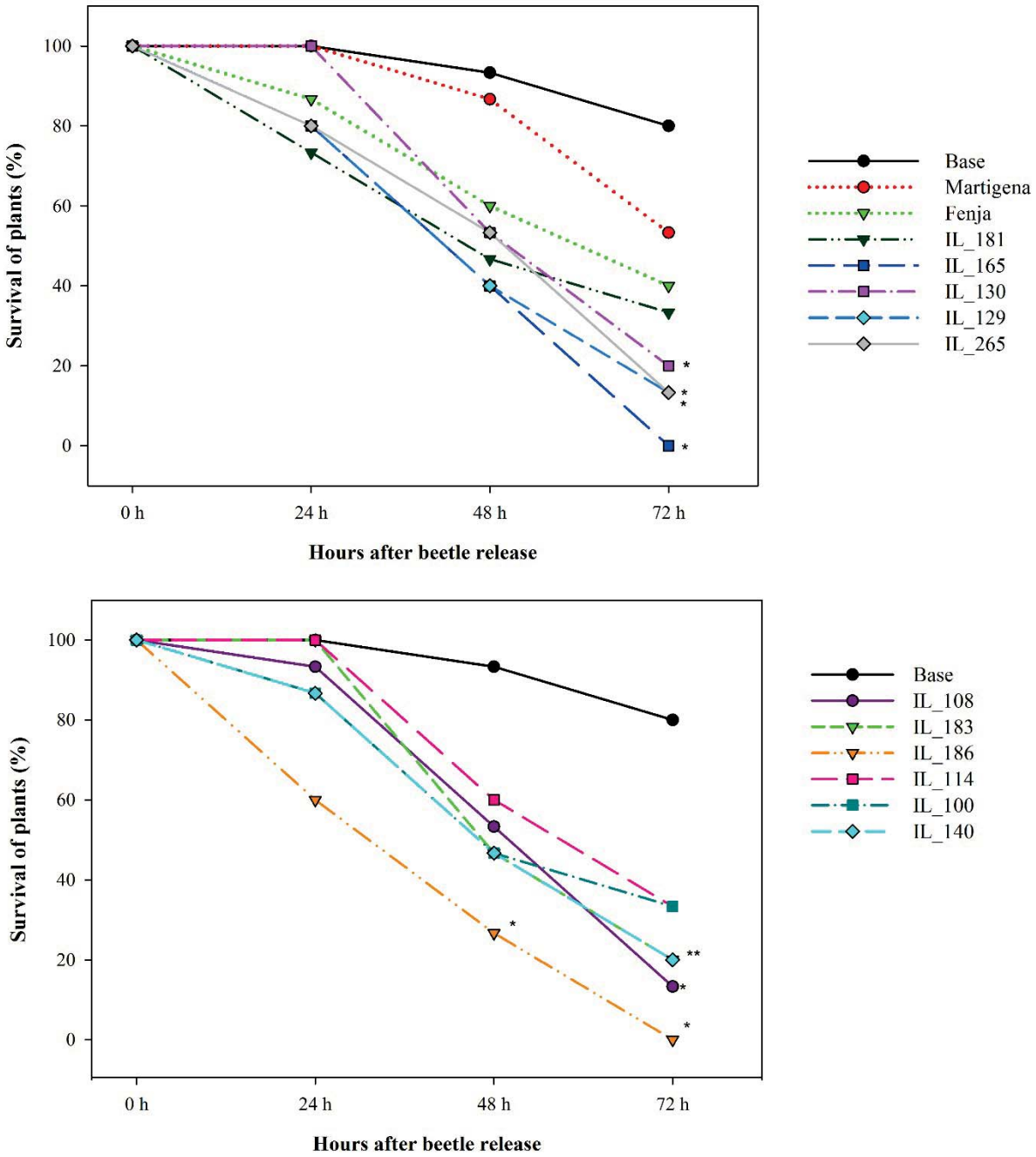


Figure 2: Plant survival (%) of 14 accessions tested in the no-choice feeding test of adult *P. chrysocephala* beetles. Differences between the *S. alba* cultivar Base and test accessions were analysed 24 h, 48 h and 72 h after beetle release. Significant differences to the *S. alba* cultivar Base are marked with asterisks (Kruskal-Wallis test, t-approximation for non-parametric data, *$p \leq 0.05$).

Discussion

The aim of this investigation is to evaluate differences in the host suitability of *B. napus*, *S. alba* and introgression lines (*S. alba* x *B. napus*) for adult CSFB feeding, with special emphasis on the relationship between GSL content in seeds and feeding activity of adult beetles. Although we did not detect any significant differences, damage as a result of adult CSFB feeding was lower for both *S. alba* cultivars, compared to the susceptible *B. napus* cultivar Fenja. The highest damage level was recorded for the introgression lines IL_165 and IL_186, with the cotyledons completely destroyed 72 h after beetle release.

In former investigations, damage of *S. alba* seedlings by cabbage flea beetles (*Phyllotreta spp*.) was significantly reduced (PALANISWAMY *et al*. 1992; BRAND & LAMB 1993; SOROKA & GRENKOW 2013) and antixenosis was considered to be the main resistance mechanism. BODNARYK & LAMB (1991) have emphasized that the higher level of tolerance of *S. alba* to flea beetle attacks is enhanced by its rapid compensatory growth. The observed differences in plant survival indicate that *S. alba* is more capable of withstanding feeding by adult cabbage stem flea beetles. *B. napus* has been described to be the least tolerant species to *Phyllotreta spp.* attack without any compensatory growth effects (BRANDT & LAMB 1993). In our study, feeding damage on the *B. napus* standard cultivar was higher than on both *S. alba* cultivars, but the differences were not significant. Moreover, plant mortality for the *B. napus* standard was not significantly higher than for the *S. alba* cultivar Base. PUTNAM (1977) has reported that feeding by *Phyllotreta spp*. on *S. alba* was initiated, but that it did not continue, indicating that herbivore-induced repellence effects might be responsible for the lower susceptibility of *S. alba* seedlings. However, this observation could not be confirmed over the course of our investigation, as feeding by CSFB on plants of *S. alba* and the introgression line IL_100 strongly increased from 48 h to 72 h (Fig. 3). These findings may be related to antixenotic properties of the cotyledons that might decrease with increasing plant age (BODNARYK & LAMB 1991).

By scoring the feeding damage of cotyledons repeatedly and in time intervals of 24 h, it was possible to distinguish the susceptibility of plants over time. Our bioassay revealed that none of the introgression lines was less damaged than the oilseed rape cultivar Fenja after 72 h. Feeding damage of the introgression lines IL_100, IL_114 and IL_183, however, was clearly lower than for the cultivar Fenja after 24 h. Nevertheless, even slight variations in the susceptibility of plants may enhance their fitness (BODNARYK 1992). Thus, accessions that suffer less attacks within the first days after seedling

emergence might have an advantage in growth and are consequently, more able to tolerate the ongoing feeding of beetles under field conditions. The lack of significant differences in the cotyledon area consumed by adults between accessions might partially be explained by the physiological state of the beetles. We used pre-aestivation beetles, which have to accumulate fat-resources before their diapause and subsequent reproduction. Consequently, their feeding behaviour might have been less selective and potential differences in host suitability might have been masked (BERNAYS & CHAPMAN 1994). Under field conditions, adult CSFBs immigrate to oilseed rape crops after aestivation, when they have already terminated the first feeding period (SCHULZ 1985). Therefore, screening for host plant suitability by using post-aestivation beetles might be more appropriate for the identification of slight variations between accessions.

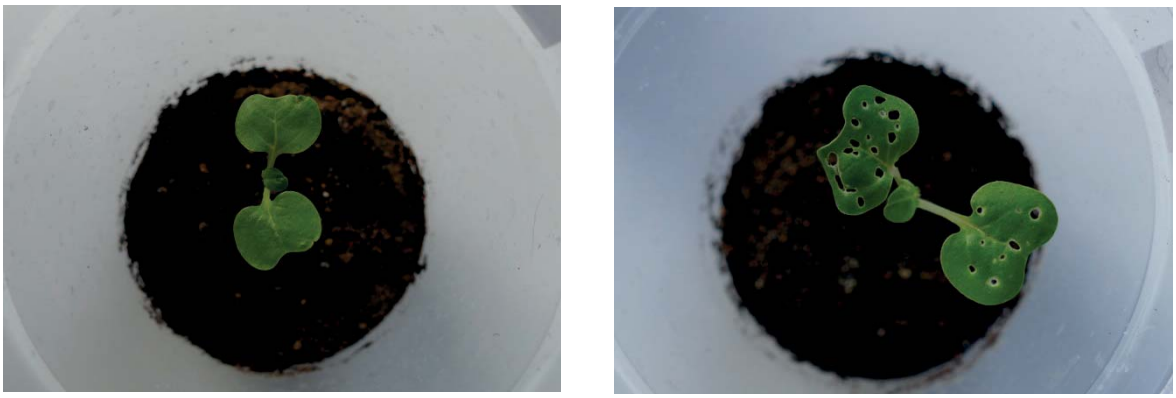


Figure 3: Seven-day-old brassicaceous seedlings. Plant without infestation of *P. chrysocephala* (left); damage pattern, 48 h after infestation by *P. chrysocephala* (right).

The content and composition of GSLs in cotyledons has been found to be quite similar to that of seeds (PALMER *et al.* 1987; MCGREGOR 1988). During subsequent plant growth, however, the content and the composition of GSLs in plant tissues changes drastically (BENNETT & WALLSGROVE 1994; VELASCO *et al.* 2007). In general, GSLs and their metabolites are known to provide important host plant stimuli for many specialist herbivores of brassicaceous crops, e.g. *Phyllotreta spp.* (LIBLIKAS *et al.* 2003; HENDERSON *et al*. 2004) and *D. radicum* (ROESSINGH *et al.* 1992; FELKL *et al.* 2005). Specific GSLs and their breakdown products are described as feeding stimuli for CSFB and are essential for host plant acceptance, although stimulating effects differ between single GSLs. Furthermore, the use of GSLs to promote resistance to CSFB has been repeatedly discussed (GIAMOUSTARIS & MITHEN 1995; BARTLET *et al.* 1999a). BARTLET & WILLIAMS (1991) have noted that adult CSFB feeding is highly stimulated by GBC, while SIN is less stimulating. Furthermore, SIB as the predominant GLS of *S. alba* has been found to be

responsible for the lower susceptibility of Brassicas to insect pests (BODNARYK 1991). According to SOROKA & GRENKOW (2013), a reduction to the SIB contents in seedlings of *S. alba* resulted in a flea beetle attack similar to that of *B. napus* and *B. rapa*. BODNARYK (1997) has further reported that lines of *S. alba* that differ in SIB concentration within the cotelydons by a thousand-fold had similar levels of antixenotic resistance to *Phyllotreta crucifera* (Goeze.) feeding, indicating that the resistance of *S. alba* is independent of the quantity of SIB in plant tissues. The GSLs GNA and GBN were detected in the seeds of Fenja as well as in the introgression lines, but not in *S. alba*. Furthermore, we did not identify SIB in the seeds of the cultivar Fenja and found only small amounts in 9 out of the 11 introgression lines. The observation that introgression lines combine the GSLs of *B. napus and S. alba* has also been made by BROWN *et al.* (1997).

The content of aliphatic GSLs in seeds was positively correlated to the feeding of adult CSFBs. This observation confirmed previous reports on the stimulating effects of aliphatic GSLs on the feeding of CSFBs (BARTLET 1996; BARTLET *et al.* 1999a). In contrast, the content of GNL in seeds of the introgression lines IL_183 and IL_100 was negatively correlated with the feeding damage caused by adult CSFBs. This is in accordance with previous data documenting a negative relationship between the GNL content of the leaf lamnia of various oilseed rape accessions in the five leaf stage and the feeding activity of adult cabbage stem flea beetles (HENNIES 2012, unpubl. MSc thesis, Goettingen). The GSL levels in seeds are quite similar to the GSL levels of the young seedlings the beetles encountered at the beginning of the experiment. However, according to KORITSAS *et al*. (1991) and BARTLET *et al.* (1999b), the content and composition of GSLs changed due to the feeding of CSFBs and subsequent myrosinase activity. Thus, future research should analyse the GSL content of cotyledons at the start and at the end of the experiment. It is well known that GSLs are important stimuli for the CSFB (e.g. BARTLET *et al.* 1994), but the influence of other secondary and primary plant compounds should also be investigated. Secondary substances such as alkaloids, phenol-derivates and saponines have been found to be involved in plants' resistance mechanisms to flea beetles (NIELSEN 1989; BARTLET &WILLIAMS 1991; BODNARYK 1997). Moreover, the sugar content of plant tissues has been repeatedly described as a feeding stimulant to Brassica specialists (BARTLET *et al.* 1994; HERVÉ 2014). Therefore, variations in the sugar content of cotyledons of our accessions on CSFB feeding should be investigated in further experiments.

While no trichomes were detectable on the majority of genotypes, the accessions IL_183, Fenja, Martigena and Base exhibited different levels of hairiness. Trichome density was negatively correlated with feeding damage caused by adult beetles. It has further been shown that high trichome densities can have adverse effects on pest species, like *Pieris rapae* L. (Lepidoptera: Pieridae) (SOUTHWOOD 1986; AGREN & SCHEMSKE 1994; SCHOONHOVEN *et al.* 2005). According to SOROKA *et al.* (2011), adult cabbage flea beetles preferred hairless accessions of canola over pubescent plants. Host acceptability of *Phyllotreta spp.* is not only influenced by the number of trichomes but also by the trichome type, e.g. stiff or soft (LAMB 1980). Our study revealed that the dry weight of seedlings is negatively correlated to the feeding damage caused by CSFBs on cotyledons. Conversely, no interaction between the cotyledon area and the feeding damage was found. In future experiments, these plant characteristics should be measured directly at the beginning and at the end of the experiment, to validate their influence on the feeding of adult CSFBs.

In field trials, screening for flea beetle resistance can be accomplished by assessing beetle abundance, measuring plant damage or determining plant survival. These methods, however, do not allow to discriminate between mechanisms of plant resistance, i.e. between antixenosis, antibiosis and tolerance. But this differentiation is mandatory for an effective screening of plant resistance (PALANISWAMY *et al.* 1992). In our experiment, we focused on no-choice tests for host acceptance and feeding suitability of adult beetles, as a no-choice situation is similar to the situation in fields where only one cultivar is available. Moreover, no-choice laboratory experiments under controlled conditions are expected to provide the most reliable results with regard to plant-pest interactions (HERVÉ 2014). Additionally, no-choice tests help to identify potential key plant traits for host plant acceptance or resistance, as the attractiveness of test plants for feeding is not biased by the attractiveness of other plants (HERVÉ 2014). In field trials, we tested an identical set of introgression lines (HENNIES 2015, unpublished data) and the feeding damage by CSFBs revealed the same trend as in our no-choice experiment, indicating the latter approach to be suitable for the evaluation of differences in plant resistance. Additional multi-choice experiments in the laboratory, however, may provide further information on host plant preference and host plant recognition by CSFBs.

In the present study, we confirmed that the *S. alba* cultivars inhibited the feeding of flea beetles, which is in accordance with previous findings. However, we could not detect any strong source of resistance to *P. chrysocephala* feeding within the set of introgression

lines tested. Nevertheless, our no-choice experiments provide useful information on the susceptibility of different genotypes for further screening and indicates the possible influence of pubescence as well as the content and composition of GSLs on infestation by *P. chrysocephala*.

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Chapter V

Screening of *Brassica napus*, *Sinapis alba* and introgression lines for antibiotic resistance to cabbage stem flea beetle larvae (*Psylliodes chrysocephala* L.)

Abstract

The cabbage stem flea beetle (*Psylliodes chrysocephala* (L.)) is an important pest in European winter oilseed rape production (*Brassica napus* (L.)). Although host plant resistance is considered a key element of integrated pest management (IPM) systems, no genotypes resistant to *P. chrysocephala* attacks have been developed thus far. The brassicaceous species *Sinapis alba* (L.) is known to be an inappropriate host for several insect pest species of oilseed rape and might therefore be a potential source of plant resistance in further breeding programs.

The objective of this study is to evaluate the host plant suitability of seven brassicaceous accessions (one *B. napus* cultivar, two *S. alba* cultivars and four introgression lines (*S. alba* x *B. napus*)) to infestation by *P. chrysocephala* larvae in a no-choice laboratory experiment. To determine differences in larval performance between accessions, various parameters of larval development (recovery and survival rate of larvae, larval weight, larval instar) were assessed. Morphological plant traits (tissue toughness, trichome density, petiole dry weight) and the glucosinolate contents of plants were analysed as potential factors affecting host suitability for larvae. We found lower recovery rates of larvae, lower larval weights and prolonged development times of larvae for both *S. alba* cultivars when compared to the oilseed rape cultivar Fenja, indicating antibiotic mechanisms of resistance against *P. chrysocephala*. In contrast, larval performance did not differ between the introgression lines and the cultivar Fenja. Glucosinolate contents exhibited induced changes and increased due to feeding by *P. chrysocephala* larvae.

Introduction

The univoltine cabbage stem flea beetle (*Psylliodes chrysocephala* (L.)) (Coleoptera: Chrysomelidae) (CSFB) can be found throughout the maritime regions of Northern Europe (ALFORD *et al.* 2003). Between late August and early September adults colonize newly emerged crops of winter oilseed rape. After a period of maturity feeding on cotyledons and the leaves of young oilseed rape plants, the females begin to lay eggs in the soil (WILLIAMS 2004). Oviposition may continue throughout autumn and winter if weather conditions are favourable (high relative humidity and 4-16°C) (SCHULZ 1985). A developmental threshold of about 5°C has been determined (MATHIASEN *et al.* 2015). Neonate larvae bore into the leaf petioles near the nodality and mine within the pith tissue; during their development, larvae move to younger leaves and later into the stems (GODAN

1950; SCHULZ 1985). Larval feeding may cause growth reduction and wilting of plants and plant losses may occur if the vegetation point is damaged or if water penetrates the plants via injuries caused by larvae and freezes during the winter (SCHULZ & DAEBLER 1984; NILSSON 2002). The risk of plant loss decreases during stem elongation, as it is difficult for the larvae to reach the elevated vegetation point (SCHULZ 1993; JOHNEN 2002). Moreover, injuries due to larval feeding promote the infection of fungal diseases, such as stem canker (*Leptospheria maculans* (Ces. & De Not)) (BROSCHEWITZ *et al.* 1993). Mature third instar larvae move into the soil to pupate (DOBSON 1960). New generation adults emerge beginning in May, feed on oilseed rape and other *Brassicas* and in midsummer they enter a period of aestivation before they migrate to winter rape crops (ALFORD *et al.* 2003).

To minimize the crop damage caused by larval feeding, the foliar application of pyrethroids is used as soon as the control threshold of three to five larvae per plant is exceeded (ULBER 2007). The efficacy and the non-target risks of these frequently used insecticides, however, are currently under debate. Recent studies have documented the increasing number of CSFB populations with a genetically manifested "knock down resistance" (kdr) to pyrethroids (HØJLAND *et al*. 2015). Thus, alternative control strategies to reduce the use of synthetic insecticides and to minimize their negative impact on the ecosystem are needed (WILLIAMS 2004; COOK *et al.* 2006). Improving insect pest management via resistant cultivars may largely contribute to the establishment of sustainable crop production systems (COOK *et al*. 2006). Traditional plant breeding methods, however, focus more on attaining high yields and quality than on producing pest resistant cultivars and consequently, no cultivars resistant to insect attacks on oilseed rape are currently available (FRAUEN 2011).

Host plant resistance to insect pests generally relies on the plant's being perceived as less attractive for colonization, feeding and egg deposition (antixenosis) and/or on defence-responses of the plant via inhibitory plant secondary metabolites (antibiosis). Antibiotic resistance traits may negatively affect pest survival and development. The larval offspring of insect herbivores may be influenced by both morphological and biochemical resistance traits, and especially neonate larvae are highly susceptible (FARRELL 1977; SCHOONHOVEN *et al.* 2005). For instance, the toughness of the plant tissue may act as a physical barrier interfering with chewing and sucking insects (FÜRSTENBERG-HÄGG *et al.* 2013). This morphological trait, however, has been largely ignored as a factor of resistance interfering with CSFB larvae. It has been reported that CSFB larvae prefer young plant

tissues; this behaviour might have been caused by differences in the nutritional quality of plants or may be related to less developed surface structures (cell walls and surface waxes) (KORITSAS *et al*. 1991). Since hatching larvae must bore into the petioles, it can be assumed that the capacity of larvae to establish in plants with a tougher petiole surface is reduced, resulting in a higher mortality of neonate larvae, as has been described by BERGVINSON *et al*. (1994) for larvae of the European corn borer (*Ostrinia nubilalis* (H.) (Lepidoptera: Crambidae)). Moreover, many studies have reported on the effects of plant pubescence on feeding and oviposition of insect pests on brassicaceous crops (e.g. SCHOONHOVEN *et al*. 2005; SOROKA *et al*. 2011), which may be based on the density, length and stability of trichomes (DALIN *et al.* 2008). Nevertheless, there is currently no information about the impact of trichomes on the performance of CSFB larvae. Furthermore, differences in the host suitability of plants for insect pests may also be linked to differences in biochemical plant characteristics, such as the content and the composition of glucosinolates (GSL) (AHUJA *et al.* 2009).

The objective of this study is to evaluate the host plant suitability of seven brassicaceous accessions for infestation by CSFB larvae. In a number of no-choice laboratory experiments, we evaluated the survival and performance of larvae for a set of seven accessions consisting of a susceptible *B. napus* cultivar, two resistant *S. alba* cultivars and four introgression lines (*S. alba* x *B. napus*). Furthermore, morphological plant traits of the petioles and the GSL content of petiole tissue were analysed as potential factors affecting larval development.

Material and Methods

Insects: New generation adults of the CSFB emerging from soil after pupation were collected from oilseed rape fields in mid-July and kept in plastic boxes (17.5 cm x 13 cm x 6 cm) during their summer aestivation. Moisture and food was provided via fresh leaves of oilseed rape (cultivar Mozart). Termination of aestivation was indicated by an increased feeding activity of adult beetles and female oviposition began approximately one month later. Eggs were transferred to autoclaved sand and stored at 2˚C. Before the experiments began, the eggs were incubated at room temperature; larvae hatched within 7-10 days. The neonate larvae were transferred to a moistened filter paper and stored at 2˚C for a maximum of 24 h, until they were used in the experiments. The vitality of larvae was assessed via their physical activity and only vital larvae were used in the experiments.

Experimental setup: A set of seven accessions were tested: 1 *B. napus* cultivar (Fenja), 2 *S. alba* cultivars (Martigena, Base) and 4 introgression lines (*S. alba* x *B. napus*). The set of introgression lines was selected based on their varying levels of resistance to the cabbage root fly larvae (three "resistant" introgression lines and one "susceptible" introgression line). The *B. napus* cultivar Fenja was used as a susceptible standard.

All plants were sown and raised in a soil substrate consisting of potting soil (Fruhsdorfer Erde, Typ 25), loamy soil and sand in a 2:1:1 ratio [vol:vol]. One week following germination, plants were transplanted into 11 cm plastic pots. Plants were grown under controlled conditions at 19°C ± 2°C, with a photoperiod of 16:8 h (L:D), using artificial light (7000 lux). The plants were fertilized weekly with 75 ml of 0.2% HakaPhos Blau® (15% N, 10% P_20_5, 15% K_20) from the fourth leaf stage onward (15% N, 10% P_20_5, 15% K_20). The experiment consisted of three subsequent runs with an identical experimental setup for the performance of the CSFB larvae (7 accessions; 3 runs x 5 infested replicate plants per accession). Within each run, replicate plants were arranged in a randomized complete block design. Moreover, plants were grown to investigate the GSL content (7 accessions; 5 infested plants, 5 non-infested plants per accession) as well as the petiole toughness, trichome density and dry weight (7 accessions; 10 non-infested plants per accession).

Larval performance: The experiment began when the plants reached the five leaf stage. Seven accessions with five replicate plants per accession were positioned at 25 cm distance from each other to prevent larval migration between plants. Only intact, i.e. undamaged plants, were used in the experiments to avoid induced changes in host plant cues. Five neonate larvae per plant were placed on the petiole of the third leaf at a distance of 1.5 cm to the leaf axil (see Appendix Fig. 2). To provide optimal conditions for larval penetration, plants were sprayed with H_20 (every hour, for a period of > 5 h) and the light was switched off (for a period of > 5 h). Inoculated plants were kept at 19°C ± 2°C with 65-85% relative humidity and a photoperiod of 16:8 h (L:D) (artificial light 7000 lux) for 14 days. To avoid pupation of third instar larvae in the soil, experiments were terminated 14 days after inoculation.

The petioles and the stem of each plant were dissected 14 days after inoculation. The larvae were counted and their vitality (dead, vital) and the weight of the vital larvae were recorded (Satorius micro scale, MC5, Germany). The head capsule width of intact larvae was measured using a stereo microscope (Wild, M3Z, Switzerland) and larval

instars were classified in first instar (L1), second instar (L2) and third instar (L3) according to DOBSON (1960). Based on the number of larvae per plant, the recovery rate (%) (defined as the ratio of larvae burrowing into the plant tissue to inoculated larvae*100) and the survival rate (%) (defined as the ratio of vital larvae feeding in the plant tissue to inoculated larvae*100) were calculated to express differences in larval performance between accessions.

Morphological characteristics: All morphological plant characteristics were assessed for five non-infested replicate plants per accession. The petiole toughness was measured at the five leaf stage of plants using a texture analyser (TA.XT2, Stable Micro Systems Ltd., UK). The petioles of the third and fourth oldest leaf were cut off at the axil and shortened starting from the axial end to 3 cm length. The prepared petiole sections were positioned in the centre of the texture analyzer (see Appendix Fig. 1). A commercial sewing needle (size no. 9, Ø 0.6 mm) was used for penetration. The penetration depth was standardized to 1 mm. Results are the maximum force needed (N/cm²) to fracture the petiole surface (cuticle, layer of wax and epidermis) and the integral of penetration force inside the petiole tissue (up to 0.8 mm penetration depth). The data were analysed using the texture expert program (version 1.10, Stable Micro Systems Ltd., UK). For the same petioles, the number of trichomes on a 1 cm section was recorded under a microscope (Zeiss, Stemi 2000-C, Germany). The width of the petiole at a distance of 1.5 cm from the axial end was measured to calculate the number of trichomes per cm². We additionally determined the dry weight (mg DW) of the petioles. For this measurement, petiole sections were dried for 36 h until they reached a constant weight. The width of the petiole at a distance of 1.5 cm from the axial end was used to calculate the dry weight per cm² of the petiole surface.

Glucosinolate analysis: The GSL contents of the petioles were analysed for five non-infested replicate plants per accessions at the beginning of the experiment (five leaf stage of plants). Due to technical problems, no GSL analysis for the *S. alba* cultivar Martigena was conducted. To consider differences in GSL profiles due to larval damage, five infested and five non-infested replicate plants per accession were analysed at the end of the experimental period (14 days after inoculation). Due to technical problems, no data were available for the introgression line IL_114 at the end of the experiment. Plant samples for the GSL analyses were taken from the petioles of the third and fourth oldest leaf. These plant samples were immediately frozen in liquid nitrogen and stored at -20˚C. The samples

were then freeze dried for 72 h and bulk samples were generated and milled (KM 75, Krups, Germany). Desulfoglucosinolates were extracted and the GSLs were analysed using high-performance liquid chromatography, as described by CLEEMPUT & BECKER (2011). The GSL content is expressed in µmol/g dry weight.

Statistical analysis: The variation in recovery rate, survival rate, larval weight, head capsule width, petiole toughness, petiole dry matter and proportion of third instar larvae between accessions was tested using a mixed model analysis of variance (ANOVA) (PROC Mixed) (SAS 9.4, SAS Institute, USA). To remove the potential bias of unequal sample size between accessions, a weighing factor for the parameters head capsule width and larval weight was included in the model. Normality and homogeneity of variance were verified by inspecting the residuals (QQ-plots). Recovery rates and survival rates of the larvae as well as the proportion of third instar larvae were arcsine transformed and larval weights, head capsule widths and petiole toughness were square-root transformed to meet the assumption of the statistical models. To identify differences between accessions, the a-posteriori Tukey test was used and p-values of multiple comparisons were adjusted, following Tukey. To test for differences between the recovery rates and survival rates of larvae within each accession, a paired t-test was used. Since bulk samples were generated for the GSL analyses, results are presented in a descriptive form. Likewise, data related to the density of trichomes could not be analysed and are presented in a descriptive form.

Spearman's rank-order correlations were used to analyse the relationship between parameters of larval performance (i.e. recovery rate, survival rate, larval weight, head capsule width) and individual plant traits (i.e. petiole toughness, dry weight, GSL content).

Results

Larval performance: At the end of the experiment (14 days after inoculation), we counted on average 3.47 ± 0.22 larvae (vital larvae 3.42 ± 0.22) in plants of the standard *B. napus* cultivar Fenja and 2.73 ± 0.27 larvae (vital larvae 1.47 ± 0.26) and 2.69 ± 0.22 larvae (vital larvae: 1.94 ± 0.27) for the *S. alba* cultivars Martigena and Base, respectively. With regard to introgression lines, the lowest number of larvae was recorded in plants of the introgression line IL_183, with 3.25 ± 0.31 larvae (3.05 ± 0.31 vital larvae), and the highest number of larvae developed in plants of the introgression line IL_129, with 3.75 ± 0.23 larvae (3.75 ± 0.23 vital larvae). The recovery rate of larvae differed significantly between accessions (F 2.17, $p = 0.052$) (Fig. 1), with significantly lower values for plants of the *S. alba* cultivar Base (53.75% ± 5.33%) than for the introgression line IL_129 (75.00% ± 4.56%). There were no differences in recovery rates between single accessions and the standard *B. napus* cultivar Fenja.

There was a positive correlation ($r = 0.93$, $p < 0.001$) between the number of larvae and the number of vital larvae (Tab. 3). The survival rate was significantly lower for both *S. alba* cultivars Martigena (29.33% ± 5.12%) and Base (38.75% ± 5.31%) than for the introgression lines (varying between 61.00% and 75.00%) and the standard cultivar Fenja (68.42% ± 4.41%) (Fig. 1). Within the standard cultivar Fenja, the recovery rate of larvae did not differ from the survival rate, whereas the recovery rate was significantly higher than the survival rate for the introgression line IL_114 ($p = 0.030$) and the *S. alba* cultivars Base ($p = 0.013$) and Martigena ($p < 0.001$) (Fig. 1).

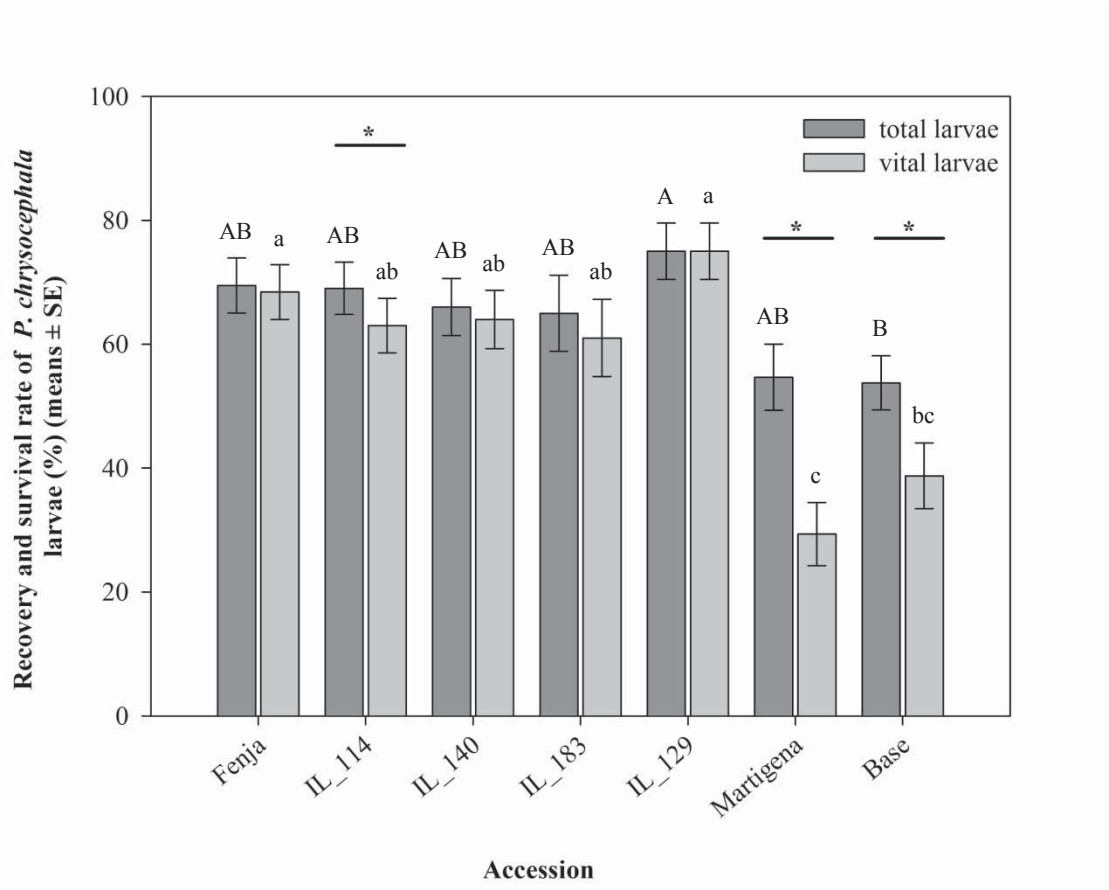

Figure 1: Recovery rate (%) and survival rate (%) of *P. chrysocephala* larvae for different brassicaceous accessions (no-choice test), 14 days after release of five larvae per plant. Different letters indicate significant differences in recovery rates (%) (upper case letters) and survival rates (%) (lower case letters) (ANOVA, Tukey test, $p \leq 0.05$). Significant differences between recovery rates and survival rates of larvae within single accessions are marked with asterisks (t-test, $*p \leq 0.05$). Data are presented as arithmetic means ± SE.

The weight of larvae differed significantly among accessions (F 22.41, $p < 0.001$). The larval weight was significantly lower for the *S. alba* cultivars Martigena and Base than for the cultivar Fenja or the introgression lines (Tab. 1). The highest larval weight was recorded in plants of the introgression line IL_140, which was significantly higher than the larval weight of the *B. napus* cultivar Fenja ($p = 0.034$). The larval weight of the introgression lines IL_183, IL_129 and IL_114 did not significantly differ from the standard cultivar Fenja. Larval weights were positively correlated with the number of vital larvae ($r = 0.79$, $p = 0.01$) (Tab. 3). The head capsule width of larvae differed significantly between accessions (F 15.63, $p < 0.001$), with significantly lower values for larvae recovered in plants of the *S. alba* cultivars than the head capsule width of single introgression lines and the standard cultivar Fenja. For all introgression lines, the head capsule width was higher than for the standard cultivar Fenja (Tab. 1).

Table 1: Weights and head capsule widths of *P. chrysocephala* larvae recovered 14 days after release of five larvae per plant. Different letters indicate significant differences between accessions (ANOVA, Tukey test, $p \leq 0.05$). Data are presented as arithmetic means ± SE.

Accession	Larval weight (mg) (means ± SE)	Head capsule width (mm) (means ± SE)
Fenja	1.69 (± 0.19) B	0.47 (± 0.02) B
IL_114	2.09 (± 0.15) AB	0.52 (± 0.01) AB
IL_140	2.38 (± 0.16) A	0.53 (± 0.01) A
IL_183	1.58 (± 0.16) B	0.49 (± 0.01) AB
IL_129	2.09 (± 0.14) AB	0.51 (± 0.01) AB
Martigena	1.03 (± 0.23) C	0.42 (± 0.02) C
Base	0.62 (± 0.12) C	0.39 (± 0.02) C

In the *B. napus* standard cultivar Fenja and all of the introgression lines, the majority of the larvae had developed into L3 larvae within 14 days (Fig. 2). The proportion of L3 larvae differed significantly between accessions (F 23.08, $p < 0.001$): significantly lower amounts of L3 larvae were found in plants of the *S. alba* cultivars Martigena and Base than in single introgression lines and the standard cultivar Fenja. Moreover, significantly more larvae developed into L3 larvae in plants of the introgression lines than in those of the standard cultivar Fenja, with the introgression line IL_140 exhibiting significantly higher proportions of L3 larvae than all of the other accessions. In plants of the *B. napus* cultivar Fenja, the proportion of L1, L2 and L3 larvae was 7.35%, 35.30% and 57.35%, respectively. For the introgression lines, the proportion of L1 and L2 larvae ranged between 0.00% and 4.69% and between 10.45% and 26.56%, respectively, with a high proportion of L3 larvae (on average > 68.75%). In plants of the *S. alba* cultivars Base and Martigena, less than 30.00% of the larvae reached the third instar stage, with consequently high proportions of L1 (Base: 29.27% and Martigena: 17.50%) and L2 larvae (Base: 51.22% and Martigena: 55.00%).

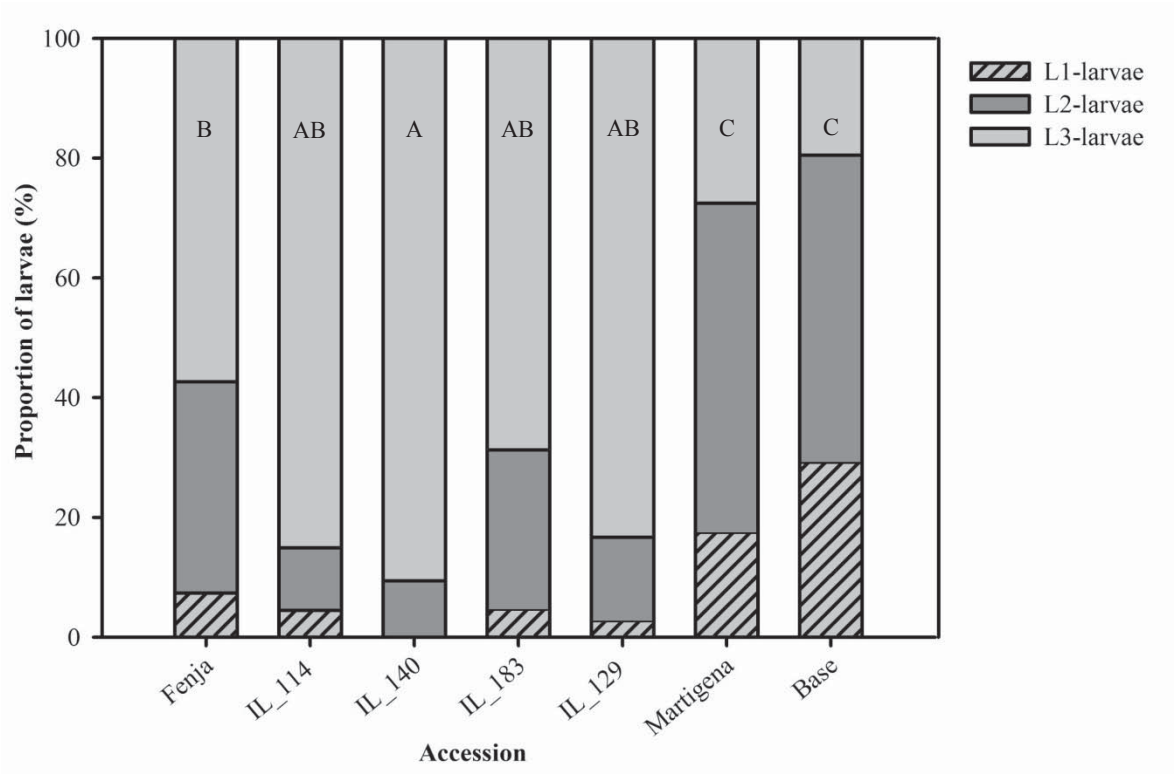

Figure 2: Proportion of the three larval instars of *P. chrysocephala* for different brassicaceous accessions 14 days after the release of five larvae per plant (L1 = first instar, L2 = second instar, L3 = third instar). Different letters indicate significant differences in proportions of L3 larvae between accessions (ANOVA, Tukey test, $p \leq 0.05$).

Morphological plant characteristics: We found no significant differences between accessions concerning both parameters of petiole toughness (force needed to fracture, integral of force within the plant tissue) (Tab. 2). We detected no trichomes on the petioles of the *B. napus* standard cultivar Fenja nor on three out of the four introgression lines, whereas a sparse hairiness was found for the introgression line IL_ 140 (0.07 trichomes/cm²). In contrast, both *S. alba* cultivars exhibited a relatively higher hairiness, with > 17 trichomes/cm². Moreover, the dry weight of the petioles did not differ among accessions (Tab. 2).

The larval recovery rate was positively correlated with the integral of penetration force inside the petiole tissue ($r = 0.79$, $p = 0.0362$) (Tab. 3), but not with the maximum force needed to fracture the petiole surface. There was also no dependence of larval performance on the dry weight of the petioles.

Table 2: Morphological features (dry weight, force to fracture and integral of force) of petioles for different brassicaceous accessions. Different letters indicate significant differences between the accessions (ANOVA, Tukey test, $p \leq 0.05$). Data are presented as arithmetic means ± SE.

Accession	Dry weight (g/cm²) (means ± SE)	Force to fracture (N/cm²) (means ± SE)	Integral of force (means ± SE)
Fenja	4.90 (± 0.12) A	2.83 (± 0.38) A	0.47 (± 0.01) A
IL_114	6.55 (± 0.54) A	3.43 (± 0.46) A	0.52 (± 0.01) A
IL_140	4.86 (± 1.04) A	2.93 (± 0.32) A	0.53 (± 0.01) A
IL_183	5.31 (± 0.53) A	3.62 (± 0.38) A	0.49 (± 0.01) A
IL_129	6.20 (± 0.53) A	3.29 (± 0.44) A	0.51 (± 0.03) A
Martigena	6.62 (± 0.52) A	2.71 (± 0.27) A	0.42 (± 0.03) A
Base	4.85 (± 0.27) A	2.88 (± 0.31) A	0.39 (± 0.01) A

Table 3: Spearman rank correlation coefficients for different parameters of larval performance and plant parameters (*$p \leq 0.05$).

	No. larvae	No. vital larvae	Recovery rate larvae	Survival rate larvae	Fresh weight larvae	Head capsule width	Petiole DW	Force to fracture petiole	Integral of force in petiole
No. larvae									
No. vital larvae	**0.93***								
Recovery rate larvae	**0.96***	**0.89***							
Survival rate larvae	**0.86***	**0.69***	**0.79***						
Weight larvae	**0.79***	**0.71***	0.64	**0.79***					
Head capsule width	0.68	0.54	0.57	0.57	**0.93***				
Petiole DW	0.31	0.08	0.25	0.08	0.48	0.42			
Force to fracture petiole	0.39	0.32	0.50	0.25	0.35	0.57	0.07		
Integral of force in petiole	**0.75***	**0.82***	**0.79***	0.68	0.32	0.21	0.43	0.39	

Glucosinolate content: At the start of the experiment, aliphatic GSLs, mainly glucoiberin (IBE), progoitrin (PRO) and gluconapin (GNA), were dominant in non-infested plants of the *B. napus* cultivar Fenja and the introgression line IL_183 (> 49%) (Tab. 4), whereas indolic GSLs, mainly glucobrassicin (GBC) and neoglucobrassicin (NEO), were dominant in petioles of the introgression line IL_114 (> 60%). For the introgression line IL_140, proportions of aliphatic and indolic GSL compounds were balanced. The content of aromatic GSLs was low (< 4%) for all introgression lines and Fenja. The initial GSL content of the *S. alba* cultivar Base was on a higher level than for all other accessions (Base: 6.04 µmol/g vs minimal 0.65 µmol/g (IL_129) and maximum 5.03 µmol/g (IL_183)). In petioles of the *S. alba* cultivar Base, the aromatic GSL sinalbin (SIB) accounted for > 90% of total GSLs.

Table 4: Glucosinolate (GSL) contents (µmol/g DW) and proportion of GSL groups (%) of petioles of different brassicaceous accessions at the five leaf stage (start of the experiment). For single GSL belonging to groups, see Appendix Tab. 1. Data derived from bulk sample analyses and are presented as means.

Accession	Total GSL (µmol/g)	Aliphatic GSL (µmol/g)	Indolyl GSL (µmol/g)	Aromatic GSL (µmol/g)	I/A	Aliphatic GSL (%)	Indolyl GSL (%)	Aromatic GSL (%)
Fenja	3.43	2.02	1.34	0.07	0.66	58.94	39.12	1.93
IL_114	1.17	0.43	0.74	0.04	1.73	36.59	63.41	3.02
IL_140	0.68	0.33	0.34	0.01	1.03	48.63	49.98	1.39
IL_183	5.03	3.98	1.00	0.05	0.25	79.18	19.92	0.90
IL_129	0.65	0.07	0.55	0.02	7.38	11.50	84.89	3.61
Base	6.04	0.24	0.02	5.77	0.10	3.96	0.41	95.63

I/A = Ratio of Indolyl GSL/Aliphatic GSL.

We found a positive correlation between the initial content of GBC in petioles and the number of larvae ($r = 0.93$, $p = 0.002$), the number of vital larvae ($r = 0.81$, $p = 0.05$) and the recovery rate of larvae ($r = 0.84$, $p = 0.037$). The content of the GSL NEO was positively correlated with the recovery rate ($r = 0.93$, $p = 0.008$). The proportion of indolic GSLs was positively correlated with the number of larvae ($r = 0.94$, $p = 0.005$), the number of vital larvae ($r = 0.82$, $p = 0.04$), the recovery rate of larvae ($r = 0.82$, $p = 0.04$) and larval weights ($r = 0.82$, $p = 0.04$). The content of the aliphatic GSL gluconapoleiferin (GNL) was negatively related to recovery rate ($r = -0.85$, $p = 0.034$). The content of the aromatic GSL SIB was negatively correlated with larval weights and head capsule width of larvae ($r = -0.94$, $p = 0.005$). The total GSL content was negatively related to the number of larvae ($r = -0.83$, $p = 0.04$), the survival rate of larvae ($r = -0.83$, $p = 0.04$) and larval weights ($r = -0.94$, $p = 0.001$).

The GSL content in petioles of non-infested and infested plants at the end of the experiment (14 days post-inolculation) is presented in Table 5. The total GSL content of all accessions was distinctly higher in infested plants than in non-infested plants, with aliphatic GSLs being the dominant group in non-infested petioles of the cultivar Fenja and the introgression lines (IL_140, IL_183, IL_129). Conversely, infested plants of the cultivar Fenja and the introgression lines had high amounts of indolyl GSLs, indicating a shift in the I/A ratio due to the feeding of CSFB larvae. The feeding of CSFB larvae in petioles of the *S. alba* cultivar resulted in a higher total GSL content, mainly due to a higher content of SIB. High proportions of the aliphatic GSL sinigrin (SIN) were found in petioles of the introgression line IL_183 in the five leaf stage (start of the experiment) as well as in non-infested and infested plants at the end of the experiment.

Table 5: Glucosinolate (GSL) contents (µmol/g DW) and proportions of GSL groups of petioles of non-infested plants (○) and infested plants (+) of different brassicaceous accessions at the end of the experiment (14 days after release of five *P. chrysocephala* larvae per plant). For single GSL belonging to GSL groups, see Appendix Tab. 1. Data are derived from bulk sample analyses and presented as means.

Accession and treatment	Total GSL (µmol/g)	Aliphatic GSL (µmol/g)	Indolyl GSL (µmol/g)	Aromatic GSL (µmol/g)	I/A	Aliphatic GSL (%)	Indolyl GSL (%)	Aromatic GSL (%)
Fenja ○	2.01	1.46	0.46	0.09	5.05	72.80	22.70	4.49
Fenja +	7.93	5.39	2.50	0.05	51.39	67.93	31.46	0.61
IL_140 ○	0.54	0.39	0.14	0.01	13.48	71.77	26.28	1.95
IL_140 +	2.29	0.07	2.21	0.01	310.76	3.05	96.64	0.31
IL_183 ○	3.42	3.19	0.20	0.03	7.76	93.26	5.97	0.77
IL_183 +	9.04	5.76	3.26	0.02	133.88	63.70	36.03	0.27
IL_129 ○	0.21	0.04	0.15	0.02	8.45	17.80	73.50	8.70
IL_129 +	3.37	0.09	3.25	0.03	126.75	2.56	96.68	0.76
Martigena ○	7.52	0.11	0.19	7.21	0.03	1.53	2.57	95.90
Martigena +	8.63	0.25	0.06	8.32	0.01	2.93	0.69	96.38
Base ○	2.93	0.09	0.06	2.79	0.02	2.95	2.09	94.96
Base +	5.30	0.09	0.15	5.06	0.03	1.69	2.76	95.55

I/A = Ratio of Indolyl GSL/Aliphatic GSL.

Fourteen days after inoculation of the larvae, the content of GBC in petioles of non-infested plants was positively correlated with the number of larvae ($r = 0.75$, $p = 0.052$), larval weights ($r = 0.82$, $p = 0.023$) and the head capsule width ($r = 0.86$, $p = 0.014$). The proportion of indolyl GSLs was positively related to the number of larvae ($r = 0.86$, $p = 0.014$), the number of vital larvae ($r = 0.86$, $p = 0.014$), the recovery rate ($r = 0.75$, $p = 0.052$), the survival rate ($r = 0.93$, $p = 0.003$) and also the larval development parameters larval weight ($r = 0.93$ $p = 0.003$) and head capsule width ($r = 0.93$, $p = 0.003$). The content of the indolyl GSL NEO was negatively correlated with the number of vital larvae ($r = -0.78$, $p = 0.04$), the survival rate of larvae ($r = -0.89$, $p = 0.002$), the larval weight ($r = -0.93$, $p = 0.003$) and the head capsule width ($r = -0.78$, $p = 0.036$). The content of SIB was negatively correlated with the head capsule width ($r = -0.92$, $p = 0.003$). We found no relationship between the total GSL content and the recovery rate and survival rate of larvae, respectively.

Fourteen days after inoculating the larvae, the proportion of indolic GSLs in the petioles of infested plants was positively correlated with the number of larvae ($r = 0.86$, $p = 0.014$), the number of vital larvae ($r = 0.86$, $p = 0.014$), the recovery rate ($r = 0.75$, $p = 0.052$) and the survival rate ($r = 0.93$, $p = 0.003$). Furthermore, the content of indolic GSLs was positively correlated with the larval weight ($r = 0.93$, $p = 0.002$) and the head capsule

width (r = 0.93, p = 0.002). Contrarily, the content of SIB was negatively related to the larval weight (r = -0.93, p = 0.003) and the head capsule width of larvae (r = -0.93, p = 0.003).

Discussion

Our larval performance test demonstrated that the host plant quality for CSFB larvae differed among test accessions. To determine the host plant suitability of the introgression lines for CFSB larvae, developmental rates and various parameters of larval performance were assessed. Whereas the four introgression lines were as suitable as the *B. napus* reference for the development of larvae, larval performance on *S. alba* was significantly less than that of the *B. napus* cultivar Fenja and the introgression lines. In plants of both *S. alba* cultivars, we determined a higher mortality of larvae, lower larval weights and a prolonged development time of larvae, indicating strong mechanisms of antibiotic resistance. The performance of larvae was influenced by the content of single GSLs in plant tissues. Moreover, we found that CSFB larval feeding induced major changes in the GSL composition of the introgression lines and Fenja, but not in the GSL composition of the *S. alba* cultivars.

The observed differences in the mortality of larvae between accessions cannot be explained by a shortage of food, as the number of larvae recovered in petioles at the end of the experiment was positively correlated with the number of vital larvae and larval weights, respectively. Moreover, the survival rate was positively related to larval weights, again indicating that competition over food can be excluded as a possible reason.

The recovery rate of larvae was higher in plants of the standard *B. napus* cultivar Fenja and the introgression lines than in the *S. alba* cultivars, reflecting the low suitability of white mustard for CSFB larvae. Furthermore, survival rates were significantly lower than recovery rates in plants of the *S. alba* cultivars and the introgression line IL_114, but not in the other accessions. We therefore assume that neonate larvae began to feed within the plant tissue of Martigena, Base and the introgression line IL_114 but died during their subsequent development. This is in accordance with DOERING (2012), who has documented a higher mortality of CSFB larvae in plants of *S. alba* as compared to a *B. napus* cultivar.

The toughness of leaf petioles may interfere with CSFB larvae since neonate larvae have to bore into the petiole tissue and later, larval instars leave the petioles and move to younger leaves, where they have to penetrate the petiole surface again. In contrast to the findings of BERGVINSON *et al.* (1994) for *O. nubilalis* on maize, we did not detect any

significant interaction between the force needed to fracture the petiole surface and the different parameters of larval performance of the CSFB. Nevertheless, we found a tendency of decreased larval performance in softer tissue, which might be related to the fact that the larvae have to feed on a greater amount of tissue to fulfil their nutritional needs (SCRIBER & SLANSKY 1981; SCHOONHOVEN *et al*. 2005). However, no information from the literature about the effects of petiole toughness on CSFB larvae is presently available. Moreover, leaf pubescence may contribute to plant resistance against herbivory (DALIN *et al*. 2008), although higher densities of trichomes were only found on the petiole surface of the *S. alba* cultivars. Adverse effects of leaf trichomes on insect herbivores have been reported for the larvae of *Pieris rapae* (L.) (Lepidoptera: Pieridae) on *Brassica rapa* (L.) (SOUTHWOOD 1986; AGREN & SCHEMSKE 1994) and for adults of *Phyllotreta spp.* (Coleoptera: Chrysomelidae)) on *S. alba* (SOROKA *et al*. 2011). We therefore expect that the trichome density on petioles of the *S. alba* cultivars might have additionally contributed to the low recovery rate of larvae in plants. Conversely, DOERING (2012) has discovered a positive correlation between the feeding activity of CSFB adults and the density of trichomes on the lamina of several brasscicaceous accessions. Further experiments comprising a set of accessions with contrasting levels of petiole pubescence are required to clarify the impact of trichomes on CSFB larvae.

A significantly higher proportion of L3 larvae was recorded in plants of IL_140 than in the other introgression lines. Larval weights were also significantly higher than in the standard cultivar Fenja, suggesting that the introgression line IL_140 is a better food source for larvae than the other accessions. Compared to the standard cultivar Fenja, all of the introgression lines had a higher proportion of third instar larvae, implying that antibiotic mechanisms of resistance restricting larval development are not present or are marginal in these introgression lines. Only a few larvae reached the third instar in the *S. alba* cultivars and larval weights were lower, which is an additional sign of the insufficient host quality of these plants.

Larval performance of several other specialist herbivores was reduced when reared on *S. alba* (JYOTI *et al*. 2001; FELKL *et al*. 2005; TANSEY *et al.* 2010). ULMER & DOSDALL (2006) have reported that larval development of *Ceutorhynchus obstrictus* (Marsh.) (Coleoptera: Curculionidae) was slower and that larval weight was reduced in comparison to *B. napus.* In general, the prolonged development time of larvae may result in longer life spans of insects, reduced reproductivity and an increased exposure to natural enemies (GOODARZI *et al.* 2015).

The content and composition of GSLs in plant tissues is dependent on several factors such as species, cultivar, plant organ, developmental stage of the plant and environmental conditions (PORTER *et al*. 1991; VELASCO *et al.* 2007; AHUJA *et al.* 2009). Moreover, infestation by insect herbivores or mechanical wounding may induce changes in total and individual GSL concentrations (reviewed by HOPKINS *et al.* 2009). The non-infested petioles of the *B. napus* cultivar Fenja and the introgression lines had high amounts of aliphatic and indolic GSLs and only low amounts of aromatic GSLs. In the *S. alba* cultivars, however, SIB was the predominant GSL that accounted for > 90% of the total GSL content in both non-infested and infested plants. Larval feeding increased the total GSL content in all of the accessions. This increase was mainly based on the generation of indolyl GSLs in the introgression lines and Fenja, whereas the GSL content in the *S. alba* cultivar predominantly increased due to a higher content of SIB. An increase of indolic compounds and a concomitant decrease of aliphatic GSLs due to herbivore infestation have been reported in previous studies (BODNARYK 1992; MEWIS *et al.* 2005; AGERBIRK *et al.* 2009). Similarly, KORITSAS *et al.* (1991) have documented that *B. napus* plants infested by CSFB larvae had higher contents of indolyl GSLs, especially NEO and GBC, and that the total GSL content and the content of SIB in *S. alba* increased as a result of wounding. Several studies have investigated the stimulating effect of GSLs on the feeding of adult CFSBs. In more recent work, the influence of a single GSL and its relative proportions are expected to be of higher importance than the total GSL content (MITHEN 1992; BARTLET *et al.* 1994; GIAMOUSTARIS & MITHEN 1995; BARTLET 1996; BARTLET *et al.* 1999). Within the literature, results of the impact of single GSL compounds on oilseed rape pests are often contradictory, as identical compounds differ in their effect on different pest species (ULMER & DOSDALL 2006; HOPKINS *et al*. 2009; TANSEY *et al*. 2010).

In our study, a positive correlation was found between the initial content of the indolyl GSLs GBC and NEO in petioles and the recovery rate and survival rate of CSFB larvae. This finding is in agreement with previous results that have reported on the stimulating effect of single GSL substances. BARTLET *et al.* (1994) have noted that the indole GSL GBC stimulated the feeding of CSFB adults and ROESSINGH *et al.* (1992) have found stimulating effects of GBC on oviposition by *D. radicum*. Whereas oviposition of *Meligethes aeneus* (Fab.) (Coleoptera: Nitidulidae) was stimulated by NEO, NEO negatively influenced the oviposition of *D. radicum* (MEIER 2013, unpublished MSc thesis; TANSEY & DOSDALL 2011). We also found a negative relation between the content of NEO and larval performance (i.e. survival rate, larval weight and head capsule width) by

comparing the GSL content of non-infested plants 14 days after inoculation with various parameters of larval performance, but this association seems uncommonly wide.

The poor performance of CSFB larvae on plants of the *S. alba* cultivars might partially be related to the high content of aromatic GSL SIB, which has been proven to protect *S. alba* cotyledons against flea beetle attacks (BODNARYK 1991; BODNARYK & LAMB 1991). MCCAFFREY *et al.* (1999) have considered SIB as a major factor of antibiotic resistance to *C. obstrictus,* although other studies have found that the resistance of introgression lines to this insect pest is independent of SIB (TANSEY *et al*. 2010). The aliphatic GSL SIN was only detected in petioles of the introgression line IL_183, which had the lowest larval performance of the introgression lines tested. AGRAWAL & KURASHIGE (2003) have reported that volatile breakdown products of SIN, i.e. allyl-isothiocyanate, have antibiotic effects on the larval performance of *P. rapae*. Therefore, we assume that SIN might have also negatively affected the CSFB larvae. However, results on the impact of a single GSL substances are not consistent (BARTLET *et al.* 1999; ULMER & DOSDALL 2006; TANSEY 2009). Our results reveal that the content of GSL in petioles changed during plant development and that they were additionally altered by larval feeding, indicating a continuous change of GSLs in plant tissues over time.

Tests on the antibiotic mechanisms of resistance are typically conducted under no-choice conditions (DENT 2000) and mainly focus on insect larvae because of their limited mobility. Information about the suitability of choice tests for CSFB larvae, however, are missing. Our experimental design could be improved by considering the whole larval development (i.e. by extending the experimental period until mature third instar larvae leave the plants to pupate in the soil) to analyse differences in the developmental time and weight of mature third instar larvae (DOSDALL & KOTT 2006).

Our experiments revealed that larvae of the CSFB are able to develop in plants of *S. alba*, but that larval performance in these plants is restricted. None of the tested introgression lines, however, was less suitable than the *B. napus* cultivar Fenja. As plant resistance to insect pests is rarely dependent on a single mechanism (DENT 2000), further investigations are needed to clarify the mechanisms of resistance and the potential synergistic plant traits involved in the resistance of *S. alba* before these can be transferred into elite varieties of *B. napus.*

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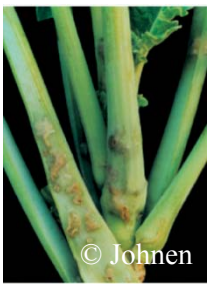
Chapter VI

General Discussion



General Discussion

During its growing season, winter oilseed rape (*Brassica napus* (L.)) is attacked by several insect pests. The most important pest species in autumn are the cabbage stem flea beetle (*Psylliodes chrysocephala* (L.) (Coleoptera: Chrysomelidae)) and the cabbage root fly (*Delia radicum* (L.) (Diptera: Anthomyiidae)) (FINCH 1993; BARARI *et al*. 2005; AHUJA *et al.* 2009). These two pests invade the fields shortly after the plants emerge, i.e. their colonization coincides with a highly vulnerable developmental stage of oilseed rape plants. Infestation by *P. chrysocephala* and *D. radicum* can cause major economic damage (DOSDALL *et al.* 2000; ALFORD *et al.* 2003). Until presently, control strategies have relied upon synthetic insecticides used as spray applications or seed treatments (WILLIAMS 2010). As the resistance to pyrethroids is continuously increasing in several oilseed rape pest species (HEIMBACH & MÜLLER 2013), alternative pest management strategies are urgently needed (HØJLAND *et al*. 2015), especially since the seed treatment of oilseed rape with neonicotinoids has been withdrawn in 2013 (BAROSO 2013). Plant resistance to insects should become a key component of integrated pest management (IPM) systems (WISEMAN 1994; WILLIAMS 2004) and has been already defined as a goal in oilseed rape breeding (FRIEDT 2011). Nevertheless, the development of cultivars resistant to insect pests is still in its inception (FRAUEN 2011).

Since the gene pool of *B. napus* currently used for breeding is limited (FRIEDT 2011), the use of different resistance and quality traits from progenitors and related plant species is a promising approach for breeding (BROWN *et al.* 1997; GIRKE 2002). *S. alba* has been widely accepted as resistant to infestation by *D. radicum* (JYOTI *et al.* 2001), *P. chrysocephala* (DOERING 2012) and several other pest species (HENDERSON *et al.* 2004; DOSDALL & KOTT 2006). Concerning the development of pest-resistant cultivars in the last decade, progress has been made in Canada by introgressing *S. alba* DNA into *B. napus.* A number of these introgressions carried genes for resistance to *Ceutorhynchus obstrictus* (Marsh.) (Coleoptera: Curculionidae), *D. radicum* and *Phyllotreta spp.* (Coleoptera: Chrysomelidae) from the *S. alba* parent (GAVLOSKI *et al.* 2000; DOSDALL & KOTT 2006; TANSEY *et al.* 2010). Studies on the resistance of introgression lines against *D. radicum* attacks were conducted under field conditions and resistance was evaluated based on root damage ratings (KOTT & DOSDALL 2004).

In this study, the susceptibility of several introgression lines (*S. alba* x *B. napus*) to infestation by both *D. radicum* and *P. chrysocephala* was evaluated under laboratory

conditions for the first time. Furthermore, we screened three resistant *S. alba* cultivars (Martigena, Base and Sirte) and three *B. napus* cultivars (Fenja, Visby and Campino), which are known to be highly susceptible to both insect pests as references. An additional objective of this study is to identify morphological and biochemical plant traits as potential factors of host plant resistance that negatively affect host acceptance by adults and larval performance of *D. radicum* and *P. chrysocephala.*

Laboratory screening methods for host plant resistance

Antixenotic resistance is based on plant traits that prevent the conolonization of insects (for feeding and oviposition) on a potential host plant, whereas antibiotic resistance refers to plant traits that adversely affect the performance of herbivores (SCHOONHOVEN *et al.* 2005). In order to differentiate between antixenotic and antibiotic plant defence, we investigated the host acceptability and oviposition preference of adult insects as well as the performance of the larvae of *D. radicum* and *P. chrysocephala.*

Our screenings were conducted under controlled conditions in a laboratory, which made it possible to control plant growth stage, timing and the intensity of pest infestation. By using insects that had been reared in a laboratory, it was possible to standardize individuals with regard to their physiological condition and stage of development. In all experiments, intact plants were offered to insects to avoid a potential bias triggered by host plant cues that changed due to wounding (FARELL 1977; AGERBIRK *et al.* 2009; HERVÉ 2014). Field experiments that examine the natural colonization processes of insect pests face several difficulties, e.g. unevenly distributed colonisation by pests, differences in growth stages of plants and furthermore, field crops are commonly colonized by several insect pest species at the same time. These restrictions reduce the reliability of field trials and make it difficult to distinguish between the mechanisms of plant resistance, i.e. antixenosis and antibiosis, and can be avoided in laboratory screenings (PALANISWAMY *et al.* 1992; HERVÉ 2014).

Antixenosis experiments: Screening methods based on multi-choice comparisons are able to detect differences in host plant preference and acceptance (SCHOONHOVEN *et al.* 2005; EICKERMANN 2008) and have been proven to be an appropriate method by which to assess the host suitability for *D. radicum* (JYOTI *et al.* 2001). In our experimental design, *D. radicum* females were able to choose between a number of alternatives and subseuquently, lay eggs according to their preference. As the number of eggs deposited on

a test accession depends on the relative attractiveness of the other accessions, minor differences in host suitability were also revealed (DEGEN 1998). Our no-choice bioassay for antixenosis resistance towards *P. chrysocephala* relied on the acceptance and suitability of a test accession for the feeding of adult beetles. No-choice bioassays are more similar to field situations, as just one cultivar is simultaneously available. Furthermore, no-choice tests help identify potential key plant traits for host plant acceptance or resistance, as the attractiveness for feeding on a test plant is not related to the attractiveness of other plants (SCHOONHOVEN *et al.* 2005; HERVÉ 2014). Laboratory feeding experiments have been conducted for several insect pests, including *P. chrysocephala* (BARTLET & WILLIAMS 1991; DOERING 2012). In our experiments, plants in the cotelydone stage were offered to beetles, as this growth stage is most vulnerable to feeding by adult *P. chrysocephala* in the field (WILLIAMS 2010).

Antibiosis experiments: Bioassays based on no-choice tests were used to investigate the performance of *D. radicum* larvae on roots and *P. chrysocephala* larvae within the petioles of brassicaceous accessions. Thereby, special emphasis was placed on the neonate larvae of both insect pests. Former studies have also successfully used no-choice tests to investigate the host plant quality of various brassicaceous accessions for *D. radicum* (MCDONALD & SEARS 1992; FELKL *et al.* 2005) and *P. chrysocephala* (DOERING 2012). Antibiotic traits of host plants may have direct lethal effects on neonate larvae or may lead to an increasing mortality during larval development. Individuals that survive antibiosis may suffer from reduced weight and prolonged developmental times (JOYTI *et al.* 2001; SAFRAZ *et al*. 2006). To study the antibiotic effects of test accessions on *D. radicum*, the larval development and performance as well as larval damage to roots were assessed by infesting plants with eggs. To screen for the antibiotic resistance of *P. chrysocephala* to larvae we infested plants with neonate larvae and assessed their survival and performance within the petioles.

Response of *D. radicum* to test accessions

Oviposition of D. radicum females: In this study, a subset of 10 accessions was screened in a multi-choice experiment for their attractiveness to *D. radicum* females for oviposition. Unexpectedly, the *S. alba* cultivars were as suitable for oviposition as the *B. napus* cultivar Fenja. Two introgression lines (IL_140 and IL_165) and the *B. napus* cultivar Visby were less attractive to oviposition than was Fenja. *D. radicum* females deposited twice as many eggs on two of the introgression lines compared to the standard

cultivar Fenja. Our observation that the resistant *S. alba* cultivars are equally attractive for oviposition contradicts the results of previous investigations that have claimed that antixenotic resistance is the main resistance mechanism against *D. radicum* attacks (DOSDALL *et al.* 1994; KERGUNTEUIL *et al.* 2014).

Effect of morphological plant traits on oviposition by D. radicum: The size of the host plants has been shown to influence the oviposition preference of *D. radicum females* (MAACK 1977; MCDONALD & SEARS 1992). While the effect of the leaf size was of minor importance in our study, the stem base diameter strongly affected oviposition by accounting for 76% of the variation in location of deposited eggs. These results indicate that the growth stage and the size of plants are key factors for host plant choice of gravid females. The preference of *D. radicum* for plants with a greater stem diameter is in accordance with observations previously made by GRIFFITHS (1986).

In addition to morphological plant traits, host plant location and acceptance by *D. radicum* is mediated by biochemical cues, especially by the content and composition of glucosinolates (GSLs) and their hydrolytic products, respectively (ROESSINGH *et al*. 1992; BAUR *et al.* 1996; TANSEY & DOSDALL 2011). Therefore, differences in oviposition between accessions might have been additionally linked to differences in biochemical plant traits that affect the host plant choice of female flies, a fact that we did not account for within this experiment.

Performance of D. radicum larvae: In no-choice experiments, 31 accessions were screened for their host suitability to *D. radicum* larvae and the results reveal differences in host plant quality between accessions for *D. radicum*. Larval performance on the two *S. alba* cultivars (Martigena and Sirte) was significantly reduced due to less third instar larvae and pupae as well as lower weights of the surviving larvae and pupae. Moreover, feeding damage on root tissues was lower on *S. alba* compared to the susceptible *B. napus* cultivar Fenja. Only 1 of the 26 screened introgression lines exhibited a lower performance of *D. radicum* larvae in terms of a significantly lower number of larvae and pupae surviving on roots. The feeding damage on roots of this introgression line (IL_183), however, was higher than on *S. alba*. Antibiotic resistance of *S. alba* to *D. radicum* infestation has also been demonstrated by JYOTI *et al*. (2001). Antibiosis has been described as the prominent mechanism of resistance against *D. radicum* in several other brassicaceous species, e.g. *Brassica fructilosa* (Cirillo), as less larvae survived and larval

weights were lower compared to *Brassica oleracea* (L.) (ELLIS *et al*. 1999, FELKL *et al.* 2005).

Effect of morphological plant traits on larval performance of D. radicum: Morphological plant characteristics may have a significant impact, especially on the success of the establishment and infestation of insect pests (SCHOONHOVEN *et al*. 2005; PRICE *et al*. 2011) In our study we did not find increased tissue toughness (i.e. the maximum force needed to fracture the root surface) to be associated with lower larval damage. Our results are in accordance with BIRCH (1988), who did not detect an effect of root toughness on larval damage of *Delia floralis* (L.) (Diptera: Anthomyiidae). Future investigations are needed to determine how the root toughness of brassicaceous accessions changes during plant development and whether an increase in root toughness is induced by larval feeding and might consequently, explain the lower suitability of *S. alba* and the introgression line IL_183, respectively.

Effect of D. radicum infestation on root glucosinolates: A subset of four accessions was analysed for the content and composition of GSLs in roots. Overall, we found a higher total content of GSLs in infested roots than in roots of non-infested plants. Infested roots of two introgression lines (IL_114 and IL_183) and Fenja had a higher content of indolyl GSLs compared to undamaged plants, whereas the content of aliphatic GSLs remained unaffected. An increase in indolyl GSLs induced by the feeding of *D. radicum* has also been observed in *B. oleracea* (VAN DAM *et al*. 2006). Our results are in agreement with previous results, as the infestation by insect pests resulted in an increase in indolyl GSLs, whereas the content of aliphatic GSLs was stable or reduced (KORITSAS *et al*. 1991; MEWIS *et al.* 2005; AGERBIRK *et al.* 2009). The content of aromatic GSLs was low in the introgression lines and Fenja. Conversly, the aromatic GSL sinalbin was predominant in both the infested and non-infested roots of the *S. alba* cultivar Martigena. Sinalbin has been shown to negatively affect insect herbivores and might also be responsible for the reduced larval performance and lower damage of root tissue of the *S. alba* plants in our experiments. Antibiotic effects of sinalbin, however, are more common for generalist insect herbivores, whereas antixenotic effects of this GSL have been reported for specialist insect pests, e.g. *Phylloreta spp.* (BODNARYK 1991; HOPKINS *et al.* 1998).

Response of *P. chrysocephala* to test accessions

Feeding of adult P. chrysocephala: A set of 14 accessions was screened for antixenotic resistance to the feeding of adult *P. chrysocephala* in a no-choice experiment. Feeding activity of beetles on the cotyledons of the *S. alba* cultivars (Martigena and Base) was delayed and consequently, the feeding damage was less compared to the *B. napus* standard cultivar Fenja, resulting in a higher proportion of plants surviving the attack of adult beetles. The consumed cotyledon area of four introgression lines was lower than for those of Fenja 48 h after the beetles were released, but did not differ between these introgression lines and Fenja at the end of the experiment (after 72 h). In former investigations, seedling damage of *S. alba* plants by flea beetles (*Phyllotreta spp*.) was also significantly lower in comparison to *B. napus* (PALANISWAMY *et al.* 1992 and 1997). In laboratory choice assays, seedlings of *S. alba* were damaged only half as much by *Phyllotreta spp.* as *B. napus* seedlings and antixenosis was considered the main mechanism of resistance (PALANISWAMY *et al.* 1997). BODNARYK & LAMB (1991) have emphasized that the higher tolerance of *S. alba* to *Phyllotreta spp.* attack is enhanced by its rapid compensatory growth. In our experiment, the differences in plant survival suggest that plants of *S. alba* are able to better tolerate the infestation of *P. chrysocephala*. As feeding damage was additionally negatively linked to seedling dry weight, the "perfect seedling" might combine antixenotic plant traits that deter flea beetles from feeding in combination with a high dry matter content and a rapid compensatory growth.

Effects of glucosinolates on adult P. chrysocephala feeding: The GSL content of seeds was analysed for the 14 accessions screened during the no-choice feeding tests with seedlings, as the seed GSL content is similar to the GSL content in 7-day-old seedlings of brassicaceous plants (PALMER *et al.* 1987).

In the introgression lines and the standard *B. napus* cultivar Fenja, aliphatic GSLs was the predominant group (mainly glucobrassicanapin and gluconapin), followed by indolic compounds (mainly glucobrassicin and 4-hydroxyglucobrassicin), whereas the content of the aromatic GSLs was below 10% of the total GSL content. Seven out of eleven introgression lines also contained small amounts of the aromatic GSL sinalbin, which is typically found in seeds of *S. alba* (HOPKINS *et al.* 1998). Highest total GSL contents were found for the *S. alba* cultivars Base and Martigena, with sinalbin accounting for more than 90% of the total GSL content in seeds. It is assumed that individual GSLs in plant tissues and the ratios among them are more important than the total level for the host

plant-pest interaction (GIAMOUSTARIS & MITHEN 1995). Specific GSLs and their breakdown products may act as feeding stimuli and are essential for host plant acceptance by *P. chrysocephala* (BARTLET & WILLIAMS 1991; BARTLET *et al.* 1999). MEIER (2011, unpublished BSc thesis) and DOERING (2012) have found a positive correlation between the content of 4-hydroxyglucobrassicin in leaves of different oilseed rape accessions and feeding by adults of *P. chrysocephala*. In our study, the seed content of aliphatic GSLs was positively related to the severity of feeding, whereas the content of the aliphatic GSL gluconapoleiferin was negatively related to feeding scores. In earlier experiments, we also found a negative association between the gluconapoleiferin content in leaves of different brassicaceous accessions and feeding by adults of *P. chrysocephala* (HENNIES 2012, unpublished MSc thesis). Former studies have highlighted the importance of indolic GSLs, especially the content of glucobrassicin (BARTLET & WILLIAMS 1991; BARTLET *et al.* 1999) for adult feeding of *P. chrysocephala*, but we could not detect any significant interaction between the content of glucobrassicin in seeds and adult feeding on cotyledons. The content of gluconasturtiin in seeds and the feeding activity of beetles, however, was positively correlated. A stimulating effect of gluconasturtiin on the oviposition of *Ceutorhynchus napi* (Gyll.) (Coleoptera: Curculionidae) has been reported by SCHÄFER-KOESTERKE *et al.* (2016). Sinalbin, the predominant GSL of *S. alba,* has been described as a factor that mitigates the susceptibility of *Brassicas* to insect pests (BODNARYK 1991) and we also found a negative correlation between the content of sinalbin in seeds and feeding scores of *P. chrysocephala*. According to SOROKA & GRENKOW (2013), *S. alba* accessions with low contents of sinalbin are as susceptible to *Phyllotreta attacks* as the reference *B. napus,* refering to the antixenotic properties of this aromatic GSL.

Effect of morphological plant traits on feeding of adult P. chrysocephala: Although the majority of accessions had no trichomes, the introgression line IL_183 and the cultivars Fenja, Martigena and Base exhibited different levels of hairiness, with trichome density negatively related to the feeding damage caused by adult beetles. High trichome densities are known to adversely affect insect pests, e.g. *Pieris rapae* (L.) (Lepidoptera; Pieridae) and *Phyllotreta spp.* (SOUTHWOOD 1986; AGREN & SCHEMSKE 1994; PALANISWAMY *et al.* 1992). LAMB (1980) and SOROKA *et al.* (2011), have found that adult *Phyllotreta spp.* prefer hairless accessions over pubescent plants. Furthermore, we found significant differences in the dry weight of seedlings between accessions and furthermore, results of the multiple regression analyses revealed that 78% of the variation in cotyledon damage by adult beetles could be explained by the cotyledon dry weight and the number of trichomes.

Performance of P. chrysocephala larvae: To assess the host plant suitability of the introgression lines for *P. chrysocephala* larvae, developmental rates and different parameters of larval performance were assessed on seven test accessions (one *B. napus* cultivar, four introgression lines and two *S. alba* culivars) in a no-choice experiment. Larval performance in plants of the *S. alba* cultivars Base and Martigena was poor: high mortality of larvae, low larval weights and a prolonged development time. This poor performance suggests that antibiotic plant traits are the major mechanism of resistance. Larval performance did not significantly differ between any of the introgression lines and the *B. napus* cultivar Fenja. In the *S. alba* cultivars and the introgression line IL_114 however, the survival rate was significantly lower than the recovery rate of larvae, indicating that larvae started to feed on the plant tissue, but failed to develop within the petioles. Similarly, DOERING (2012) has reported that the larval mortality of *P. chrysocephala* was greater in *S. alba* plants compared to a susceptible *B. napus* cultivar. Larvae in plants of the introgression line IL_140 achieved significantly higher mean larval weights in comparison to Fenja and had the highest ratio of L3 larvae, indicating that this introgression line is a high-quality food source for larvae. In all of the introgression lines more larvae developed into the third instar than in plants of the *B. napus* cultivar Fenja, whereas only few larvae reached the third instar in the *S. alba* cultivars. The poor conditions for larval development in plants of *S. alba* are also reflected by significantly lower larval weights. A slower development of larvae may result in a longer life span, reduced productivity, increased exposure to natural enemies and may result in a higher exposure to negative abiotic environmental impacts (GOODARZI *et al.* 2015).

Effects of glucosinolates on performance of P. chrysocephala larvae: We analysed the initial GSL content within the petioles of six accessions. Non-infested petioles of the *B. napus* cultivar Fenja and the introgression lines had high amounts of aliphatic and indolyl GSLs and only a small ratio of aromatic GSLs. In the *S. alba* cultivars, however, the aromatic GSL sinalbin was predominant and accounted for > 90% of the total GSL content in both non-infested and infested plants. A significant, positive correlation was found between the content of both the aggregated group of indolyl GSLs and neoglucobrassicin and the recovery rate of larvae, whereas the total GSL content was negatively correlated with the survival rate of larvae. The sinalbin content, as the typical GSL compound of *S. alba*, negatively influenced the head capsule width of *P. chrysocephala* larvae, which is in accordance with the negative effects of sinalbin as described above.

Effects of Glucosinolates on feeding of P. chrysocephala larvae: The GSL sinigrin was only detected within the petioles of one introgression line (IL_183), which had the poorest larval performance, i.e. a lower proportion of L3 larvae and the lowest larval weights among all introgression lines tested. BARTLET *et al.* (1994) have reported that sinigrin did not stimulate feeding of adult *P. chrysocephala*, but the volatile hydrolyses product of sinigrin (allyl-isothiocyanate) has been found to have an antibiotic effect on the larval performance of *P. rapae* (AGRAWAL & KURASHIGE 2003).

Effect of P. chrysocephala larval feeding on glucosinolates: In all accessions, the GSL content of the petioles of infested plants was higher than the content of non-infested plants. In plants of Fenja and the introgression lines, we found higher proportions of indolyl GSL within the petioles of infested plants. A shift towards higher production of indolyl GSLs, especially the content of neoglucobrassicin and glucobrassicin in conjunction with stable or reduced aliphatic GSLs, in plants infested by *P. chysocephala* has been documented by KORITSAS *et al.* (1991). The authors have also reported a higher total GSL content in damaged plants of *S. alba*, mainly due to an increased content of sinalbin. This is in agreement with our observations as well as with the effect of *D. radicum* larvae feeding on roots (see: *Effect of D. radicum infestation on glucosinolates*). Herbivore-induced changes to the content and composition of GSLs in plant tissues may affect the primary pest species itself and may further alter the host plant acceptance of other herbivores (generalists as well as specialists). Furthermore, volatile compounds, such as isothiocyanates (hydrolyses products of aliphatic and aromatic GSLs), which are released at higher concentrations in response to herbivory are known to be attractive to parasitoids and predators (AHUJA *et al*. 2009).

Effects of petiole morphology on P. chrysocephala larval performance: In contrast to the petioles of Fenja and the majority of introgression lines, which had no trichomes, the petiole surface of the *S. alba* cultivars Martigena and Base exhibited a hairiness with > 17 trichomes/cm². A sparse hairiness was only detected on the petioles of the introgression line IL_140 with 0.07 trichomes/cm². Trichomes may have adverse effects on insect herbivores, as it has been reported for *P. rapae* on *Brassica rapa* (L.) (AGREN & SCHEMSKE 1994) and they may further hamper the movement of neonate larvae before they bore into the petioles (SCHOONHOVEN *et al*. 2005). Unexpectedly, our analyses revealed that a higher petiole toughness (i.e. the integral pentration force inside the petiole tissue) was associated with a better larval performance. The weaker larval performance in

softer tissue may be related to the fact that larvae have to feed more on soft tissues to fulfil their nutritional needs (SCRIBER & SLANSKY 1981; SCHOONHOVEN *et al*. 2005). However, there is currently no available information from the literature about the effects of petiole toughness on *P. chrysocephala*. The effect of trichomes as well as the influence of tissue strength should be analysed in more detail in future experiments, by using a set of accessions with contrasting morphological traits.

The preference-performance hypothesis

A theory widely used to explain the host plant choice of insect herbivores is the preference-performance hypothesis, also known as the "mother knows best" principle (VALLADARES & LAWTON 1991; JOHNSON *et al.* 2006). This theory is based on the assumption that the larvae of insect herbivores are limited in their mobility and therefore, females must choose the optimal host plant for the survival and development of their offspring. In the multi-choice oviposition experiment, *D. radicum* females in general preferred larger plants (with larger stem diameter) as host plants, a choice which might be associated with high amounts of food and increased survival of larvae, respectively. The *S. alba* cultivars were as attractive for oviposition as the majority of the introgression lines and the standard *B. napus* cultivar Fenja. However, the development of larvae in *S. alba* was restricted due to a pronounced antibiotic resistance. We therefore conclude that *D. radicum* females do not follow the pattern predicted by the "mother knows best" principle.

In contrast, *P. chrysocephala* appears to be able to better maximize the fitness of its larval offspring, since the feeding of adult beetles on seedlings of *S. alba* and larval performance were low in comparison to the introgression lines and Fenja. Adults and larvae of *P. chrysocephala* accept the same range of plants as food, although the adults are more selective (BARTLET & WILLIAMS 1991). Since females of *P. chrysocephala* tend to feed and oviposit near plants that are better suited to the feeding needs of their larvae, the basic concept of the "mother knows best" principle can be confirmed. In this context, it should be noted that both *P. chrysocephala* adults and larvae feed aboveground. Females of *D. radicum* interact with the aboveground parts of plants, whereas their larvae develop belowground and might therefore be affected by other plant traits and stimuli than the female flies (AHUJA *et al*. 2009).

Conclusion

Our results confirm that *S. alba* is a source of resistance against the two autumn pests *D. radicum* and *P. chrysocephala*. *S. alba*, however, did not adversely affect the oviposition of *D. radicum*, but clearly reduced the survival of their larvae. It can be assumed that antixenotic and antibiotic mechanisms of resistance of the *S. alba* were partially transferred to the screened introgression lines. Antibiotic resistance to *D. radicum* feeding on roots was identified in one introgression line and appeared to be the most powerful resistance mechanism against this insect pest. Our bioassays related to *P. chrysocephala* reveal that *S. alba* exhibited pronounced antixenotic resistance to feeding by adults as well as antibiotic defence mechanisms against their larvae. Moreover, feeding by *P. chrysocephala* adults on four introgression lines was delayed, indicating that antixenotic traits were transferred from *S. alba* to these introgression lines. Biochemical and morphological plant characteristics that interfere with the host plant choice of adults and the performance of larvae highlight the importance of GSLs, especially sinalbin, as well as the density of trichomes on cotyledons and petioles, respectively.

Further breeding programs should focus on the identification of genes and quantitative trait loci (QTLs) that are involved in the resistance of *S. alba,* before these can be transferred to high-yielding *B. napus* cultivars. Nevertheless, phenotyping will still be essential (PANGULURI *et al.* 2013). In this context, mechanisms of tolerance such as compensatory plant growth should be also taken into consideration. As antibiosis seems to be the strongest factor of resistance to *D. radicum* attacks, plant traits that influence larval development and performance should be investigated in more detail. To identify these characteristics, roots of accessions that exhibit contrasting levels of larval performance should be analysed for GSL content, volatile hydrolytic products and primary plant compounds, such as sugars and lignin content. With regard to mechanisms of resitance to *P. chrysocephala*, plant traits that affect host acceptance of adults appear to be of particular interest. To identify these factors, accessions with contrasting levels of feeding damage by *P. chrysocephala* need to be analysed for additional biochemical plant traits, such as volatile isothiocyanates and primary plant compounds, as well as morphological plant traits, such as the texture and composition of the wax layer. While both *P. chrysocephala* adults and larvae feed aboveground, the larvae of *D. radicum* feed belowground. Consequently, the it is difficult to develop cultivars that exhibit resistance to both autumn insect pests.

Overall, our results indicate that only moderate levels of antibiotic and antixenotic resistance are expressed in the screened introgression lines of *S. alba x B. napus*. Comparatively low levels of resistance, however, may still have benefits for crop protection (TANSEY 2009), as these can be implemented in IPM strategies in combination with agronomic practices, such as sowing date (ELLIS & KIFT 2003). Our studies reveal that the resistance of *S. alba* to *P. chrysocephala* is based on both antixenotic and antibiotic defence traits (to a varying extent). A targeted combination of the resistance mechanisms may increase the durability of resistance (DENT 2000). Moreover, a partial resistance to insect pests should be preferred over a total resistance, as this reduces the selection pressure to overcome resistance by pest populations (GATEHOUSE 2002; TANSEY 2009).

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Summary

Evaluation of resistance mechanisms against *Delia radicum* L. and *Psylliodes chrysocephala* L. in brassicaceous accessions

The cabbage stem flea beetle (*Psylliodes chrysocephala* (L.)) and the cabbage root fly (*Delia radicum* (L.)) represent two of the most damaging pests to winter oilseed rape (*Brassica napus* (L.)) throughout Europe. Management of these pests strongly relies on synthetic insecticides, which are often applied without regard to pest incidence. Because this strategy may have harmful side effects on natural biocontrol agents and has increased the risk of insecticide resistance, alternative approaches (including host plant resistance) need to be explored.

As white mustard (*Sinapis alba* (L.)) is known to be widely resistant to the attack of several insect pests, it was chosen as a potential source of pest resistance in *B. napus*. In this study, a total of 26 introgression lines derived from hybrids of *S. alba* x *B. napus* were screened for resistance to *P. chrysocephala* and *D. radicum* under controlled laboratory conditions. The infestation levels of these lines were compared to the infestation of *S. alba* and *B. napus* cultivars, respectively. Potential mechanisms of resistance were investigated, including biochemical and morphological plant traits.

In multi-choice oviposition screening tests for antixenotic resistance, females of *D. radicum* laid significantly fewer eggs on roots of two of the introgression lines than on roots of the reference *B. napus* cultivar Fenja. The *S. alba* cultivars Martigena and Base were as attractive for oviposition as the *B. napus* reference, indicating no antixenotic resistance of *S. alba* to females of *D. radicum*. Screening for antibiotic resistance revealed a strong resistance to the feeding and development of *D. radicum* larvae on roots of *S. alba*. Resistance was expressed via significantly lower development rates of third instar larvae and pupae, lower weights of larvae and pupae as well as a reduction in the damage to root tissue caused by larval feeding, compared to the susceptible *B. napus* cultivar Fenja. Only one of the 26 introgression lines exhibited a significantly lower performance of *D. radicum* larvae on roots in comparison to the reference *B. napus* cultivar Fenja. The stem base diameter of plants strongly affected the host plant choice of *D. radicum* females for oviposition: egg numbers significantly increased with increasing stem diameters of plants.

The no-choice screening test of antixenotic resistance to the feeding of adult *P. chrysocephala* on seedlings revealed a moderate resistance in *S. alba*, as feeding on cotyledons was initiated delayed and less severe than for *B. napus*. Feeding damage on the 11 screened introgression lines was not different than that found on the *B. napus* cultivar Fenja, but feeding was initiated delayed on four of the introgression lines. To determine the host plant suitability of the introgression lines for *P. chrysocephala* larvae, development rates and different parameters of larval performance were assessed. The four introgression lines were as suitable as the *B. napus* reference for the development of larvae within petioles, whereas larval performance on *S. alba* was significantly reduced compared to the reference *B. napus* cultivar Fenja and the introgression lines, indicating an antibiotic resistance. Feeding damage caused by *P. chrysocephala* adults was negatively correlated with seedling dry weight and density of the trichomes found on cotyledons. Glucosinolate content exhibited induced changes and increased due to feeding by *D. radicum* as well as *P. chrysocephala* larvae, indicating an influence of biochemical plant traits on insect-plant interactions.

The performance of *D. radicum* and *P. chrysocephala* on the same set of different brasscicaceous accessions has not been investigatedin detailed laboratory studies before. Improved knowledge on the resistance mechanisms has been gained, which is a prerequisite for further attempts at breeding resistant cultivars of oilseed rape.

Appendix

Table 1: Names of individual glucosinolates and chemical groups of glucosinolates detected within the petioles of brassica accessions according to VELASCO & BECKER (2000) and FAHEY *et al.* (2001).

Systematic name	Trivial name	Abbreviation	Group
2-hydroxy-3-butenyl	progoitrin	PRO	aliphatic
2-hydroxy-4-pentenyl	napoleiferin	GNL	aliphatic
3-butenyl	gluconapin	GNA	aliphatic
4-pentenyl	glucobrassicanapin	GBN	aliphatic
2-propenyl	sinigrin	SIN	aliphatic
3-methylsulphinylpropyl	glucoiberin	IBE	aliphatic
4-methylthiobutyl	glucoerucin	ERU	aliphatic
4-methylsulphinylbutyl	glucoraphanin	RAA	aliphatic
4-methylsulfinyl-3-butenyl	glucoraphenin	RAE	aliphatic
2-phenylethyl	gluconasturtiin	NAS	aromatic
p-hydroxybenzyl	sinalbin	SIB	aromatic
4-hydroxy-3-indolylmethyl	4-hydroxyglucobrassicin	4OH	indolyl
3-indolylmethyl	glucobrassicin	GBC	indolyl
4-methoxy-3-indolylmethyl	4-methoxyglucobrassicin	4ME	indolyl
n-methoxy-3-indolylmethyl	neoglucobrassicin	NEO	indolyl

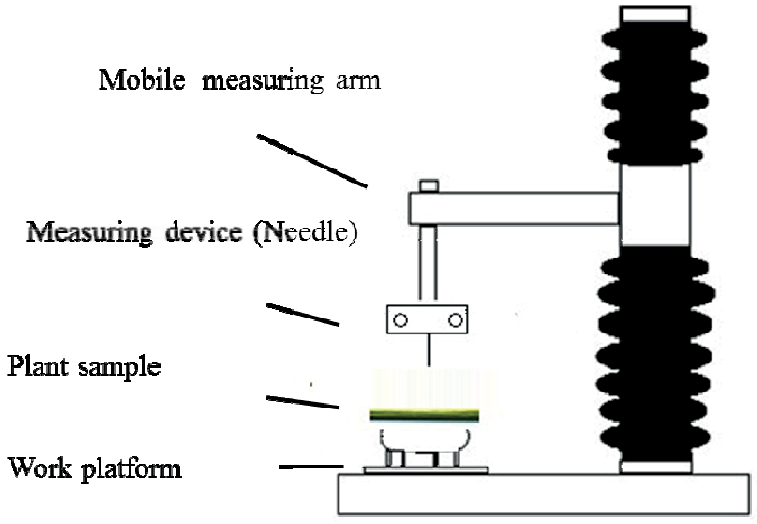

Figure 1: Schematic diagram of the measuring unit of the texture analyser used for force measurements (figure modified to Stable Micro Systems Ltd. 1996).

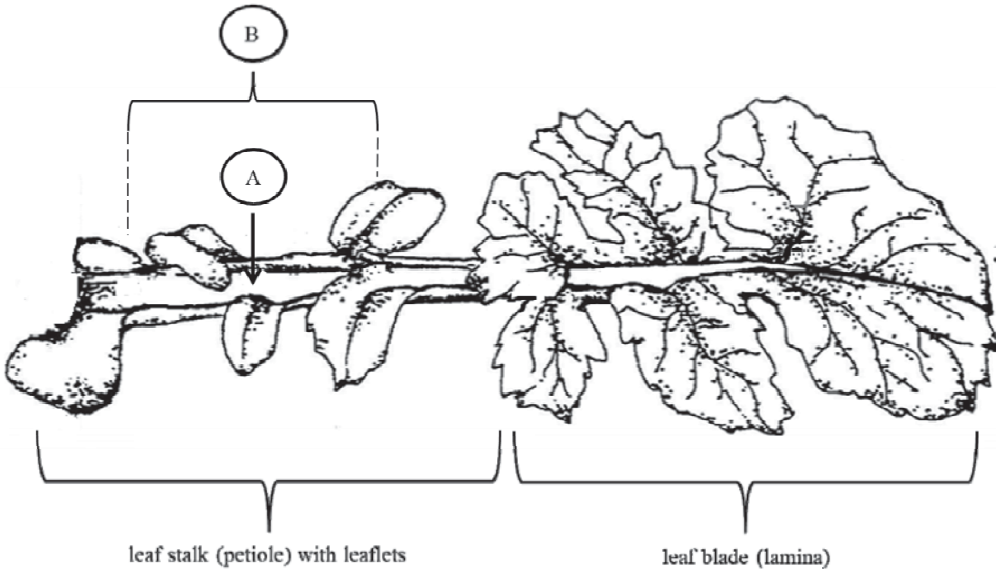

Figure 2: Release point of the neonate *P. chrysocephala* larvae on the leaf petiole (A) and the petiole segment used for toughness measurements and dry weight determination (B) (figure modified to NISSEN 1997).

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Acknowledgements

I would like to thank Prof. Dr. Stefan Vidal for giving me the opportunity to be a part of his working group and for reviewing this thesis.

Many thanks to Prof. Dr. Andreas von Tiedemann for co-reviewing this thesis.

I am especially grateful to Dr. Bernd Ulber for enabling me to explore the world of oilseed rape pest, supporting me in my experiments and answering my questions over the last few years.

A very special thanks to Claudia Nordmann and Dorothea Mennerich for their excellent technical assistance, support and sense of humour.

This project was only possible thanks to the cooperation of the following divisions: Plant Breeding, Quality of Plant Products, Agronomy in the Tropics and General Plant Pathology and Crop Protection:

> I would like to thank Dr. Inga Smit and Birgit Bode for allowing me to use the texture analyser and the leaf area meter. Thanks to Dr. Uwe Ammermann for his help in analyzing the glucosinolates.
>
> I also want to thank Maik Knobel, Hubertus Reintke, Evelyn Vorbeck and Gerald Miotke for their technical assistance in the greenhouse as well as in supplementary field experiments.

I am very grateful to Dr. Katharina Lohaus, Dr. Ines Vollhardt and Dr. Mario Schumann for their support and helpful comments. I would also like to thank Dr. Christian Kluth for his advice concerning statistical questions.

Special thanks to my PhD colleagues in the working group for providing a pleasant working atmosphere, both professionally and personally.

Finally, I am deeply grateful to my family and friends, especially Andreas, who has supported and motivated me over the years.

This research was funded by the scholarship program of the German Federal Environmental Foundation (DBU).

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