Preference-Performance Relationships in Herbivorous Insects Feeding on Oilseed Rape Inoculated with Soil-borne Fungi

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Summary

There is increasing evidence that plant-associated microorganisms are able to mediate plant-herbivorous insects-interactions. In this study, I evaluated the impact of two plant colonising fungi on three herbivorous insect species, using mycological, entomological and plant biochemistry techniques. In detail, my research focused on the mediation of two soil-borne fungi, the endophyte *Acremonium alternatum* and the fungal plant pathogen *Verticillium longisporum* on the preference and performance of three herbivorous insects with different feeding modes, *Delia radicum* L.(Diptera: Anthomyiidae), *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Brevicoryne brassicae* L. (Hemiptera: Aphididae). Two different cultivars of oilseed rape (*Brassica napus* and *Brassica napus* var.) were used. Host-preference of the herbivorous insects was tested by using mechanically damaged and intact plants either with endophyte infection (E+) or controls (E-) and plants inoculated with Verticillium (V+) or control (V-). Performance of insect feeding on infected /uninfected plants was evaluated by recording mortality of larvae, development time, pupal weight and fecundity. A field experiment was implemented to test the impact of endophyte inoculations on insect preference/performance relationships with regard to cabbage aphid population dynamics. Volatiles released from aerial plant part of infected or control plants were collected and analyzed qualitatively and quantitatively to explain host preference of adult herbivorous insects. The content and relative composition of four typical phytosterols, namely Sitosterol, Campesterol, Brassicasterol and Stigmasterol was quantified from the leaves and roots of infected and control plants to understand mechanisms of a fungal infection on insect performance.

The results of the experiments can be summarized as follows:

- Nymphs of *B. brassicae* significantly preferred control (V- and E-) host leaf-discs, and *P. xylostella* females preferred to oviposit on V- host plants in cage experiments. However, nymphs and larvae of the three insect species tested did not show a significant host plant preference for infected or control host plants as has been demonstrated in the field.
- More volatiles were released by *V. longisporum* infected oilseed rape plants as compared to control plants. Particularly, the significantly higher amount of

deterrent volatile might explain the host plant preference of *P. xylostella* larva and adult to V- host plants.

- A higher mortality was calculated from larval to adult development in *P. xylostella* reared on V+ leaf-discs in Petri-dishes only. However, *A. alternatum* or *V. longisporum* infections in host plants did not result in a significant difference with regard to pupal or adult weight in *P. xylostella* and *D. radicum*.
- *A. alternatum* or *V. longisporum* infections accelerated the time needed for the development from pupae or nymphs to adults in all three insect species tested.
- I found different effects of an *A. alternatum* or *V. longisporum* inoculation on insect fecundity; it increased in *D. radicum*, decreased in *P. xylostella* and was not influenced in *B. brassicae*.
- Changes in the overall phytosterol content and the relative proportions of specific sterols partly explained differences in insect development when feeding on V+/V- or E+/E- oilseed rape plants. However, the carbon/nitrogen balance was not influenced in E+/E- cabbage leaves in the field experiment. Further experiments are needed to determine the specific parameters involved.
- My results show that the effect of an inoculation of plants with *A. alternatum* or *V. longisporum* may alter the preference/performance relationship in herbivores insects, however these differences are related to the specific insect species and plant cultivars or species involved.

Key words: *Acremonium alternatum*, *Verticillium longisporum*, *Delia radicum*, *Brevicoryne brassicae, Plutella xylostella*, preference-performance of insect, volatile profile, phytosterols profile.

Research Background

It is commonly accepted that herbivorous insect performance depends on plant nutrient supply (Harvey *et al*., 2003; Wittstock *et al*., 2004; Johnson *et al*., 2006). However, there is now increasing evidence that metabolites produced by the host plants may be altered when infected by fungi (Bowden *et al*., 1990; Costa Pinto *et al*., 2000; Arnold & Engelbrecht 2007). Under field conditions most plant species are regularly infested with these organisms resulting in more complex multispecies interactions such as herbivore-host plant-fungi interactions, rather than simple bilateral interactions typically investigated in the majority of papers (Hatcher, 1995a; Gang & Bower, 1997; Hatcher *et al*., 1997; Azevedo *et al*., 2000). The interaction between herbivores-fungi could be direct, e.g. fungi as food for insect (Martin, 1992; Sabatini & Innocenti, 2000), insects acting as vectors for spore transmission (Agrios, 1980; Palermo *et al*., 2001) and insects behavior accelerating or decelerating the invasion and colonization of fungus in host plant (Wheeler & Blackwell, 1984; Hatcher & Paul, 2000; Gange *et al*., 2002; Currie, *et al*., 2006). The interaction between herbivores-fungi could be indirect in which the insect behavior and physiology were influenced by altered primary and secondary metabolites produced in infected host plant (Hatcher, 1995b; Hatcher & Ayres, 1997). The impact of fungi infection in shared host plant on plant-associated insect could be beneficial (Mondy *et al*., 1998; Johnson *et al*., 2003), detrimental (Hatcher *et al*., 1994; Mesquita *et al*., 1997; Röder *et al*., 2007) or negligible (Kingsley *et al*., 1983; Saikkonen *et al*., 1996). Endophytic fungi were defined as those species that occured within the living tissues of plants, without causing visible disease symptoms at a particular time (Stone *et al*., 2000). To date, fungal endophytes were found in all plant species worldwide with an extremely high diversity and abundance in the tropical trees and herbaceous plants (Arnold *et al*., 2001; Seena & Sridhar, 2004; Gange *et al*., 2007; Li *et al*., 2007). The ecological role of endophytes colonizing in grasses and trees were well studied, to a less extent, in agricultural crops (Gaylord *et al*., 1996; Saikkonen *et al*., 1998). Many studies argued for a mutualistic role of these grass endophytes by receiving shelter and nutrients from their host plant and in return conferring host plant the advantages in herbivores or pathogen resistance (Clement *et al*., 1997; Wilson & Carroll, 1997; Arnold *et al*., 2003) and enhancing host plant the tolerance to abiotic stresses (West

et al., 1990; Bacon, 1993). However, such mutualism did not seem to be universal in terms of the negligible or antagonistic role of endophytes to host plant reported in some studies (Saikkonen *et al*., 2004; Krauss *et al*., 2007). These grass endophytes were specific to host plant with vertical transmission via plant seeds and could produce some toxic substances (e.g. alkaloids) which endowed host plant the resistance to herbivores (Faeth, 2002; Gimenez *et al*., 2007), and even to the third trophical level (Sassi *et al*., 2006). In contrast to the intensive studied grass endophyte, some unspecialized endophytes in forest and agricultural crops received less attention, among which *Acremonium spp*. was particularly noticeable in terms of their high frequency occurring in endophyte assemblage survey (Collado *et al*., 1999; Kumaresan & Suryanarayanan, 2001; Grunewaldt-Stöcker & von Alten, 2003). Some *Acremonium spp*. strains endowed host plants the resistance to pathogen infection (Wicklow *et al*., 2005; Kasselaki *et al*., 2006; Grunewaldt-Stöcker *et al*., 2007) and some prompted the growth of plant root system such as *A. kiliense* and *A. cucurbitacearum* (D'Amico *et al*., 2008). The *Acremonium spp*. strains were unspecialized fungi with horizontal transmission via spores and did not produce toxic substance which was distinct from grass endophytes (Clay, 1988; Raps & Vidal, 1998).

In current study, the endophyte fungus *Acremonium alternatum* was used. It infects and restricts mainly in the root of plant and does not produce toxins and does not influence the plant growth and carbon/nitrogen balance in leaves (Raps & Vidal, 1998).

Due to inhabiting the intercellular spaces of host plants, the associations between endophytes and their host plants are less intimate as compared to pathogenic fungi (Carlile *et al*., 2001). Increasing evidence documented the transmission from asymptomatical endophyte to pathogenic fungi which showed the highly variable relationship between plant and specific fungus (Hammon & Faeth 1992; Saikkonen *et al*., 1998; Tagne *et al*., 2002). It indicated the indirect impacts of overt plant pathogens on phytophagous insect populations and communities were as important as those of reported endophytic fungi (Johnson *et al*., 2003). Lots of pathogens rendered obvious alteration in morphology and/or physiology in host plant, thereby influenced host-choice and performance of insect (Pfunder & Roy, 2000; Kellogg *et al*., 2005).

In this study, a host-specific vascular fungal pathogen *Verticillium longisporum* was used which was proved as the casual agent of Verticillium wilt on oilseed rape (*Brassica napus* L. spp. *oleifera*) (Karapapa *et al*., 1997; Zeise & von Tiedemann, 2001). The typical symptoms infected by *V. longisporum* in host plant included necrosis and chlorosis of leaf, stunting plant, ripening and premature senescence (Veronese *et al*., 2003; Babadoost *et al*., 2004; Zhou *et al*., 2006). Verticillium wilt is an increasing problem on Brassica oilcrops in northern European countries (Svensson & Lerenius, 1987; Zielinski & Sadowski, 1995). Presently the relative intensive cultivation and continuous crop rotation of OSR rendered this disease spreading quickly and resulted in dramatic economic loss (Svenson & Lerenius, 1987).

In terms of the accumulative finding that chewing insects performed worse than phloem-sucking insects on plants infected by mycorrhizae, Gange and West postulated that the effect of microorganism on insect performance related to insect feeding pattern (Gange & West, 1994; Gange *et al*., 1994; Gange, 2001). In this study, three types of insect species with different mouthpart (chewing insect: *Plutella xylostella*; phloem-sucking insect: *Brevicoryne brassicae* and soil-dwelling insect: *Delia radicum*) were incorporated with either *A. alternatum* or *V. longisporum* inoculated oilseed rape (*Brassica napus*, *Brassica napus* var.) to test the impact of soil-borne fungi on insect preference and performance.

Preference – performance hypothesis predicted the strong selection pressure imposed on the gravid adult to oviposite on plants or plant parts with high nutritional quality in order to maximize the offspring fitness (Jaenike, 1978). In some cases, even host plant was infected by fungus, the preference of ovipositing female did favor the offspring performance. Adults of the herbivorous beetle *Cassida rubiginosa* (Müller) preferred to consume and lay eggs on healthy creeping thistles over those infected by the fungus *Phoma destructiva* (Plowr.) and subsequently it was demonstrated the larvae developed better on these healthy plants (Kruess, 2002). Females of the stem-boring weevil *Apion onopordi* (Kirby) preferred to deposit eggs on systematically rust-infected (*Puccinia punctiformis*) creeping thistles and it was proved that feeding on these infected plants increased the fecundity of adult (Friedli & Bacher, 2006). However, some confusing phenomena were also reported that indicated the poorly correlated between insect preference and performance (Cronin & Abrahamson, 2001). Greenhouse whiteflies *Trialeurodes vaporariorum* (Westw.)

preferred to probe on tomato plants infected by *A. strictum*, however the larval suffered from a high mortality (Vidal, 1996). The gravid females of *Helicoverpa armigera* (Hübner) preferred to lay eggs on tomato plants inoculated with *A. strictum* (Jallow *et al*., 2008), however it was demonstrated that the offspring suffered from reduced growth rate, high mortality and low fecundity (Jallow *et al*., 2004). In this study we combined the host preference of nymph and gravid adult with the performance of offspring to test whether fungal infection interrupted the relationship of preference/performance of insect.

Concerning the soil-dwelling insect, the 'mother knows best' principle postulated that these insects should be taken into account the relationship of preference – performance of insects (Johnson *et al*., 2006). The subterranean larvae spent the whole development stage in the soil and had a low capacity to make mobility, therefore adult underwent a severe pressure to select an appropriate oviposition site with high nutrient and maximizing the offspring fitness (Hunt *et al*., 2001). Cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae)—is widespread, extremely destructive specialist insect on cruciferous crops. By depositing eggs around host plant stems, hatched maggots damage or destroy root system of many crucifers, including oilseed rape (Dosdall *et al*., 2000; Jensen *et al*., 2002). Feeding damage results in water stress and provides routes for fungi infection (Ridsdill Smith, 1977). The damage to the roots results in seriously reduction both in yield and quality and causes considerable economic losses for oilseed rape (Griffiths, 1991). Many studies investigated the visual and olfactory factors that influence the host choice of gravid adult of cabbage root fly (Tuttle *et al*., 1988; Roessingh & Städler, 1990; Degen & Städler, 1997; De Jong & Städler, 1999). It was demonstrated, out of the soil the newly hatched cabbage root fly larvae were capable of orientating to favored host plant (Kostal, 1992). However, viewing the difficulty to monitor the host choice of soil-dwelling larvae within the intransparent soil matrix, to date, as for this aspect, little report was presented, to a less extent, the impact of phytopathogenic fungi (Johnson *et al*, 2004; Zhang *et al*., 2003). In this study, we exerted the host choice test of larvae to mechanic damaged root/intact root of infected/uninfected host plant with man-made apparatus. The oviposition preference of gravid female was tested in cage experiment. Cabbage root fly performance (i.e. the fecundity, longevity, mortality and the development time of every stage) was tested in three consecutive

generations. The results of endophyte or pathogen on cabbage root fly preference and performance were shown in Chapter 1 and Chapter 2 individually.

With respect to the chewing insect, significant negative impact from *A. alternatum* or *A. strictum* inoculation on insect performance were documented majority in Lepidoptera insects, such as Diamondback moth, *Plutella xylostella* L. and polyphagous moth, *Helicoverpa armigera* (Raps & Vidal, 1998; Dugassa-Gobena *et al*., 1998, 2003; Jallow *et al*., 2004, 2008). Scant literature documented the impact of *Verticillium* spp. on insect performance. *Verticillium albo-atrun* infection in alfafa had negligible impact on southern armyworm *Spodoptera eridania* (Cram.) (Kingsley *et al*., 1983) whilst *Verticillium dabliae* infection in cotton seedlings delayed the development of the spider mite *Tetranychus urticae* (Karban *et al.*, 1987). In this study, we used *Verticillium longisporum* to test its impact on preference/performance of diamondback moth. The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a highly mobile, the most destructive oligophagous pest in crucifers world-wide and it attacks host plants from seedlings until harvest stage and causes heavy loss to cruciferous crops each year (Talekar & Shelton, 1993; Yang *et al*., 1993). We tested the host preference of diamondback moth larva/gravid females to *V. longisporum* infected/uninfected host plant in dual-choice bioassay. The insect performance was fulfilled as allowed insect larvae to feed on the leaf-discs/intact host plant inoculated/noninoculated with pathogen. Some indices were recorded including weight gain of larvae, mortality rate, pupa weight, adult weight and length of hind tibia. The results were presented in Chapter 3.

As for the sucking-sap insect, many researches showed the positive impact of microorganism infection on aphid performance such as the accelerated development time, improved weight and enhanced reproduction (Gange & West, 1994; Gange, 1996; Moran, 1998; Gange *et al*., 1999; Johnson *et al*., 2003). However, the development of aphids, *S. graminum* was not influenced by mycorrhizal fungi (*Glomus fasciculatum*) on sorghum (Pacovsky *et al*., 1985). By inoculation plant with *A. strictum*, a close fungus strain to *A. alternatum*, Vidal (1996) demonstrated that the greenhouse whitefly, *Trialeurodes vaporariorum* (Westw.), a sucking-sap insect encountered high mortality and retarded development. Based on these inconsistent reports, we questioned the effect of soil-borne fungi (*A. alternatum* and *V.longisporum*) on phloem-sucking insect. The cabbage aphid, *Brevicoryne brassicae* (L.) (Homoptera: Aphididae) is important specialist pest on plants of the

family Brassicaceae (=Cruciferae) grown in temperate and warm climatic zone (Minks & Harrewijn,1988; Cole & Lynn, 1996; zhang & Hassan, 2003). They prefer feeding on newly emerged plant tissues and weaken plants by removal of sap that cause bleaching and distortion of the leaves and prevent the development of a marketable product and cause serious loss of yield (Costello & Altieri, 1995; Kelm, & Godomsk, 1995; Gabrys *et al*., 1998). Not only feed, aphids can bring plant virus disease (Hodgson, 1981; Masterman, *et al*., 1994; Nebreda *et al*., 2004). It was demonstrated the honeydew produced by aphids could be easily consumed by epiphytic microorganism and promoted the growth of microbes (Stadler & Müller, 2000). It coexisted with the fact that the aphid population was dramatically suppressed by some fungal pathogens (Steinkraus, 2006). These complicated interactions depicted a close association between aphids and microbes in nature. In chapter 4, I tested the host preference of cabbage aphid nymphs to leaf-discs/intact foliar from infected/uninfected host plants and monitored the insect development (development time from nymph to adult, nymphs number produced). Two cultivars of oilseed rape (*Brassica napus*, *Brassica napus* var.) were used as host plant since plant genotype could influence the effect of fungi on associated insect performance (Kellogg *et al*., 2005). It was demonstrated that the effect of grass endophyte on reducing aphid population *Rhopalosiphum padi* and *Metopolophium dirhodum* last four generations (Meister *et al*., 2006). Therefore in this study, the insect performance of cabbage root fly and cabbage aphids were monitored in three consecutive generations.

With respect to the interaction of herbivores-host plant-fungi, only few study integrated the laboratory and field environment (Gange & West, 1994; Gange, 1996; Moran, 1998; Röder *et al*., 2007). In this research, a field experiment on endophyte mediation the interaction between host plant and herbivores was fulfilled to compare with the results obtained from the laboratory with emphasis of the impact on cabbage aphid population.

The underlying mechanisms of preference/performance of insect mediated by phytopathogenic fungi were involved in the alteration of in metabolisms in infected plant (Sticher *et al*., 1997; Miller *et al*., 2002; Wurst *et al*., 2004). Visual and olfactory cues (volatile and/or leaf-surface chemicals) were two main crucial factors for insects to land and oviposition (Renwick *et al*., 1992; Bernays & Chapman, 1994; Renwick & Chew, 1994). Pathogen-induced alternation in morphological characteristics of host

plant and quantitatively and qualitatively variation of volatile and non-volatile secondary metabolisms had been documented (Pfunder & Roy, 2000; Huang, *et al*., 2003) which urged insects to select a favorable host plant between infected/uninfected host plant (Cardoza *et al*., 2003; Jallow *et al*., 2008). Higher volume of volatiles could be released in crushed plant tissue than in intact tissue (Kirk, 1991). Therefore in this study, we tested whether the orientation effect of insect to infected/uninfected host plant was more apparent when provided host plant was mechanic damaged. Volatiles emitted from the aerial part of infected/uninfected host plant were collected to detect and analyze the variations in quality and quantity so as to explain the insect host preference.

The development of insect relies on not only the qualitative but also quantitative composition of foodstuff, and any departure from the normal composition might cause pests to suffer from impaired metabolitics and other nutritional troubles (House, 1969). Some changes in primary metabolisms in host plant due to fungal infection rendered the problem of the nutritional suitability of the host plant to insect. Fungi infection in host plant resulted in some changes in the balance of carbon/nitrogen and carbohydrate levels (Gange & West, 1994; Hatcher, 1995b); protein, amino acid and sucrose content (Smith *et al*., 1985; Costa Pinto *et al*., 2000); production of some toxic substance in endophytic grasses (e.g. alkaloids) (Dahlman *et al*., 1991; Gimenez *et al*., 2007) and fluctuation of phytosterols content and relative proportion (Bernays, 1993; Dugassa-Gobena *et al*., 1998, 2003). All these alteration had impressive influence on insects development attributes.

Cholesterols are essential substance for insects in structure of membrane and in the production of ecdysteroid, the molting hormones (Clayton, 1964; Svoboda & Thompson, 1985). However, insects can not biosynthesize their sterols *de novo,* the unique source is from diet or from symbionts (Ritter, 1984; Douglas, 1988). Compared with the detailed studies on the sterol metabolism in some insect taxa, such as grasshoppers (Orthoptera), beetles (Coleoptera) and caterpillars (Lepidoptera) (Svoboda & Lusby, 1994; Svoboda & Weirich, 1995; Nes *et al*, 1997; Behmer & Elias, 1999, 2000), the sterol metabolism in the dipteran insects (cabbage root fly) and homoptera (especially: Aphididae cabbage aphid) are virtually unknown. The roles of phytosterols in the development of diamondback moth was tested using artificial diet or the infected host plant, however the conclusions were divergent (Behmer & Grebenok, 1998; Duguassa, *et al*., 1998). It is consensus that alteration

in phytosterols composition impaired insect development (Richter *et al*. 1987; Bodnaryk *et al*., 1997; Nes, *et al*., 1997). In this study, the quantity and composition of three dominant phytosterols in flowering plants (i.e. sitosterol, campesterol and stigmasterol) (Nes, 1977; Behmer & Nes, 2003) and one specific phytosterol in *Brassicaceae* family (i.e. brassicasterol) (Amar, 2007) were analyzed in infected and uninfected plant roots/leaves to elucidate the underlying mechanism of the impact caused by soil-borne fungi on insect performance.

After carrying out this study, we are able to address the following questions:

- whether soil-borne fungus inoculation in host plant changed host preference of larvae /nymphs and gravid adults.
- what kind of cascade effect on insect performance brought about when insect was reared with infected/uninfected host plant.
- whether soil-borne fungi had distinct effect on insects with different mouthparts.
- whether soil-borne fungi altered the content and relative proportion of dominant phytosterols in root/leaves of host plant.
- whether soil-borne fungi altered volatile profiles qualitatively and quantitatively.
- whether plant genotype influenced the effect of soil-borne fungus on insect preference/performance.

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Chapter 1

Preference-performance of the cabbage root fly (*Delia radicum* (L.)) feeding on oilseed rape inoculated with an endophytic fungus

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Abstract

The effect of an unspecialized, soil-borne endophytic fungi, *Acremonium alternatum* Gams (Ascomycotina, Clavicipitacea) on the preference and performance relationship in the cabbage root fly, *Delia radiucm* L. (Diptera: Anthomyiidae), on oilseed rape were evaluated in laboratory and glasshouse experiments. The preference of larvae to root segments (arena experiment) and intact roots of endophyte inoculated plants (E+) and control plants (E-) was tested in dual-choice experiments. The oviposition preference of females was tested in cage experiments. Cabbage root fly larvae of three generations genetically linked (F_1-F_3) were fed on E+/E- host plants using different incubation times per generation to test for potential accumulative effects of the endophyte on successive herbivore generations. The content and composition of the four prevailing phytosterols sitosterol, campesterol, brassicasterol and stigmasterol in E+/E- plant roots were analyzed to understand potential underlying mechanisms. The preference results showed that the endophyte inoculation did not have a significant impact on host preference of cabbage root fly maggots and adults. The performance results showed the endophyte had a negligible impact on the development of cabbage root fly larvae within three generations. However, the developmental duration from pupae to adults was significantly reduced in the F_3 on E+ *B. napus* var. plants, and the fecundity significantly increased in the F_2, demonstrating an accumulative effect of the endophyte on insect performance within generations.

Fecundity and female longevity increased when pooling the data of all three generations on E+ *B. napus* var. No obvious effect by the endophyte inoculation was observed on insect performance on *B. napus*. Sitosterol was the most prevailing sterol followed by campesterol, stigmasterol and brassicasterol. The content of the phytosterols differed between the two treatments with a significant increase in the brassicasterol and slightly increase in campesterol in the roots of E+ *B. napus* var. In contrast, a remarkable difference was found with respect to the relative composition of phytosterols, caused by the endophyte inoculation. The proportion of brassicasterol and campesterol considerably increased while sitosterol decreased in E+ *B. napus* var plants. No significant difference with regard to the phytosterol content and relative composition was detected in E+/E- *B. napus* plants.

In summary, our results demonstrate that although the inoculation by the endophyte *A. alternatum* did not change the host preference of cabbage root fly larvae and adults, this fungus did increase the fecundity and longevity of the flies on *B. napus* var. plants with an accumulative effect within successive generations. These results may be interpreted with regard to the significant changes in the phytosterol proportions in endophyte infected as compared to control plants. The effect of endophytic fungus on the performance of the herbivorous insect depended on the host cultivar tested. The complex consequences of microorganisms mediating herbivore-plant interactions are discussed.

Key words: *Acremonium alternatum*, *Delia radicum* L., *Brassica napus* L., *Brassica napus* var. preference-performance relationship, phytosterols

1.1 Introduction

There is now increasing evidence that microorganisms may have profound effects on plant-herbivore interactions (Clay 1988; Barbosa *et al*., 1991; Hammon & Faeth, 1992; Azevedo *et al*., 2000). Endophytes are microorganisms colonising healthy plant tissues causing inconspicuous inoculations (Wilson, 1995). To date fungal endophytes have been found to be ubiquitous in all plant species worldwide, with extremely high diversity and abundance, specifically in the tropics (Petrini, 1986; Arnold *et al*., 2000, 2001; Seena & Sridhar, 2004; Li *et al*., 2007).

In the past two decades, researches largely focused on the seed-transmitted, host-specific clavicipitaceous endophytes in grass species, since infected plants became less susceptible to herbivorous insect attack (Johnson *et al*. 1985; Breen, 1994; Saikkonen *et al*., 1996). Recent papers even demonstrated grass endophytes mediating interactions with the third trophic level (Bultmann *et al*., 2003; Sassi *et al*., 2006). Many studies argued for a mutualistic role of grass endophytes, on one hand by receiving nutrition (e.g. carbon) and protection from their host plants (Barbosa *et al*., 1991); on the other hand increasing plant growth and tolerance to abiotic and biotic stresses such as drought (West *et al*., 1990; Bacon, 1993;) and herbivore feeding (Clay, 1987; Kanda *et al*., 1994; Clement *et al*., 1997). However, such mutualism did not seem to be universal due to the negligible or antagonistic role of endophytes to host plant reported in some studies (Faeth, 2002; Saikkonen *et al*., 2004; Krauss *et al*., 2007). These host-specific grass endophytes are different from the endopyhtes found in trophical trees and agricultural crops which are unspecialized and transmitted horizontally via spores. It is proved that the grass endophytes represent only a small fraction among the whole fungal endophytic assemblage colonizing leaves, roots, stems and bark in herbs, shrubs, trees, and cultivated crops (Schulthess & Faeth, 1998; Arnold *et al*., 2001). Although the diversity and abundance of these endophytes are higher as compared to the grass endophytes, they have received less attention. Only few study surveyed the ecological role these unspecialized endophytes on their host plants for example, Arnold & Engelbrecht (2007) found that the minimum leaf conductance increased in endophyte infested trees, accentuating water loss during droughts and Costa Pinto *et al*. (2000) showed that photosynthetic efficiency decreased in endophyte

colonized banana and maize leading to reduced carbonhydrate synthesis in banana and maize. Among the least studied taxa are the soil-borne endophyte fungi of the genus *Acremonium* spp., which is particularly remarkable given their ubiquity in forests and crops (Kumaresan & Suryanarayanan, 2001; Grunewaldt-Stöcker & von Alten, 2003; Wicklow *et al*., 2005). Some *Acremonium* spp. strains enhanced host plants the resistance to pathogen infection (Bargmann & Schönbeck, 1992; Wicklow *et al*., 2005; Grunewaldt-Stöcker *et al*., 2007) and some species prompted the growth of plant root system such as *A. kiliense* and *A. cucurbitacearum* (D'Amico *et al*., 2008). In this study, the soil-borne fungus *A. alternatum* was used. This endophyte invaded host plant from the root and was mainly restricted there.

By altering the physiology of host plant, soil-borne fungi may alter the host preference/performance relationship of the associated herbivorous insects and resulted in beneficial, detrimental or negligible effects on the insect's development (Barbosa 1991). Recent studies in agriculture crops showed that adults of greenhouse whitefly *Trialeurodes vaporariorum* (Westw.) and *Helicoverpa armigera* preferred to oviposite on host plants inoculated either with *Acremonium alternatum* or *A. strictum* while the offspring suffered from a retarded development and high mortality (Vidal, 1996; Raps & Vidal, 1998; Jallow *et al*., 2008). The negative effects resulting from endophyte inoculations were reported in other studies as well (Rabin & Pacovsky, 1985; Gange *et al*., 1994; Gange, 2001; Miller *et al*., 2002). However, Lappalainen & Helander (1997) found the endophytic fungi colonizing mountain birch leaves had a negligible effect on leaf beetle larval performance under natural conditions. The beneficial effects caused by the endophytic fungus *Rhytisma acerinum* or the arbuscular mycorrhizal fungi *Glomus intraradices* on the weight and fecundity of two aphid species were reported (Gange, 1996; Gange *et al*., 1999).

The mechanisms of an endophyte inoculation causing effects on herbivores include the production of mycotoxins by the infected plant (e.g., alkaloids or other toxic compounds) (Dahlman *et al*., 1991; Faeth, 2002; Findlay *et al*., 2003; Arimura *et al*. 2005; Gimenez *et al*., 2007), alteration of the plant's nutritional quality (e.g. phytosterol content and composition) (Bernays, 1993; Dugassa-Gobena *et al*., 1998), fluctuation in carbohydrates product (Farrar, 1992) and changes in the carbon-nitrogen balance (Jones & Last, 1991; Gange, 1996; Faeth & Fagan, 2002).

The cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae) is a widespread and destructive specialist pest of cruciferous crops. The eggs are deposited around the

stem basis of the host plants, and the hatching maggots damage or destroy the root systems of many crucifers, including oilseed rape (Dosdall *et al*., 2000; Jensen *et al*., 2002). Feeding damage results in water stress and provides routes for fungal infections, such as *Verticillium* wilt pathogens and *Fusarium* rot fungi (Ridsdill Smith, 1977). The damage to the roots results in serious reductions both in yield and quality and causes considerable economic losses for oilseed rape production (Griffiths, 1991).

In this study, a soil-borne endophyte (*Acremonium alternatum*), the cabbage root fly (*Delia radicum* L.) and two cultivars of oilseed rape were used to asses

- whether an endophyte inoculation alters host preference of adults and larvae of the cabbage root fly
- whether an endophyte inoculation impacts the performance of larvae
- whether the interaction between the endophyte inoculation and the plant-associated herbivore is influenced by plant genotype

Sterols are indispensable substances used for the insect's membrane and as a molting hormone; its amount and composition in plant tissues directly influence the performance of herbivorous insects (Zandee, 1962; Clayton, 1964; Nes *et al*. 1997). Insects are unable to synthesize these sterols needing the unique resource either from host plants or symbionts (Ritter, 1984; Douglas, 1988; Ikekawa, 1993; Mondy & Corio-Costet, 2000). Previous studies found infection by micro-organisms changed the phytosterol content and composition in host plants (Dugassa-Gobena *et al*., 1998, 2003). In this study, by analyzing the content and the composition of phytosterols in endophyte infected and control plants, we attempt to understand how endophyte changes nutrient suitability of host plant by altering phytosterol and discover the mechanisms of the impact of endophyte on insect performance.

1.2 Material and Method

Plants

Oilseed rape plants (*Brassica napus* L. (cv Falcon) and *Brassica napus var*.(cv Licosmos)) were sown and grown in plastic pots (diameter: 11 cm) in the greenhouse [24 ± 3°C, 80% relative humidity (r.h.) under 16L : 8D photoperiod] for one week.

Young seedlings were transplanted to plastic pots of the same size with a substrate comprising soil and sand (ökohum®-Rindentorfsubstrate, Type RTS; Ratio: 2:1) and were grown for 3 weeks in the greenhouse. Plants were watered daily and fertilized weekly with a liquid fertilizer (**Wuxal® Super (10-10-8 w/v)**)

Insects

The flies originally were provided by the JKI (Julius-Kühn-Institut, Braunschweig, Germany) in November 2003 and reared in a climate controlled culture room (21 ± 1°C, 70 ± 5% r.h. and LD 16h : 8 h photoperiod) following the method of Finch & Coaker (1969) with some modifications. Adult flies were held in cubic screen cages (28cm × 28cm × 28 cm) and fed with artificial food. A cubic of Kohlrabi (*Brassica oleracea* var. *gongylodes*) was placed on a layer of quartz sand in a Petri-dish to attract the females for oviposition. Eggs were collected by floating with water and filtrated on a filtrate paper in a funnel and transferred to a fresh kohlrabi with shallow cut-surface. The kohlrabi was incubated with sand for 3 weeks, thereafter the pupae were collected out of the sand by floating. Two types of food were provided to the adults, i.e. dry powder and sticky honey. The powdery food was made of dextrose, organic skim milk powder, soy flour and brewer's yeast (10g:10g:1g:1g); the sticky honey was made of commercially available honey, soy flour and brewer's yeast (5g:5g:1g). Tap water was offer separately in a glass vial, the opening directed downwards to a filter paper on a Petri-dish.

Fungi

A strain of the root endophyte *Acremonium alternatum* (CBS 831.97) from the Netherlands was maintained on potato dextrose agar (PDA) in the laboratory. For the production of a spore suspension, the autoclaved potato dextrose broth (PDB) was inoculated with two pieces of PDA agar containing the fungus mycelia. The flasks were kept at 23°C on a rotary shaker at 100 rpm for approximately three weeks to ensure fungal growth and sporulation. The spore suspension was filtrated under vacuum and the concentration of the spore suspension was cerified using a Thoma counting chamber (Marienfeld, Germany) (depth 0.1mm, square width:

0.05mm) under a microscope. The due spore suspension was adjusted by adding tap water to 10^6 conidia ml^{-1}.

Protocol for the dual-choice experiment using cabbage root fly larvae and E+ or E- root-segments of the host plants

Three weeks old plants were watered with 70ml of a conidial suspension (10^6 conidia mL^{-1}); the same amount of sterilized culture filtrate was used for control plants. One week after inoculation the plants were used for the experiments. Plants were cut at the interface between above-ground and below-ground. The roots were gently washed to maintain the fine root hairs. Extra water adhering to the root hairs was removed using a filter paper. Endophyte-infected (E+) and uninfected (E-) roots were arranged in the test area as described below.

The experiment was conducted in Petri-dishes (diameter: 14 cm) divided into an E+ area, an E- area and two searching areas, respectively. The distance between the E+ and E- area was 6 cm. E+ and E- root pieces with comparable hair root amount were placed in the corresponding E+ / E- areas in the Petri-dishes. Before starting the experiment, the root fly larvae were kept in darkness for 2-3 hours with a little piece of Kohlrabi at room temperature. One larva previously feeding for 12 days on kohlrabi was placed at the starting point, opposite to the root segments. The Petri-dishes were filled with Vermiculit to simulate the soil (Figure 1). The experiment was performed in complete darkness for 120 min at room temperature. Larvae entering the E+ or E- area were recorded as responding to the treatment, otherwise they were regarded as not responding. The experiment was replicated 30 times.

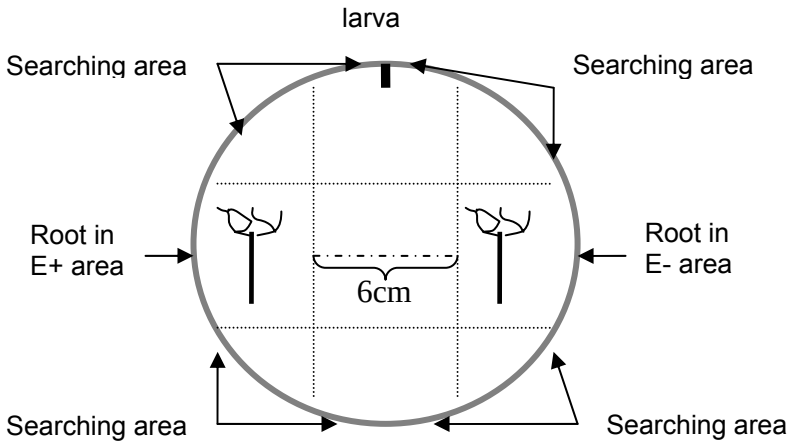

Figure1 Dual-choice experimental setup for cabbage root fly (*Delia radicum* L.) larvae offered *Acremonium alternatum* infected or control root segments of *Brassica* spp..

Protocol for dual-choice of cabbage root fly larva to intact root of host plant

In this experiment, host plants were grown in special pots with a hole (diameter: 2.0 cm; hole position: 2.5 cm to the bottom of the pot) blocked using a solid stopper (diameter: 2.0 cm; length: 3.0 cm). Four weeks old *B. napus* var. plants were inoculated with *A. alternatum* for one week by watering with 70ml of a spore suspension (10^6 conidia mL^{-1}); the same amount of culture filtrate was added to control plants.

During the experiment a two-arm transparent tube was inserted serving as a larval passage to the host plants. Each arm consisted of two equally detachable compartments (diameter: 2.0 cm, length: 2.5 cm) with a detachable cup at the ends. Using this two-arm test tube, root fly larvae could be easily probed in the arms either filled with vermiculite or soil or left empty. The larval entrance was plugged to avoid larvae escaping.

When starting the dual-choice experiment, the arms were inserted ca. 2.5 cm into the pots close to the plant roots. Before starting the experiment, the root fly larvae were kept in darkness for 2-3 hours with a little piece of Kohlrabi at room temperature. One larva which was incubated for 12 days on Kohlrabi was placed at the entrance of the tube using a fine tweezer, thereafter entrance was sealed with a plug. The experiment was performed in a climatic chamber (25°C, 70% r.h.) in darkness for 15 min. If larvae moved more than 2/3 of the tube length, this was recorded a positive choice, otherwise they were recorded as making no-choice. The experiment was replicated 29 times.

A preliminary experiment tested the ability of cabbage root fly larvae to discriminate between substrate soil and host plants. That experiment was set up using three weeks old *Brassica napus* var. plants and pure soil in 20 replications.

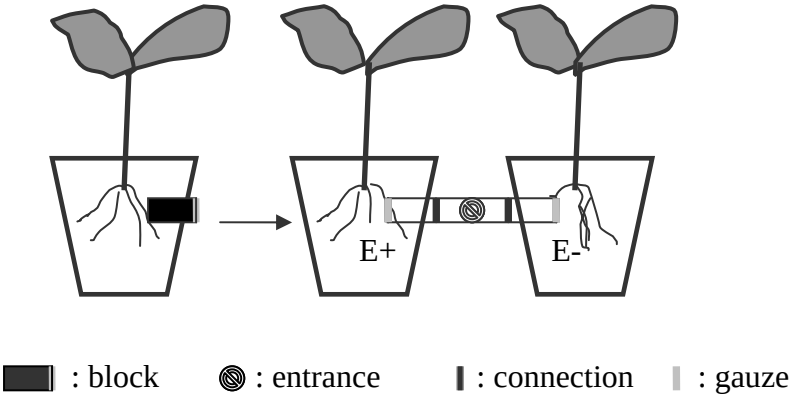

Figure 2 Dual-choice experimental setup for cabbage root fly (*Delia radicum* L.) larvae to discriminate between *Brassica spp. roots* infected by *Acremonium alternatum* and controls

Cabbage root fly oviposition on E+ / E- cabbage plans

Four weeks old *Brassica napus* var. plants were inoculated with *A. alternatum* for three weeks by watering with 70ml of a spore suspension (10^6 conidia mL^{-1}); the same amount of culture filtrate was used to water control plants. Twelve plants, 6 inoculated and 6 control plants, were arranged in 3 by 4 blocks in each of six gauze cages (area:75cm×55cm, height:75cm), alternating control and inoculated plants. 80 ml of sand was added to each plant (sand particle: ca. 1mm) on the surface of the pot soil (depth: ca. 15mm) for the flies to oviposit on. Ten pairs of newly hatched females and male were released into one cage for 9 days. Water and artificial food was supplied according to the root fly rearing procedure (21±1°C, 70±5% r.h. and LD 16h : 8 h photoperiod). Thereafter eggs were collected from the sand by floatation in water and counted. Following the experiment, 11 to 13 pots of E+/E- host plants, randomly selected, were cut at the interface of the above-ground and below-ground. Roots were gently washed and root and shoot parts of the plants were enclosed in paper bags and dried at 60°C for one week to measure dry weight.

Insect performance fed on E+ / E- host plant

In this experiment, three successive generations of cabbage root flies were reared on either inoculated or control plants. The origin of eggs and the incubation time of *A. alternatum* infected host plant differed.

Three weeks old *B. napus* and *B. napus* var. were inoculated with *A. alternatum* for three weeks by watering with 70ml of a spore suspension (10^6 conidia mL^{-1}); the same amount of culture filtrate was added to control plants. The larvae of the first (F_1) and the third generation (F_3) were fed on E+/E- host plants for 10 days, whereas the larvae of the second generation (F_2) was maintained on E+/E- host plants for 21 days (Figure 3).

F_1: Healthy eggs were incubated for 11 days on Kohlrabi, then 3 larvae were transferred to the roots of E+/E- host plants using a fine tweezer; development continued for 10 days. Pupae were collected from the pot soil, weighted and placed in Petri-dishes lined with a moist paper and incubated in a climatic chamber (21 ±1°C, 70% r.h., LD 16h : 8 h). After emergence of adult flies, a male and a female was released into a transparent plexiglass cylinder (height: 35cm, diameter: 12cm) with access to artificial food and tap water, as used for permanent rearing. The fecundity experiment took place in an environment-controlled room (21 ±1°C, 70% r.h., LD 16h : 8 h) and developmental indices were recorded (e.g. the duration from pupa to adult, from eclosion to the first batch of eggs deposited, sex). During the oviposition time, dead males were replaced with new males for maximizing fecundity of females. Eggs were collected every 5 days. Fecundity and longevity of males and females were recorded. Ten plants were used (3 larvae/plant).

F_2: To increase the number of root fly adults in the fecundity experiment, 5 eggs laid by F_1 female were placed on the roots of E+/E- host plants to obtain eggs of F_2. Twenty plants were used (5eggs/plant).

F_3: Eggs from F_2 females were incubated on Kohlrabi for 11 days and 5 larvae were placed on each root of E+/E- host plants for 10 days. Twenty plants were used (5 larvae/plant).

High humidity is regarded critical for the viability of eggs and hatching (Finch & Coaker, 1969). Therefore, after transferring eggs into the soil near to the roots (i.e. F_2), it was very important to irrigate host plants with extra water as compared to the doses regularly applied (100-120ml / pot). After 3-4 days of hatching, water was reduced and applied two times per week (60-80ml / pot) to maintain the development of larvae. Larvae not pupating within three weeks were not used in the fecundity experiment.

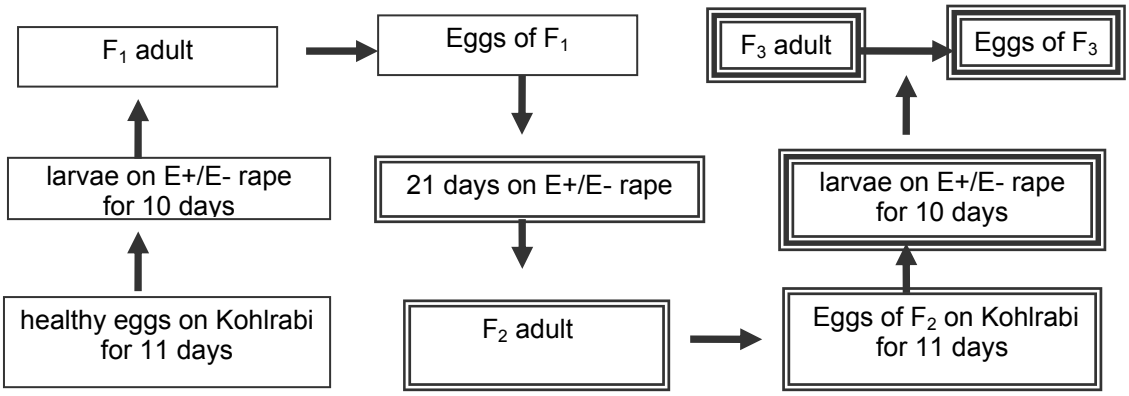

Figure 3 Experiment process of cabbage root fly (*Delia radicum* L.) feeding on oilseed rape inoculated/uninoculated with *Acremonium alternatum* within three generations

Phytosterol profiles in plants

Three week old *B. napus* and *Brassica napus* var. plants were inoculated with *A. alternatum* for three weeks by watering with 70ml of a spore suspension (10^6 conidia mL^{-1}); the same amount of culture filtrate was added to control plants. Three weeks later, ten plants from each treatment were used to analyse the phytosterol profiles. Plants were cut at the interface between above-ground and below-ground and roots were gently washed to maintain as much root hair as possible.

Roots were grinded to fine powder in liquid nitrogen using a mortar and a pestle. 0.5-0.6 g plant material was transferred to a glass bottle (volume: 40 ml) with a screw-cup. Phytosterols were extracted following the procedure described by Newton (Newton, 1989) with modifications as follows: 20ml mixed solvent (5 ml 10 M KOH, 15 ml 96% ethanol and 0.06 g pyrogallol was added to each bottle containing plant material. An ultrasonic homogenizer (Model: Sonoplus HG 2200 / UW 2200, 200 W, 20 kHz, Bandelin GmbH, Germany) was used for 30 sec for cracking cells completely and degassing. Thereafter bottles were placed in a shaker water bath at 80°C for 2.5 h. After cooling the samples to room temperature, 40 µl cholesterol (Concentration: 5 µg / µl chloroform, Merck GmbH, Germany) was added as an internal standard. Phytosterols were extracted with 10 ml hexane for 3 times. After thoroughly shaking for 20 sec and depositing for 5-10 min for separating phases, the supernatant was transferred to a rotary glass. One ml distilled water was added to wash the fractions and the separated lower phase was sucked out. Samples were distilled with a rotary-evaporator at 42°C. The concentrated sterols were resolved in

1.5ml hexane and transferred to 2 ml Eppendorf cups. After centrifuging with 10,000 rpm for 10 min, the supernatant was transferred to a GC-vial and the hexane was evaporated overnight at 50°C in a thermal block.

The concentrated sterols were resolved in 240 µl hexane and 80 µl N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Fluka/Riedel-deHaen GmbH, Germany) and incubated in the thermal block at 70°C for 20 min. After cooling down to room temperature, 100-150 µl of the extracts were transferred to GC-vials equipped with 300 µl inserts.

Phytosterol identification and quantification was based on the internal standard method (peak area and retention time). Sterols were analyzed using a gas chromatograph (Shimadzu GmbH, Model: GC14/15A) with a flame ionization detector. The samples were run on a fused silica column (SPB-1; 1.3m x 0.32mm, 0.25µm film thickness, Supelco Inc./ Sigma-Aldrich, Germany). Helium was used as a carrier gas, the make-up gas was synthetic air with a linear velocity of 35cm/s. The temperature program was initially 3 min at 180°C then increased to 290°C at a heating rate of 10°C/min to keep 36 min. The total analyzing time was 50min. The detector temperature was 300°C. Peak areas were calculated using an integrator and the internal standard. A set of typical peaks indicating phytosterols, such as brassicasterol, campesterol, stigmasterol and sitosterol had been individually identified beforehand using GC-MS with synthetic sterols as a reference. The phytosterol content in one gram plant material was calculated following the formula:

$$G = \frac{200 \times p_2}{p_1 \times g}$$

G: weight of unkown phytosterol in one gram plant material

p_1: peak area of cholesterol

p_2: peak area of unkown phytosterol

g: weight of plant material

The total phytosterol content in one sample was calculated by summing up all four phytosterols.

Statistical analysis

The results of the larval feeding preference to root segments and intact roots were analyzed using a binomial probability given the null hypothesis that larvae had an even choice with regard to the two treatments. The larvae that did not make a choice were not included in the analyses. The mean number of eggs laid by adult females in the oviposition preference experiment was analyzed by a one-way analysis of variance (ANOVA; PROC GLM; SPSS 10.0) with inoculation status (E+, E-) as the main effect. Within the root fly performance experiment, the data on the developmental time (from pupa to adult, from eclosion to first batch of eggs and longevity of males and females) were analyzed by one-way analyses of variance (ANOVA). For the F_2 from pupa to adult on *B. napus* var., data were log10 transformed to meet a normal distribution. On *Brassica napus* data on the developmental time from pupae to adults were analyzed using a Mann-Whitney test. Data on the developmental time from eclosion to egg laying in F_3 on *B.napus* var. were analyzed using a Mann-Whitney-test. Because eggs were collected every five days, repeated measure ANOVA was used for the fecundity analysis. One-way ANOVA was used to analyze the phytosterol content and composition. All analyses were performed using SPSS 10.0.

1.3 Results

1.3.1 Dual-choice of cabbage root fly larvae to E+/E- root-segments and intact roots

Although more larvae chose root segments or intact roots of uninfected host plants, this difference was not significant (Table 1). In a preliminary dual-choice test, root fly larvae were able to detect their host plants as compared to soil alone (14 larvae orientated towards plants; 3 larvae orientated to plant soil, and 3 no-choice, binomial test p=0.013). Thus larvae were capable of choosing their host plants by means of olfactory cues.

Table 1 Feeding preference of *Delia radicum* larvae to root segments and intact roots in laboratory dual-choice tests among *Acremonium alternatum* infected/uninfected *Brassica napus* / *B.napus* var.

Cultivar in exp.	E+	E-	No-choice	Sig
B. napus (RS)	11	18	1	n.s
B.napus var.(RS)	9	19	2	n.s
B.napus var.(RI)	11	14	5	n.s

RS: root segment experiment; RI: intact root experiment; n.s: no significant difference

Preference of root fly female adult to E+/E- *B. napus* var.

The inoculation of plants with endophytes did not significantly influence plant size (dry shoot and root weight) (Table 2). On average 47.7% of eggs were laid on infected plant as compared to 52.3% on healthy plants (paired t-test: t=-0.794 df=5, p=0.463, n=6). A mean of 39.28 ± 25.94 eggs per plant were laid on inoculated rape plants as compared to 49.25 ± 31.98 on healthy plants. Because of the high variation in egg numbers, even a LOG10- transformation of the data did not result in significant differences at the 5% level (ANOVA, $F_{(1,70)}$=3.591, p=0.062). Although not significant, adults consistently choose control plants, which was also found in a field experiment (Li & Vidal, unpubl.).

Table 2 Dry weight of roots and shoots of *Brassica napus*/*Brassica napus* var. inoculated with *Acremonium alternatum* for one week or control. E+: with *A. alternatum*. E-: controls.

cultivar	N	dry weight	E+	E-	F	P
B. napus	11	dRW	0.57± 0.21	0.71± 0.27	2.008	n.s
	12	dSW	2.81± 0.90	3.03± 0.73	0.401	n.s
B.napus var.	13	dRW	0.21± 0.054	0.24± 0.071	2.051	n.s
	13	dSW	1.65± 0.37	1.89± 0.37	2.269	n.s

N: the number of plant, dRW: dry root weight, dSW: dry shoot weight; One-way ANOVA test. n.s: no significant difference

1.3.2 Effect of the endophyte on the development of the root fly

The endophyte inoculation increased mortality of eggs and larvae when fed on E+ *B. napus* in the F_2 and F_3 generation; however this was reverse when fed on E+ *Brassica napus* var. When feeding on healthy plants larvae gained more pupal weight within three generations and on two cultivars tested. The mortality of pupae

were higher on endophyte inoculated plants except in F_1 on *B. napus* and F_2 on *B. napus* var. Males always spent less time to eclose than females on the two cultivars tested (on *Brassica napus*: females: 12.59 ± 3.72 days, males: 10.19 ± 2.05 days; $F_{(1,66)}$= 11.675, p=0.001<0.01; on *Brassica napus* var.: females: 9.7 ± 2.67, males: 8.63 ± 2.99, $F_{(1,144)}$= 5.135, p= 0.025<0.05) within the three generations. Concerning the days from pupa to adult, females and males feeding on infected host plants spent less time, which was particularly obvious for the females in the F_3 on E+ *B. napus* var. (P= 0.002<0.01). However, endophyte inoculation neither had an impact on the developmental time of the females from eclosion to laying the first batch of eggs nor between the generations (on *Brassica napus*: E+ females: 5.34 ± 1.68 days, E- females: 5.68 ± 1.06 days, $F_{(1,24)}$=0.417, p=0.525; on *Brassica napus* var.: E+ females: 4.72 ± 1.19 days, E- females: 4.87 ± 0.95 days, $F_{(1, 35)}$= 0.185, p= 0.67), nor on the sex ratio (Table 3). Three cabbage root fly adults showed deformations (E+: 2 females, 1 male) when fed on inoculated *B. napus* or fed on *B. napus* var. (E+: 2 females, E-: 1 female).

Table 3 Development of cabbage root flies (*Delia radicum*) feeding on *A. alternatum* infected/ uninfected host plants within three successive generations. [Mortality rates (%, from egg to pupa and from pupa to adult), pupal weight (mg, total pupal weight/total number of pupae), sex ratio (♀:♂),development time (mean ±SD, days)]. E+: *A. alternatum*-infected plants, E-: control plants.

Host plant cultivar	Treatment		Mortality of egg + larva (%)	pupa weight (mg)	Mortality of pupa (%)	sex ratio (♀:♂)	Pupa-adult (days) ♀	Pupa-adult (days) ♂	Eclosion-laying eggs (days)
B.napus	F_1	E+	53.3	13.2	21.4	0.38	9.60±2.20	8.75±1.58	4.21±0.36
		E-	63.3	13.4	23.1	0.67	12.4±2.36	10.71±2.21	5.09±0.42
	F_2	E+	86.0	14.0	15.4	0.83	13.4±0.55	11.67±1.37	3.96±0.49
		E-	74.0	14.4	3.70	0.40	15.0±2.61	11.33±1.44	4.83±0.81
	F_3	E+	82.5	13.0	47.4	0.75	9.50±3.87	7.33±1.53	7.17±1.45
		E-	70.7	13.6	22.2	1.85	13.43±5.26	8.67±1.37	6.36±0.79
B.napus var.	F_1	E+	56.7	12.4	7.60	1.25	9.29±2.43	8.60±2.41	5.30±0.61
		E-	56.7	13.4	0.00	1.67	8.00±1.80	7.80±2.17	5.06±0.37
	F_2	E+	54.0	13.3	10.9	1.41	11.65±1.56	11.18±1.70	4.28±0.44
		E-	65.0	13.5	31.5	0.41	12.71±1.38	11.76±1.25	4.04±0.49
	F_3	E+	61.9	9.80	23.2	0.79	6.80±1.57**	5.91±1.24	5.33±1.35
		E-	66.0	14.8	18.7	0.67	9.22±1.78	6.86±1.70	5.41±1.21

F_1: first generation, F_2: second generation, F_3: third generation; **: significantly different (F_3, development days from pupa to adult, ANOVA, $F_{(1, 22)}$=12.11, P=0.002<0.01);

Effect of endophyte inoculation on fecundity of females

More eggs were laid by E+ female than by E- females in each generation on the two cultivars tested; however, these differences were not significant for E+ *B. napus* in each generation (Table 4). By pooling all data within the three generations on *Brassica napus*, endophyte inoculation did not significantly influence fecundity and longevity of females, but endophyte inoculation significantly decreased the longevity of males. Females exhibited a comparable longevity as compared to males (females: 41.83 ± 17.42, males: 40.64 ± 15.46, ANOVA, $F_{(1,61)}$= 0.081, p= 0.777).

Table 4 Mean number of eggs (±SE) laid per 5 days, mean fecundity (±SD) and mean longevity (±SD) (days) of *Delia radicum* adults out of larvae feeding on *A. alternatum* infected/ uninfected host plant roots within three successive generations (F_1-F_3); E+: *A. alternatum*-infected plants, E-: control plants.

culivar	F_1-F_3	treatment	Eggs/5 days	fecundity	longevity (day)	
					♀	♂
B.napus	F1	E+	20.33±8.11	178.5±79.9	47±25.98	30.4±13.58
		E-	15.53±6.55	146.5±12.02	29.5±10.61	41.25±15.54
	F_2	E+	12.89±4.65	193.1±52.2	42.8±12.2	35.8±14.4
		E-	10.21±3.33	153.0±29.3	54.2±19.6	40.5±13.6
	F_3	E+	14.26±4.35	168.33+34.8	40.3+23.0	34.8±12.1
		E-	13.59±4.85	148.6±85.0	54.2±10.6	30.5±34.7
B. napus var.	F_1	E+	10.01±3.5	160.2±66.6	45.8±21.4	59.0±3.2
		E-	9.11±3.58	145.4±44.4	44.3±8.3	57.5±16.5
	F_2	E+	11.63±3.78	154.4±30.3**	45.3±13.1	43.6±9.5
		E-	9.01±3.64	117.1±21.6	35.71±5.3	46.5±7.8
	F_3	E+	19.13±4.38	178.4±67.7	40.3±12.8	42.6±14.3
		E-	16.93±6.35	149.9±51.9	21.0±11.7	35.3±10.3

**: significantly different (F2, fecundity on *B. na*pus var. ANOVA; $F_{(1,20)}$=8.502 P=0.009<0.01).

On *Brassica napus* var., significantly more eggs were laid by F_2 females on E+ *B. napus* var. plants (E+: 154.4 ± 30.3:, E-: 117.1 ± 21.6, ANOVA, $F_{(1,20)}$= 8.502 P= 0.009<0.01**). By pooling all data within the three generations, the endophyte inoculation increased fecundity and longevity of females. Females exhibited a significantly shorter longevity as compared to males (females: 38.72 ± 14.76, males: 43.83 ± 12.80, ANOVA, $F_{(1,120)}$= 4.172, p= 0.043<0.05*) (Table 5).

Table 5 Impact of *Acremonium alternatum* infection on fecundity and longevity of cabbage root fly adults (*Delia radicum* L.) on *Brassica napus* / Brassica napus var. (mean±SD; one-way ANOVA, n.s: no significant difference)

cultivar			E+	E-	F	p
B. napus	fecundity	♀	170.75±82.76	150.63±64.4	$F_{(1,24)}$=0.483	n.s
	longevity	♀	43.00±18.11	41.00±17.43	$F_{(1, 27)}$=4.703	n.s
		♂	32.92±16.01	44.38±14.31	$F_{(1, 32)}$=4.703	0.038*
B. napus var.	fecundity	♀	166.23±54.95	138.04±42.51	$F_{(1,57)}$=4.261	0.044*
	longevity	♀	43.37±14.21	31.52±13.48	$F_{(1, 56)}$=10.05	0.002**
		♂	44.91±12.9	42.59±12.8	$F_{(1,60)}$=0.504	n.s

1.3.3 Phytosterol

Four major phytosterols were detected and summed up to calculate the total phytosterols content: sitosterol, campesterol, stigmasterol and brassicasterol.

In the roots of both E+ and E- *B. napus /B.napus* var. plants, sitosterol was the most abundant phytosterol followed by campesterol, stigmasterol, and brassicasterol, respectively.

For *B. napus*, *A. alternatum* inoculation decreased the quantity of campesterol, stigmasterol and sitosterol, respectively and the total amount of phytosterol content per one gram root material, however these differences were not significant. Regarding the relative sterol content, the main phytosterols comprised 77.6% sitosterol, 19.3% campesterol, 2.57% stigmasterol and 0.53% brassicasterol in the roots of inoculated host plants; in the control plants, the composition was 77.2% sitosterol, 19.9% campesterol, 2.2% stigmasterol, and 0.7% brassicasterol. There were no significant differences with regard to the phytosterol composition between the two treatments.

For *B. napus* var. the inoculation decreased sitosterol content, but increased the content of the other three phytosterols per one gram root material. However, the differences for the phytosterol content in the two treatments was not statistical significant. Concerning the relative composition of phytosterols in the roots of inoculated host plants, the main phytosterols comprised 67.7% sitosterol, 24.6% campesterol, 6.8% stigmasterol, and 0.9% brassicasterol; in control plants, the composition was 74.0% sitosterol, 19.3% campesterol, 6.2% stigmasterol, and 0.5% brassicasterol. In E+ *B. napus* var., the relative content of brassicasterol and

campesterol significantly increased, whereas the relative content of sitosterol considerably decreased (Table 6).

Table 6 Phytosterol quantity and relative composition in the roots of *Acremonium alternatum* infected or uninfected *Brassica napus* / *Brassica napus* var.

phytosterol in host plant root	**E+**	**E-**	**$F_{(1,11)}$**	***P* value**
Host plant: B.napus				
mean of brassicasterol (ug/g root)	2.22±1.20	1.99±0.83	0.24	n.s
mean of campesterol	67.32±14.16	78.14±25.66	1.25	n.s
mean of stigmasterol	8.10±5.40	10.78±5.47	1.15	n.s
mean of sitosterol	261.93±52.92	310.35±90.83	1.95	n.s
Sum of phytosterol	339.57±70.18	401.25±121.52	1.78	n.s
relative content of brassicasterol	0.007±0.003	0.0053±0.003	1.36	n.s
relative content of campesterol	0.199±0.017	0.193±0.009	0.89	n.s
relative content of stigmasterol	0.022±0.0091	0.0257±0.0065	0.88	n.s
relative content of sitosterol	0.772±0.016	0.776±0.013	0.38	n.s
***Host plant: B.napus* var.**				
mean of brassicasterol	2.91±0.98	1.87±0.17	6.45	0.027*
mean of campesterol	79.97±22.38	60.83±8.32	3.88	0.074
mean of stigmasterol	22.68±11.66	19.30±3.73	0.46	n.s
mean of sitosterol	216.87±42.07	232.93±25.20	0.66	n.s
Sum of phytosterol	322.42±74.47	314.92±33.33	0.05	n.s
relative content of brassicasterol	0.009±2.7E-03	0.005±3.5E-04	8.40	0.014*
relative content of campesterol	0.246±0.017	0.193±0.006	54.4	0.000***
relative content of stigmasterol	0.068±0.019	0.062±0.014	0.44	n.s
relative content of sitosterol	0.677±0.028	0.740±0.015	25.1	0.000***

(One-way ANOVA test, n.s: no significant, P<0.05*, P<0.01**, p<0.000***)

1.4 Discussion

Dual-choice of cabbage root fly larvae and adults

In this study, the host plant choice of cabbage root fly larvae was not influenced by the endophyte inoculation, as shown by the dual-choice experiment. However cabbage root fly larvae showed a strong tendency to uninfected plants, particularly when root segments were used. This might be attributed to a higher release of specific volatiles (e.g. isothiocyanates) by crushed cruciferous plant tissues than intact plant (Finch, 1978; Kirk, 1992). An earlier study reported a host preference

behavior of the specialist cabbage root fly larvae to turnip roots and swede root (Rygg & Sömme, 1972). Several studies proved that during host plant finding maggots were attracted by either semiochemicals released in the rhizosphere or carbon dioxide (CO_2) emitted by root respiration (Kostal, 1992; Bernklau & Bjostad, 1998; Cobb, 1999; Bernklau *et al*., 2004; Johnson & Gregory, 2006). According to personal observations, root fly larvae, when arriving in the roots, preferred to orientate towards hairy roots, and then started to bore into the main root part. This behavior might be related to the higher quantity of volatiles released by the root hairs. In this study, although not significant, adults consistently laid more eggs on control plants, a behavior, which was also found in a field experiment (Li & Vidal, unpubl.). Females test the suitability of a host plant before egg-laying by processing visual, olfactory and contact stimuli qualitatively and quantitatively. Visual cues are very important factors for host choice of cabbage root fly adult, such as foliar color, plant size, and leaf form (Tuttle *et al*., 1988; Roessingh & Städler, 1990; Degen & Städler, 1997; De Jong & Städler, 1999; Finch & Collier, 2000; Kostal *et al*., 2000). In this study, the dry weight of root and shoot when comparing E+/E- host plants did not differ significantly. We thus excluded visual cues as factors influencing the behaviour of the cabbage root fly females. Volatiles released from E+/E- host plants are regarded as the most crucial factors guiding the larvae and adult in host plant finding. Landing and oviposition of cabbage root flies are mediated by olfactory stimuli such as volatile glucosinolates and their breakdown compounds from Brassicaceae plants, and to some extent contact chemostimulation such as the recently found oviposition stimulant CIFs (cabbage identification factors) (Baur *et al*., 1996; Hopkins *et al*., 1997; De Jong & Städler, 2002; Marazzi & Städler, 2004). It has been shown that volatile profiles are altered by microorganism growing inside the host plants. Glucosinolates, accumulated in the roots and shoots of Brassicae plants inoculated by the leaf spot pathogen (*Alternaria brassicae*) or the clubroot disease fungi (*Plasmodiophora brassicae*) (Doughty *et al*, 1991, 1996; Ludwig-Müller *et al*, 1997), resulting in a host preference by Delia maggots and adults (Hough-Goldstein & Bassier, 1988). Unfortunately, in this study we just showed the preference of cabbage root fly larvae and adults. The underlying mechanism has to be evaluated in further experiments, including analyses of the volatile profiles. Consistent with the finding of larval preference, Jallow *et al*. (2004) found that *Heliocoverpa armigera* (Hübner) larva did not discriminate between tomato plants infected by *Acremonium*

stritum and uninfected plants; however *H. armigera* adults and greenhouse whitefly adults significantly preferred to lay more eggs on endophyte infected plants (Vidal, 1996; Jallow *et al*., 2008). These findings are opposite to the preference of cabbage root flies in this study. Current findings shed light on the complex diversity involved in specific plant pathogens, plants and herbivorous insect interactions (Barbosa, 1991). Moreover, it has to be evaluated further, whether plant pathogens consistently alter the insect preference to host plants leading to fitter offspring as proposed by the preference-performance principle (Thompson & Pellmyr, 1991; Awmack & Leather, 2002; Johnson *et al*. 2006;).

Larval and pupal development

The subsequent insect development experiments showed that an endophyte inoculation had no significant impact on the mortality of larvae and pupae. On the contrary, the development time from pupae to adults was considerably shortened for F_3 on E+ *B. napus* var. These results demonstrate that the endophyte inoculation had a minor effect on the development of cabbage root fly larvae and pupae.

Fecundity

Fecundity was improved in cabbage root flies when reared for three generations on two oilseed rape cultivars; specifically there was a significant increase for F_2 females reared on E+ *Brassica napus* var. The impact of the endophyte on female longevity was not consistent within each generation. When pooling the data for three generations, longevity and fecundity were significantly higher in females developing on E+ *Brasica napus* var., however the positive effect of the endophyte was not obvious on *B. napus*, except that the longevity of male root flies significantly increased. These results display that the genotype of the host plants influence the effect of the endophytes on the performance of herbivorous insects. This has been shown in other studies as well (Faeth, 2002; Bultman *et al*., 2006). Because a previously study showed that the dependence of fecundity and longevity on the food provided (Finch & Coaker, 1969), the artificial food supplied to E+/E- females was homogenized in our experiment, and the rearing environment was standardized

(temperature, photoperiod). The only possible interfering parameter was the size of the oviposition stimulant, kohlrabi and the time it stayed fresh. A fresh stimulant will be more attractive for females than a dry kohlrabi, thereby causing the typical oviposition behavior such as walking on the kohlrabi, touching the kohlrabi using their ovipositors and finally egg-laying in the sand beneath the kohlrabi (personal observation). In this experiment, kohlrabi was replaced every 5 days since the first batch of eggs was collected, which possibly did not match very well to each female's stimulant need for egg-laying, generally before laying eggs females showed the strongest reaction to the stimuli (personal observation). In addition to the increased fecundity, longevity of females was improved as well on E+ *B. napus* var.

In summary, in our experiments cabbage root fly larvae and adults chose plant without endophytes, however the subsequent development experiment showed feeding on endophyte infected host plant increased fecundity of females, and in part also longevity, especially in females fed with E+ *B. napus* var. As compared to this study the positive impact of the soil-borne fungus on insect development was also shown in other studies. For example, the arboreal endophyte (*Rhytisma acerinum*) enhanced growth of aphids (*Drepanosiphum platanoidis*) feeding on the leaves of *Acer pseudoplatanus* (Gange, 1996) and an arbuscular mycorrhizal fungus *Glomus intraradices* infection positively influenced the weight and fecundity of the aphids *Myzus ascalonicus* and *M. persicae* feeding on *Plantago lanceolata* (Gange *et al*. 1999).

In general, preference of the gravid female should lead to a better development of their offspring. The aphid *Rhopalosiphum padi* preferred healthy host leaves of cool season grasses over endophyte (*Neotyphodium coenophialum*) infected treatments because the offspring suffered from high mortality (Bultman *et al*., 2006); Adults of the herbivorous beetle *Cassida rubiginosa* (Müller) preferred to consume and lay eggs on healthy creeping thistles over those infected by the fungus *Phoma destructiva* (Plowr.) and subsequently larvae developed better on these plants (Kruess, 2002). Females of the stem-boring weevil *Apion onopordi* (Kirby) preferred to deposit eggs on systematically rust-infected (*Puccinia punctiformis*) creeping thistles and adults emerging from infected shoots had a higher fecundity than from healthy shoots (Friedli & Bacher, 2001). However, insects preference and performance parameters are sometimes poorly correlated (Cronin & Abrahamson, 2001). Some confusing phenomena were also observed; for example greenhouse

whiteflies preferred to probe on tomato plants infected by *A. strictum*, however larval development suffered from a higher mortality (Vidal, 1996). The gravid females of *Helicoverpa armigera* (Hübner) preferred to lay more eggs on tomato plants inoculated with *A. strictum* (Jallow *et al*., 2008), however it was demonstrated that the offspring suffered from reduced growth rate, high mortality and low fecundity (Jallow *et al*., 2004). The preference-performance results using *Acremonium* spp. showed that the endophytes have a potential to protect plants by disrupting insect choice to infected host plant or by increasing insect mortality on infected plants.

It is generally accepted that microorganisms cause changes to their host plants including physical, nutritional and allelochemical parameters (Barbosa, 1991). Endophytic fungi are able to produce toxic alkaloids when growing inside grasses (Clay, 1988; Johnson *et al.*, 1985; Breen, 1994; Bush *et al.*, 1997), and they alter the nutritional suitability of their host plants to insects by influencing the phytosterol content and composition (Dugassa-Gobena *et al*., 1998, 2003). They are also known to alter carbonhydrate, protein, amino acid and sucrose content (Smith *et al*., 1985; Barbosa, 1991; Costa Pinto *et al*., 2000), the carbon/nitrogen balance (Hare & Dodds, 1987; Gange & West, 1994; Gange, 1996). The last, but not the least, they are able to induce changes in the defense compound such as the composition of flavonoids of white clover and the content of glucosinolates in Cruciferous plants (Doughty *et al*., 1991; Ponce *et al*. 2004; Treutter, 2006). All these changes may influence the performance of herbivorous insects feeding on these plants. However, Raps & Vidal, (1997) have shown that the carbon and nitrogen content was not different in endophyte (*Acremonium alternatum*) infected and control cabbage leaves. The only parameter influencing insect development was attributed to a change in the phytosterol content and composition (Dugassa-Gobena *et al*., 1998, 2003).

Phytosterols

Sterols play a dual role in insects: they are essential for insect integument development and they serve as precursors for ecdysteroid, the molting hormones (Zandee, 1962; Clayton, 1964; Svoboda & Thompson, 1985). Unlike vertebrates, however, insects can not biosynthesize their sterols *de novo*; the only source comes from the diet (Hobson, 1935a, b; Ritter, 1984) or from symbionts (in some aphids

and ants) (Douglas, 1988; Ba *et al*., 1995). Flowering plants (Class Angiospermae) contain a mixture of different sterols among which the main components are sitosterol, campesterol, and stigmasterol; sitosterol is usually the most dominant sterol (Nes, 1977; Behmer & Nes, 2003). In contrast brassicasterol is especially typical for plants in the *Brassicaceae* family (Amar, 2007) while ergosterol is the main sterol produced by most fungi (Granado *et al*., 1995). When different phytosterols were provided to insects purely or as an array of proportion in artificial diet, phytosterols were classified as suitable or unsuitable according to the extent to which they prompted the insect development (Nes *et al*., 1997, Behmer & Elias, 1999a, 2000). Generally, phytosterols are converted to cholesterol by dealkylation of a side chain (Svoboda *et al*. 1985; Ikekawa *et al*., 1993), however many insect species have metabolic constraints for some phytosterols with a special configuration, especially the double bond position (Dadd, 1960; Ritter, 1984; Behmer & Grebenok, 1998; Behmer & Elias, 1999b, 2000).

Cabbage root flies are specialist insect pests on Brassica plants. Compared with the detailed studies on the sterol metabolism in other insect taxa, such as grasshoppers (Orthoptera), beetles (Coleoptera) and caterpillars (Lepidoptera) (Clark & Bloch, 1959; Ritter, 1984; Svoboda & Lusby, 1994; Svoboda & Weirich, 1995; Nes *et al*, 1997; Behmer & Elias, 1999a, 1999b, 2000; Behmer *et al*. 1999), the sterol metabolism in the dipteran insects is virtually unknown. Several studies proved that some dipteran species lacked the capacity of dealkylating β-sitosterol to cholesterol, such as larvae of the housefly (*Musca domestica*), fruit fly (*Drosophila melanogaster*) and two specialists Drosophila species on cacti (*Drosophila mojavensis* and *D. nigrospiracula*) (Kaplanis *et al*, 1965; Kircher *et al*., 1984; Svoboda *et al*. 1989). To date, no study has shown the sterol requirement and metabolism of cabbage root flies, except one paper demonstrating the extreme sensitivity of young instars of the root fly to cholesterol (Dambre-Raes, 1976). It was proposed that cabbage root flies exhibited specificity in sterol requirements due to a fundamental difference in the sterol metabolism as compared to other non-specific sterol requiring insects (Chippendale, 1971). This specific requirement in sterol was also found in *Drosophila pachea* (Dipetera, Brachycera, Drosophilidae) whose larval stages feed in the stem of Senita cacti (*Lophocereus schottii*) (Heed & Kircher, 1965). The questions whether cabbage root fly can metabolize phytosterol by using the dealkylating pathway and

whether they have specific sterol requirements are needed to be disentangled in future work.

In this study, significantly more Brassicasterol, and Campesterol were found in E+ roots of *B. napus* var., however no obvious differences for Stigmasterol and Sitosterol quantities were found. Although some papers showed the effect of specific phytosterols, such as Sitosterol or Stigmasterol, there is increasing evidence that the phytosterol composition is crucial for normal growth and development of insects. Several studies have documented the negative impact of phytosterol composition alteration on insect development (Richter *et al*. 1987; Bodnaryk *et al*., 1997; Nes, *et al*., 1997). In our research, the relative content of Brassicasterol and Campesterol in E+ *B. napus* var. increased significantly and Sitosterol decreased significantly. Campesterol was found to be of higher nutritional value than β-Sitosterol to support larvae growth and development of the housefly, *Musca domestica,* (Kaplanis *et al*, 1965). Many studies documented the positive effect of Sitosterol for supporting the growth and reproduction of insect in artificial diet (Nes *et al*, 1997, Behmer & Elias 2000). In contrast, in a different paper dealing with *Acremonium alternatum* infected leaves of Brussels sprouts, the content of Brassicasterol positively and the content of Sitosterol negatively correlated with larval weight of the diamondback moth; thus regarding Brassicasterol as a utilizable and Sitosterol as a non-utilizable phytosterol (Dugassa-Gobena *et al*., 1998). In infected roots of *B. napus* var., the significant increase of suitable phytosterols (e.g. Campesterol and Brassicasterol) and the decrease of unsuitable phytosterols (e.g. Sitosterol) in the relative composition could be the reason why higher fecundity and longevity were found in females reared on these infected host plants comparing with control host plants. However more studies are needed to understand the function of the main phytosterols and corresponding metabolism processes, which are far from being fully analyzed for the cabbage root fly.

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Chapter 2

Preference-performance of the cabbage root fly (*Delia radicum* (L.)) feeding on winter oilseed rape (*Brassica napus* (L.)) inoculated with a soil-borne fungus (*Verticillium longisporum*)

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Abstract

To date plant-associated microorganisms can mediate plant-herbivore interaction was well accepted. This study focused on the indirect effect of a systemic infection of oilseed rape (*Brassica napus*) with soil-borne fungus *Verticillium longisporum* on the preference-performance of root-bored insect, cabbage root fly (*Delia radicum* L.) via larva host-choice, larva and pupa performance and adult oviposition bioassay. The profiles of primary metabolites, phytosterols in *V. longisporum* inoculated (V+) and uninoculated (V-) plant root were quantified to elucidate the underlying mechanisms. This study integrated *B. napus*, the important economic crops, *V. longisporum*, the wide-spread specialized soil-borne pathogen and extremely destructive, root-borrowing cabbage root fly into one research system. The comprehensive experiment design will provide a broader view than most other studies to the mediating of plant-associated microorganisms on plant-herbivore interaction. A reverse orientation behavior to V+/V- root segments and intact root showed cabbage root fly larvae could not distinguish a healthy plant from pathogenic plant. *V. longisporum* infection in plants increased the number of chlorotic leaves, decreased fresh shoot and root weight individually. With the same eggs loading on the surface of plant root, larva suffered from a high mortality fed on V+ root. Pupa weight was not reduced obviously on V+ plant. However pupa underwent a significant high mortality of eclosion. Infection shortened the duration from pupa to adult females and increased the oviposition deposited within 10 days in comparison with that on V- plant.

Some significant alternations in quantity and composition of phytosterols were detected in root of V+/V- *B. napus*. The inoculation with *V. longisporum* significantly reduced campesterol meanwhile increased stigmasterol in quantity. The relative composition of sitosterol, stigmasterol and campesterol were significantly altered in root of V+/V- *B. napus*. Some significant correlations were established:

- Positive correlation between pupa weight and eclosion duration
- Positive correlation between fresh shoot weight and fresh main root weight in V+/V- plants.
- Negative correlation between fungus DNA quantity with fresh shoot weight on V+ plants
- Positive correlation between brassicasterol with pupa weight on V+ plant

- Positive correlation between fungus DNA quantity and sitosterol content.

However no correlation between fungus DNA quality and pupa weight and oviposition respetively was found. Cabbage root fly, the root-feeding insect, in spatial dimension, close to the soil-borne fungi, *V. longisporum* by sharing the same site on host plant, the effect of fungus on insect performance via host plant was not much more impressive than spatially separated insects and fungus.

Key words: *Delia radicum* L.; oilseed rape; *Verticillium longisporum*; preference; performance; phytosterols;

2.1 Introduction

Almost all plants on the earth are potentially colonized by microorganisms, acting either in a mutualistic, neutral, or antagonistic way with their hosting plants. Because these plants may also serve as food for herbivorous insects, indirect interactions between microorganisms and insects via the shared plant could be expected. However, only in the recent 20 years, these interaction between insect-plant-microorganisms have been taken into account in experimental systems (Hodgson, 1981; Butin, 1992; Breen, 1994; Gaylord *et al*., 1996; Clement *et al*., 1997; Nakai & Kunimi, 1997; Moran, 1998; Wilson & Faeth, 2001; Sabzalian *et al*., 2004; Meister *et al*., 2006).

Soil-borne fungi are very common under natural conditions. Unlike vascular-arbuscular mycorrhizal (VAM) fungi, which normally act as mutualists with their host plants by enhancing nutrient uptake and resistance to drought (Allen *et al*., 1986; Bolan, 1991), most soil-borne pathogens normally induce a series of disease-related morphological, physiological and metabolic changes in their host plants resulting in changes in plant size, color, senescence, nutrient quality, and allelochemicals profiles, repsectively (Barbosa, 1991). These changes may influence insect preference during host-finding and altered performance when feeding on these plants and result in beneficial, detrimental or neutral effects on the herbivore, depending on the pathogen, plant and insect species involved in these interaction (Barbosa 1991; Gange, 2001).

Only few insects have been tested with regard to their development when feeding on host plants infected by microorganisms, and the results reported are inconsistent. Several papers report detrimental effects on herbivorous insects when plants are infected by plant pathogens. For instance, greenhouse whiteflies (*Trialeurodes vaporariorum* (Westw.)) and diamondback moths (*Plutella xylostella* L.) suffered from a reduced relative growth rate (RGR) and higher mortality feeding on soil-borne endophyte infected cabbage plants (Vidal, 1996; Raps 1998), the leaf beetle *Cassida rubiginosa* Müller suffered from retarded development and higher mortality feeding on creeping thistle infected by pathogenic fungus *Phoma destructiva* (Plowr.) (Kruess, 2002). On the other hand, alfalfa, infected with the pathogen *Verticillium albo-atrum* had negligible effects on the growth of the southern armyworm

(*Spodoptera eridania* (Cram.)) (Kingsley *et al*., 1983). Beneficial effects have been reported for the arbuscular mycorrhizal fungus *Glomus intraradices* on aphid (*Myzus ascalonicus* and *M. persicae*) weight and fecundity (Gange *et al*. 1999).

In the studies reported previously, most insect species used were chewing or sucking insects feeding on aerial plant parts, such as leaves. There are only few papers for root-feeding larvae. Gange *et al*. (1994) and Gange (2001) showed a negative impact of arbuscular mycorrhizae (*Glomus mosseae* and *Glomus fasciculatum*) on the black vine weevil (*Otiorhynchus sulcatus* (Fab.)) feeding on infected plants such as reducing larva growth and increasing larvae mortality.

The cabbage root fly *Delia radicum* (L.) (Diptera: Anthomyiidae) is a widespread and extremely destructive specialist insect on cruciferous crops. By depositing eggs around host plant stems, hatched maggots damage or destroy the root systems of many crucifers, including oilseed rape (Dosdall *et al*., 2000; Jensen *et al*., 2002). Feeding damage results in water stress and provides routes for fungal infections, such the *Verticillium* wilt pathogen and *Fusarium* rot fungi (Ridsdill Smith, 1977). The damage to the roots results in seriously reduction both in yield and quality and causes considerable economic losses for oilseed rape (Griffiths, 1991)

Verticillium wilt is an increasing problem on Brassica oilcrops in northern European countries (Svensson & Lerenius, 1987; Zielinski & Sadowski, 1995). On oilseed rape (*Brassica napus* L. spp. *oleifera*) (**OSR**) this disease is triggered by *Verticillium longisporum*, a host-specific vascular wilt pathogen (Karapapa *et al*., 1997; Zeise & von Tiedemann, 2001). The typical symptoms caused by *V. longisporum* on host plants include necrosis and chlorosis of leaves and plant stunting, ripening and premature senescence (Bhat & Subbarao, 1999; Steventon, 2002; Babadoost *et al*., 2004; Zhou *et al*., 2006). Verticillium fungi are soil-borne pathogens and mainly spread via infested soil from field to field (Xiao & Subbarao, 1998). Presently the relative intensive cultivation and continuous crop rotation of OSR has rendered this disease spreading quickly and resulted in dramatic economic losses (Svenson & Lerenius, 1987).

Previous studies found that infection by microorganisms altered phytosterol content and composition (Dugassa-Gobena *et al*., 1998, 2003). Sterols are necessary compounds for the insect membranes and are a precursor of molting hormones; their amount and composition in plants directly effects insect development (Zandee, 1962; Clayton, 1964; Nes *et al*. 1997). Insects cannot synthesize sterols, thus the need to

take it up from their host plants or they may harbour symbionts producing these sterols (Ritter, 1984; Douglas, 1988; Ikekawa, 1993; Nes *et al*., 1997; Mondy & Corio-Costet, 2000). In this study the content and composition of four typical phytosterols namely Sitosterol, Campesterol, Stigmasterol and Brassicasterol were calculated for understand insect performance on inoculated plants.

Using *Verticillium longisporum* and the cabbage root fly (*Delia radicum* L. - **CRF**) on oilseed rape *Brassica napus* we asked the following questions:

- Do CRF larvae and adults discriminate between infected and control plants?
- Do soil-borne pathogens influence the development of larvae and subsequent adult fecundity?
- Do changes in the performance of CRF larvae relate to changes in the phytosterol profile?

To my knowledge, this is the first report on the influence of a soil-borne vascular wilt pathogen on oilseed rape on herbivorous insect performance, especially on root-feeding insects.

2.2 Material and method

Host plants

We used the winter oilseed rape variety 'Falcon', (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG) in this study. In the dual-choice experiments we used three weeks old plants which had been inoculated the previous week to avoid the symptoms of verticillum infection became visible.

Brassica napus seeds were sterilized using 70% alcohol for 2 min, then rinsed 3 times using autoclaved tap water; seeds were grown in quartz sand for 7 days. For the preference experiments we used seedlings, which were directly transferred to plastic pots (11cm diameter) containing a mixture of sand and soil (Ökohum®-Rindentorfsubstrate, Type RTS; Ratio: 1:2). Plants were grown in a greenhouse at 24±3°C, 80% relative humidity (RH) and a LD 16 : 8 h photoperiod for three weeks. Plants were watered daily and fertilized at weekly intervals with Wuxal® Super (10-10-8 w/v). Seedlings, used in the performance experiments, were inoculated using the root dipping method following Bhat and Subbarao (1999), however without

trimming the roots. In short, seedlings were thoroughly pulled out and the roots were washed using tap water; thereafter the roots were dipped into a spore suspension (10^6 conidia ml^{-1}) for 40 min; whereas the roots of seedlings serving as the control were dipped in tap water for 40 min. Thereafter seedlings were replanted into the substrates used before and grown in the greenhouse until used. Plants were arranged in a randomized block design on greenhouse benches during this period.

Fungus

Verticillium longisporum, isolate VL 43, originated from a field with *B. napus* located in the north of Germany. Isolation method and fungus cultivation has been described by Zeise and von Tiedemann (2001, 2002a, b). To maintain the pathogenicity, a spore suspension (10^6-10^7 conidia mL^{-1}) was divided into 1.5 ml Eppendorf cups with Czapek Dox medium supplemented with 25% glycerol at -20°C. For propagation, droplets of these suspensions were plated onto potato dextrose agar (PDA) and incubated for three weeks at 21°C in darkness. Spore suspensions were obtained by gently adding some distilled water to the plates, harvested using a hair-brush. Inoculum for root dipping inoculations was produced by adding 500 µL of the spore stock suspension to 250 mL potato dextrose broth (PDB), grown for three weeks in darkness at 23°C on a rotary shaker at 100rpm. The concentration of the conidia suspension was adjusted using a Thoma counting chamber (Marienfeld, Germany) (depth: 0.1mm, square width: 0.05mm) under a microscope.

Insects

The CRF stock was originally provided by the BBA (Biologische Bundesanstalt für Land und Forstwirtschaft) in November 2003 and was reared in a climate controlled culture room (temperature: 21°C±1°C, 70±5% r.h. and photoperiod: 16h light: 8 h dark). The rearing method for the CRF followed the method described by Finch and Coaker (1969) with some modifications regarding food supply. During rearing, marketable turnip cabbage was used for larvae rearing. Adult flies were kept in cubic screen cages (28cm × 28cm × 28 cm) and fed on artificial foods. A cube of turnip cabbage was placed on a layer of quartz sand in Petri-dishes to attract females to

oviposit. Eggs were collected by floating and filtrated on filtrate paper in a funnel. The eggs were transferred onto a cut surface of fresh turnip cabbage and buried in sand for 3 weeks before pupae were extracted by floating. Adults were provided with powder food containing dextrose, skim milk powder, soybean flour and brewer's yeast, respectively (10g:10g:1g:1g) and sticky food comprised of honey, soybean and brewer's yeast (5g:5g:1g). Tap water was permanently offered in a separate Petri-dish.

Dual-choice of CRF larvae using root segments

Three weeks old *B. napus* plants were inoculated with 70ml of a spore suspension (10^6 conidia /ml) by watering (subsequently named **V+**). Control plants were watered with the same volume of culture filtrate (subsequently named **V-**). Plants were used in the experiments one week later.

The experiment was set-up using Petri-dishes (Ø: 14 cm). The arena was divided into a V+ area, a V- area and a searching area, respectively. The distance between the V+ and the V- area was 6 cm along the center line. Before starting the experiment, plant roots were carefully washed in tap water. Thereafter, segments of V+ and V- roots, bearing a comparable amount of root hairs, were placed in the Petri-dishes. Larvae used in this experiment were experienced to Petri-dishes with a piece of turnip cabbage as a stimulant for 2-3 hours previous to transferring them to the experimental arena. One 12 days old larva (II-III instar) was placed on top of the central line as the starting point, faced to the root hairs (Figure 1). Vermiculit was added to simulate the soil medium. The experiment was conducted in complete darkness for 120 min. at room temperature. Larvae entering either the V+ or the V- area were recorded as a positive choice, otherwise we regarded their behavioural response as no choice. The experiment was replicated 30 times.

Dual-choice of CRF larvae using intact root systems

Seven days old seedlings were transplanted to pots with a lateral hole of 2.0 cm in diameter. These opening holes were sealed by a plug during growth in the greenhouse. Immediately before starting the experiment, a two-arm transparent tube

(5 cm in length and 2.0 cm in diameter) was attached to on pot per side, either containing inoculated or control plants. The arms were approximately 2.5 cm inserted into the soil to closely reach the plant roots (Figure 2). Larvae used in this experiment had previous experiences to Petri-dishes with a little piece of turnip cabbage as a stimulant for 2-3 hours at room temperature in darkness. One CRF larva, reared on pieces of turnip cabbage for 13 days was introduced to the tube via a central entrance hole using fine tweezers, thereafter the whole was sealed. Larvae were allowed to make a choice within 15 min in darkness in a climatic chamber (25°C, 70%RH). If larvae moved more than 3 cm in one tube, we regarded this as a positive choice, otherwise we recorded a no-choice. The experiment was replicated 27 times.

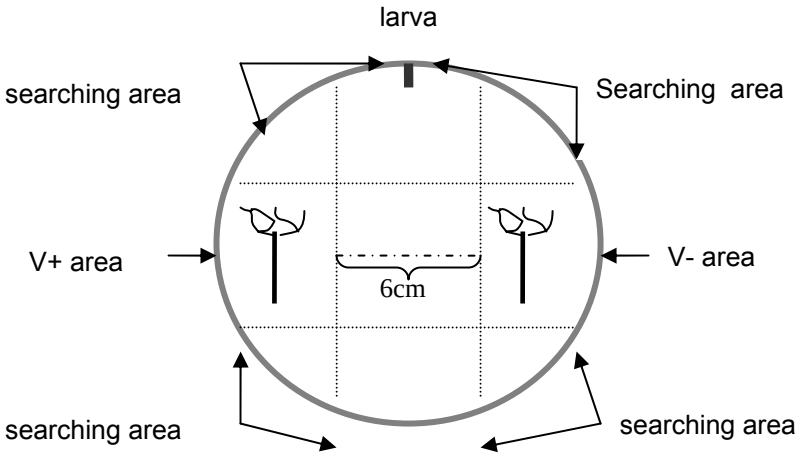

Figure1 Dual-choice experimental set up for cabbage root fly larvae (*Delia radicum* L.) to test for discrimination between root segments either infected by *Verticillium longisporum* or controls of *Brassica napus*

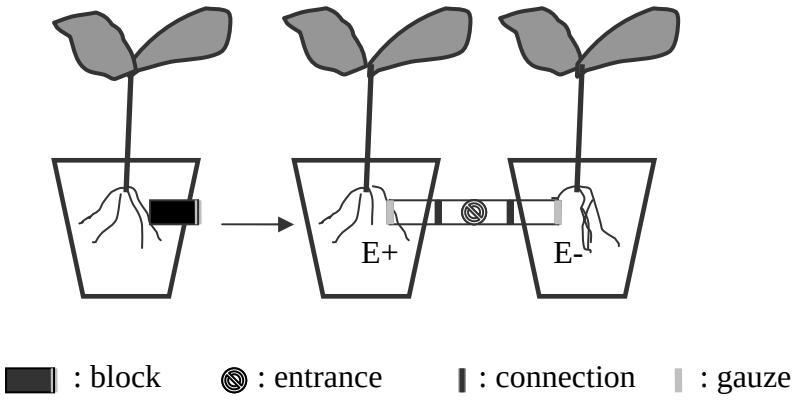

Figure 2 Dual-choice of cabbage root fly (*Delia radicum* L.) larva to intact roots of *Verticillium longisporum* infected and control plants of *Brassica napus*

Performance of root fly larvae on V+/V- *Brassica napus*

We used three weeks old *B. napus* plants inoculated using the root dipping or the control. Five CRF eggs were placed on the surface of the main root of the host plant using a fine paint-brush in the greenhouse. After 24 days, pupa were extracted from the soil by visual inspection and weighted individually. At this time plants were categorized using the parameters number of leaves, number of chlorotic leaves and the corresponding score of disease seriousness (from 0 to 5; Table 1), height and fresh weight of above-ground and below-ground biomass. The hypocotyls of the plants were cut and frozen in liquid nitrogen for subsequent DNA extraction and real time-PCR detection. The experiment was replicated 29 times.

Pupae found in the soil of the same plant were transferred to Petri-dishes with a moistened filter paper on the bottom and were placed in a climatic chamber (21 °C; 60 r.h., 16h light : 8h darkness). Tap water was added to the Petri-dishes each week to adjust humidity. After adult eclosion, one male and female were caged in one cylinder (15 cm in diameter and 30 cm in height) with artificial food, water and a Petri-dish with sand and a piece of turnip cabbage allowing oviposition. The turnip cabbage was replaced every 3 days. The experiment lasted 10 days. Eggs were collected as described previously.

Table 1. Assessment key for scoring disease symptoms in plants of *Brassica napus* inoculated with *Verticillium longisporum*

score	percentage of leaves with yellow symptoms (%)
0	0
I	0-20
II	20-40
III	40-60
IV	60-90
V	dry and dead

Real-time PCR (Polymerase Chain Reaction) Experiment

DNA extraction

Plants used for the insect performance experiments were harvested and the roots were washed carefully and immediately transferred to liquid nitrogen and stored at -

20°C. To extract DNA, the whole root (main root and root hair) was pulverized to fine powder under liquid nitrogen using a pestle and mortar. 100mg root powder was weighted and dispersed in 1ml CTAB buffer (20 mM EDTA, 10 mM Tris, 0.13 M sorbitol, 0.03 M N-laurylsarcosine, 0.02 M hexadecyltrimethylammonium bromide, 0.8 M sodium chloride, 1% (w/v) polyvinylpolypyrolidone, pH set to 8.0 with HCl) containing 2µl mercaptoethanol and 1µl proteinase K. This mixture was homogenized using a Vortex for 30 seconds followed by incubation in a water bath for 20 min at 40°C and 20 min at 60°C. After the mixture has been cooled to room temperature it was extracted with 750 ul chloroform-isoamyl alcohol (24:1). Phases were separated by centrifuging at 8.000 rpm for 20 min. The supernatant was transferred to a 1.5-ml Eppendorf cup and 500µl isopropanol was added, by slight shaking for 15 sec. After incubation at room temperature for 15min, DNA was deposited by centrifuging at 11.000 rpm for 20 min. The pellet was washed with 500 µl 70% (v/v) ethanol and dried in SpeedVac at 30°C for 10 min. Then dissolved in 30µl TE buffer (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl) and incubated over night at 4°C to ensure that the DNA was dissolved completely. The quality and quantity of DNA were assessed by electrophoresis in 0.8% (w/v) agarose gels (Biozym LE Agarose, Biozym Scientific GmbH, Oldendorf, Germany) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid) with 25ng Lambda Phage DNA as a standard. The electrophoresis was run at 65 Volt for 80 min. Double-stranded DNA was stained with ethidium bromide (ethidium bromide, 2 mg L^{-1}) for 15 min and washed with H_2O for 10 min. The gels were documented with the help of a digital imaging system (Vilber Lourmat, Marne-

Real-time PCR Amplification

Samples stored in fridge at -20°C were melted in water-bath at 42°C for 2 hours. The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *Verticillium longisporum* DNA. Forward Primers OLG70 (CAGCGAAACGCGATATGTAG) and backward primer OLG71 (GGCTTGTAGGGGGTTTAGA) (P. Karlovsky, unpublished) were used to multiply a fragment specific for *Verticillium longisporum*. The reaction mixture consisted of NH4-reaction buffer (16mM$(NH_4)_2$ SO_4, 67mM Tris-HCL, 0.01% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Luckenwalde, Germany), 3 mM $MgCl_2$(Bioline, Luckenwalde),

0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of every primer, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM fluorescein (BioRad, Hercules, CA, USA). 100,000 dilution folders 0.1x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 2 µl of template DNA and doubly distilled water (ddH_2O) filled to a total volume of 25 µl. The detection of amplification products, based on the fluorescence of SYBR Green I, was implemented with filters set at 490±10 nm for excitation and 530±15 nm for emission. The PCR amplification was run following cycling process. Initial denaturation for 2 min at 94°C, followed by 36 cycles of denaturation step at 94°C for 20 sec., then an annealing steps at 59°C for 30, and at 72°C for 40 s. The final elongation last for 5 min at 72°C. With PCR going-on, the detection of fluorescence was performed in the annealing step of each cycle. Following amplification, the melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for 1 min and then slowly increasing the temperature from 65°C to 95°C at the rate of 0.5°C 10 s^{-1} with a continuous measurement of the fluorescence.

The amount of DNA of *V. longisporum* in plant samples (in µg g^{-1}) was assessed from a PCR standard curve in iCycler software which showed genomic *V. longisporum* DNA from 0.5 to 64.0 pg. The final DNA amount in one gram plant material was calculated following the equation:

$$M = 150\, fd$$

M : final DNA amount in one gram plant material

f : DNA diluted folder for Rt-pcr

d : DNA amount in Rt-pcr

Phytosterol Analysis

Roots were cut from plants used in the larval performance experiment and pulverized into fine powder in liquid nitrogen using a mortar and a pestle. 0.5-0.6 g plant material was transferred to a 40 ml glass bottle with a screw-cup. Phytosterols were extracted following the protocol described by Newton (1989) with modification as follows: 20ml mixed solvent (5 ml 10 M KOH, 15 ml 96% ethanol and 0.06 g

pyrogallol) was added to each bottle containing plant material. An ultrasonic homogenizer (Sonoplus HG 2200/UW 2200, 200 W, 20 kHz, Bandelin GmbH, Germany) was used for 30 sec for completely cracking the cells and degassing. Thereafter the bottles were placed in a shaker water bath at 80°C for 2.5 h. After cooling the samples to room temperature, 40 µl Cholesterol was added as an internal standard (Cholesterol 5 ug/ul chloroform, Merck GmbH, Germany). Phytosterols were extracted with 10 ml hexane for 3 times. After thorough shaking for 20 sec, deposited for 5-10 min to separate phases, the supernatant was transferred to a rotary glass. One ml distilled water was added to wash the fractions, the separated lower phase was sucked out. Samples were distilled with a rotary-evaporator at 42°C. The concentrated sterols were resolved in 1.5ml hexane and transferred to 2 ml Eppendorf cups. After centrifuging with 10,000 rpm for 10 min, the supernatant was transferred to a GC-vial and the hexane was evaporated overnight at 50°C in a thermal block. The concentrated sterols were resolved in 240 µl hexane and 80 µl N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)(Fluka/Riedel-deHaen GmbH, Germany) and incubated in a thermal block at 70°C for 20 min. After cooling down to room temperature, 100-150 ul of the extract was transferred to a GC-vial equipped with 300 ul inserts.

Phytosterol identification and quantification was based on the internal standard method (peak area and retention time). 1 ul of each sample with a split of 1:50 of extracted sterols were analyzed using a gas chromatograph (Shimadzu GmbH, Model: GC14/15A) with a flame ionization detector. The samples were run on a fused silica column (SPB-1; 1.3m x 0.32mm, 0.25µm film thickness, Supelco Inc.; Sigma-Aldrich, Germany). Helium was used as a carrier gas, the make-up gas was synthetic air with a linear velocity of 35cm/s. The temperature program was initially 3 min at 180°C then increase to 290°C at a heating rate of 10°C/min to keep 36 min. The total analyzing time was 50min. The detector temperature was 300°C. Peak areas were calculated using an integrator and the internal standard. A set of typical peaks, indicating phytosterols, such as Brassicasterol, Campesterol, Stigmasterol and Sitosterol individually, had been identified beforehand using GC-MS with synthetic sterols as a reference. The phytosterol content in one gram plant material was calculated following the equation:

$$G = \frac{200 \times p_2}{p_1 \times g}$$

G : weight of unkown phytosterol in one gram plant material p_1 : peak area of cholesterol, p_2 : peak area of unkown phytosterol, g : weight of plant material

Total phytosterol in one sample was calculated by summing up all the four phytosterols.

Statistics

The result of CRF larval and adult oviposition in the dual-choice experiments were analyzed using a Binomial test. In the CRF performance experiment, the height of plant, the fresh weight of above-ground and below-ground parts and the weight of CRF pupa were analyzed by ANOVA. The amount of leaves, yellow leaves and the mean score of yellow leaves and mean eggs were analysed by a Man-Whitney test, because the data were not normally distributed. Number of pupa and adults and the mortality were analyzed using a Chi-square test.

The content and composition of phytosterols were analyzed by ANOVA. For correlation analyses, SigmaPlot 10.0 was used, the independent variable (x).and dependent variable (y) were given individually in corresponding results All other analyses were performed by using SPSS 10.0.

2.3 Result

2.3.1 Real time-PCR and dual-choice of cabbage root fly larvae to cut root / intact root systems

Real-time PCR was used to qualitatively and quantitatively assess the infection of *Verticillium longisporum*. In plant samples used for root fly larval performance, 93% of the plants were successfully inoculated. The average *verticillium* DNA amount was 7.99±5.14 ng/g fresh root tissue of *Brassica napus* at 6 weeks dpi. The yield and quality of DNA was shown (Figure 3).

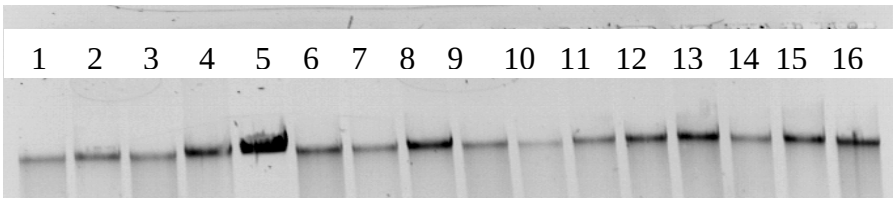

Figure 3: Yield and quality of DNA extracted from fresh root of *Brassica napus* either inoculated with *Verticillium longisporum* or control (lane 1-11: V+; lane 12: 25ng λ-DNA; lane13-16: V-)

From the 30 larvae tested, ten larvae choose V+ root segments whereas eighteen larvae choose V- root segments and two larvae did not make a choice. The preference was not significant at the statistical level of 5% (Binomial test: p=0.186). However a reverse result was obtained with regard to intact roots: seventeen larvae chose V+ plant and ten larvae chose V- plant, although this result was not significant at the statistical level of 5% (Binomial test p=0.248).

2.3.2 Morphology of V+/V- *B. napus* plants

Because of the short time since inoculation, some typical *V. longisporum* infection symptoms (Steventon *et al*., 2001, 2002) have not been obvious in infected *B. napus* plants, such as internal root discoloration or browning of the roots, which was also been reported for horseradish (*Armoracia rusticana*) roots (Babadoost *et al*. 2004). However, necrosis and chlorosis of leaves and plant stunting were visible after inoculation for 2-3 weeks (pers. observation).

In this study morphological characteristics, such as number of leaves, yellow leaves and the corresponding disease score, plant height and fresh weight were regarded as the main indices to assess the seriousness of infections. The total number of leaves on uninfected plants was significantly higher as compared to infected plants. The height and fresh weight of above-ground and the length of the main roots of non-infected plants were different from those of infected plants; only a minor difference was found with regard to the fresh weight of the main roots (Table 2). A significant correlation was found between fresh shoot weight and fresh main root weight (V+: $y=0.502+0.033x$ $r^2=0.947$, $p=0.0002$; V-: $y=0.136+0.031x$, $r^2=0.947$, $p=0.0111$ (Figure 4).

Table 2 Plant morphological parameters of *Verticillium longisporum* infected and control *Brassica napus* plants at six weeks past inoculation using the root-dipping method and cabbage root fly larvae feeding for 24 days (mean±STD; symptom score: 0-5 (from healthy to severe infection according to yellow area percentage); Fresh weight of main root is the debris of main root after root fly larvae feeding)

morphological indices	V+	V-	*p* value
total leaf amount	6.04±0.92	8.04±1.48	0.000***
yellow leaf amount	1.14±0.65	0.33±0.05	0.000***
symptom score	2.75±1.62	0.85±1.35	0.000***
height of above ground (cm)	28.86±4.88	37.20±3.21	0.000***
fresh weight of shoot (g)	21.59±7.80	36.96±5.26	0.000***
length of main root (cm)	3.34±1.05	2.78±0.81	0.031*
fresh weight of main root (g)	1.21±0.39	1.28±0.35	n.s

ANOVA test was used for analyzing height and weight of above-/below-ground plant part; total leaf amount, yellow leaf amount and socre was analysed with Mann-Whitney test (* p≤0.05; ***p≤0.0001; n.s: no significant difference).

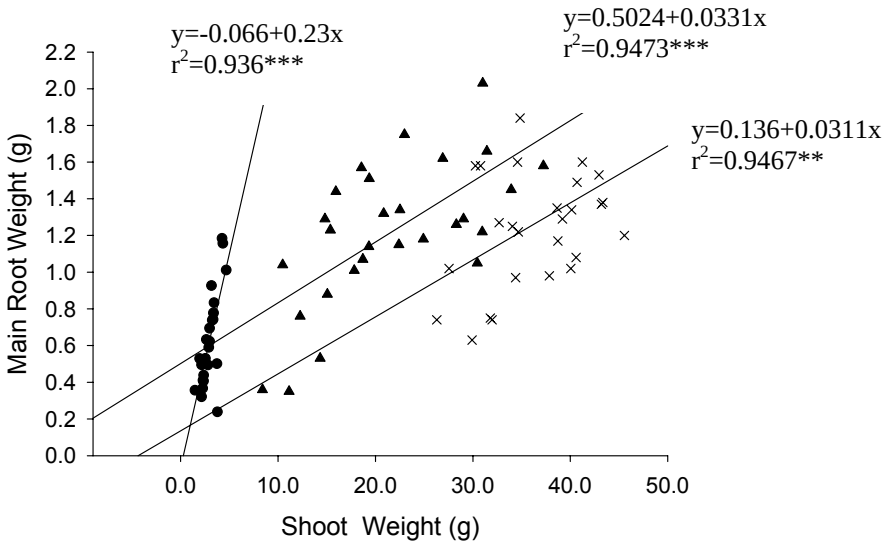

Figure 4 correlation between fresh shoot weight and fresh root weight on Verticillium infected and uninfected *B. napus* with cabbage root fly and without cabbage root fly.

● Solid black dots: plants without cabbage root fly feeding.

▲ Soild black triangle: treatment with fungus and root fly larvae.

X Thin X: treatment without fungus, but with root fly larvae.

2.3.3 Performance of cabbage root flies on V+/V- *Brassica napus*

Thirty five replications were established for the V+ treatment, however because of infections, 6 plants died during growth. Thus, twenty-nine replications for each treatment were used in the larval performance experiment and subsequent analyses.

Table 3 Performance of cabbage root fly (*Delia radicum* L.) developing on *Verticillium longisporum* infected or control *Brassica napus* plants. From egg to premature pupal stage, cabbage root fly spent in plant pots; from pupae to adult eclosion in a climatic chamber (mean ± STD)

Experiment	V+	V-	p-value
Exp. 1: Egg-Larva-Pupa Development			
Eggs	145	145	/
Number of pupae	63	76	n.s
Mortality rate during egg-pupal stage (%)	66.6%	47.6%	0.076
Pupal weight (mg)	14.26±2.22	14.37±2.20	n.s
Exp. 2: Adult Development and Oviposition			
Adult eclosed within 15 days	46	56	n.s
Eclosioin rate	73.0%	73.6%	n.s
Females	25	21	n.s
Males	31	25	n.s
Pupal weight of eclosed females (mg)	15.31±1.77	15.24±2.3	n.s
Pupal weight of eclosed males (mg)	13.75±1.73	13.61±1.41	n.s
Eclosion duration of females (day)	10.83±3.10	11.93±1.91	0.014*
Eclosion duration of males (day)	8.9+1.95	9.64±2.61	n.s
Oviposition within 10 days	70.47±31.67	53.41±41.72	0.088
Exp. 3: Adult Eclosion			
Sum of eclosed adults	48	67	0.076
Mortality during pupal-adult stage (%)	23.8%	11.8%	0.046*
Mortality from egg to adult	66.9%	53.8%	n.s
Sex ratio (♀:♂)	1:1.23	1:0.92	n.s

(* p≤0.05; ***p≤0.0001; n.s: no significant difference)

On V- plants, more pupae were found with a higher weight. The mortality from egg to pupae was higher, though not significantly, on Verticillium infected *B. napus* (p=0.076). However, the eclosion rates (V+: 73%, V-: 73.6%) within 15 days were at the same level. For females developing on V- plants, the eclosion duration was significantly longer than those on V+ plants. The subsequent oviposition was not significantly different. Adults, eclosing from pupae on V+ plants laid more eggs as

compared to those from V- plants within 10 days. Pupal weight of males was less than that of females (♀: 15.33±2.03, ♂: 13.67±1.55, ANOVA $F_{(1, 96)}$=20.37, p=0.000) and had a shorter eclosion time (♀: 11.25±2.20, ♂: 9.30±2.33, ANOVA $F_{(1, 96)}$=18.05, p=0.000). The mortality from pupa to adult and from egg to adult was higher on V+ plants. However fungal infection did not change the sex ratio significantly. Three deformed adults on V- and five on V+ were collected for each treatment; 2 deformed females and 1 male on V- plants and 3 deformed females and 2 males on V+ plant (Table 3).

2.3.4 Phytosterols

Four major phytosterols were analyzed and summed up to calculate the total phytosterols content: Sitosterol, Campesterol, Stigmasterol and Brassicasterol. In both treatments, Sitosterol was found to be the most prominent phytosterol followed by Campesterol and Stigmasterol. Brassicasterol was found the least. As for Sitosterol and Brassicasterol, Verticillium infection did not result in a significant change in the amount of the sterols analysed. However the Verticillium-infection reduced the content of campesterol and increased stigmasterol as compared to the controls. Furthermore, the fungal infection reduced the overall amount of phytosterols.

In terms of the relative sterol content, roots of Verticillium infected host plants contained 75.8% Sitosterol, 18.7% Campesterol, 4.8% Stigmasterol and 0.7% Brassicasterol; in control plants, the composition was 74.8% Sitosterol, 21.9% Campesterol, 2.6% Stigmasterol and 0.7% Brassicasterol.

The proportions of Campesterol, Stigmasterol and Sitosterol in two treatments were significantly different (Table 4; individual phytosterol content: Campesterol, $F_{(1,18)}$=12.971, p=0.002; Stigmasterol, $F_{(1,18)}$=10.852, p=0.004). No significant difference was found for Brassicasterol, Sitosterol and sum of all phytosterols. For the relative content: Campesterol, $F_{(1, 18)}$=22.936, p=0.000; Stigmasterol, $F_{(1,18)}$=12.591, p=0.002; Sitosterol, $F_{(1.18)}$=4.573, p=0.046. No significant difference was found for the relative content of Brassicasterol.

Table 4 Phytosterols content (ug/g root) and relative composition in *Verticillium longisporum* infected and uninfected roots of *Brassica napus* plants

phytosterol	V+	V-	P value
mean of brassicasterol	2.15±0.12	2.19±0.23	n.s
mean of campesterol	73.25±6.33	61.49±8.16	**
mean of stigmasterol	8.87±2.81	15.72±5.95	**
mean of sitosterol	250.63±26.49	249.01±21.04	n.s
Sum of phytosterol	334.90±33.01	328.41±27.35	n.s
relative content of brassicasterol	0.007±5.92E-04	0.007±1.05E-03	n.s
relative content of campesterol	0.219±0.01	0.187±0.019	***
relative content of stigmasterol	0.026±7.75E-03	0.048±0.018	**
relative content of sitosterol	0.748±0.013	0.758±8.03E-03	*

(* p≤0.05; ***p≤0.0001; n.s: no significant difference)

2.3.5 Correlationship of Parameters

For root fly larvae feeding on uninfected host plants, the eclosion duration positively correlated with pupal weight (Figure 5). However, for those larvae fed on infected host plants, the influence of pupal weight on eclosion duration of adults was only significantly for males. The amount of fungal DNA in host plant roots did negatively affect the growth of aerial parts. Highly infected plants (more fungal DNA in the plant), were more stunted. The amount of fungus DNA did not correlate with the total amount of pupae and larvae recoveredd. A positive correlation between pupal weight and eclosion duration was found on V- treatments; pupae heavier in weight needed longer to become adults. However, the fungal infection made this correlation reverse in males but not in females. In the two treatments, no correlation was found between the sum of phytosterols and number of pupae. pupal weight and female fecundity. Nevertheless, in infected plants a positive correlation was found between Brassicasterol content and pupal weight (Table 5).

Table 5 Correlationship between cabbage root fly development attributes, fecundity and phytosterol content in the fresh root of *Verticillium longisporum* infected/uninfected *Brassica napus*

Correlation (x, y)	treatment	regression	r^2	p value
Pupal weight *vs*. eclosion duration	V+	y=5.49+0.29x	0.059	n.s
	V-	y=5.77+0.35x	0.086	0.032*
♂ pupal weight *vs*. eclosion duration	V+	y=0.22+0.63x	0.32	0.008**
	V-	y=3.97+0.42x	0.052	n.s
♀ pupal weight *vs*. eclosion duration	V+	y=15.8-0.35x	0.066	n.s
	V-	y=10.75+0.078x	0.0088	n.s
Sum of phytosterol *vs*. pupa amount	V+	y=7.94-0.018x	0.2705	n.s
	V-	y=3.62-0.00042x	0.009	n.s
Sum of phytosterols *vs*. pupa weight	V+	y=19.13-0.014x	0.18	n.s
	V-	y=13.99-3.49E-005x	1.58E-006	n.s
Bassicasterol *vs*. pupa weight	V+	y=9.14+2.49x	0.42	0.043*
	V-	y=21.70-3.58x	0.22	n.s
Sum of phytosterols *vs*. fecundity	V+	y=118.92-0.19x	0.0073	n.s
	V-	y=234.35-0.49x	0.21	n.s

(* p≤0.05; ***p≤0.0001; n.s: no significant difference)

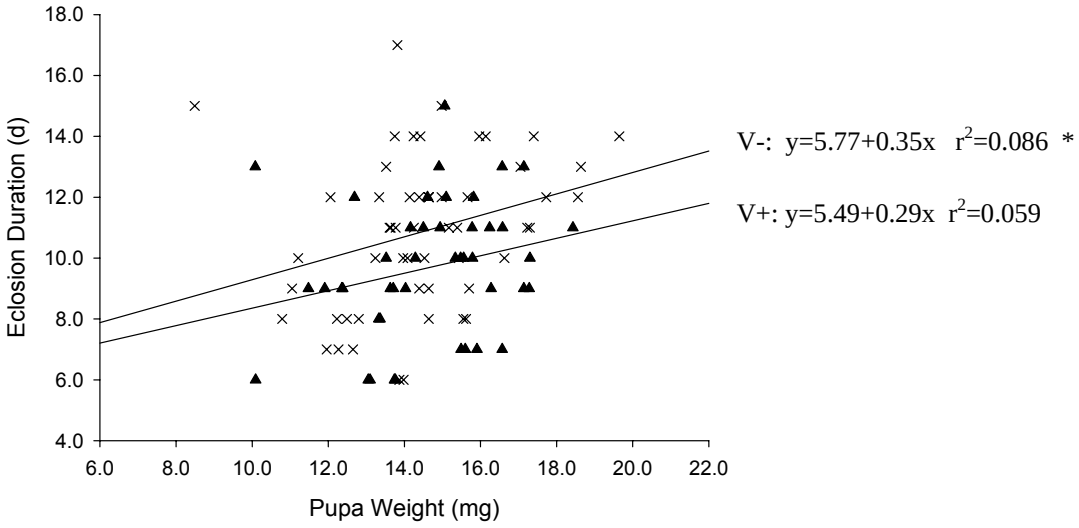

Figure 5 Correlationship between pupal weight and eclosion duration of cabbage root fly feeding on *Verticillium longisporum* infected *Brassica* spp. and control (V+:▲, V-:x)

For Verticillium infected host plants, a negative correlation was found between amount of fungal DNA and fresh weight of aerial plants, because, more seriously infected plants showed stunting symptoms more obviously (Figure 6).

From eggs to pupa, a higher mortality was found on V+ host plants, thus the fitness of host the plants effected larval development. However, no correlation was found between fungal DNA and number of pupae, pupal weight and female fecundity.

There was significant positive correlation between fungal DNA, the amount of Sitosterol and the sum of phytosterols (Figure 7, 8). In summary, Verticillium infection altered the total amount of phytosterols, especially Sitosterol and its composition (Table 6).

Table 6 Correlationship between *Verticillium longisporum* DNA contained in one gram fresh root of *Brassica napus* plant, several development attributes of cabbage root fly and the corresponding phytosterols content

Correlation (x, y)	Regression	r^2	p value
Fungal DNA & aerial fresh weight of above-ground	y=25.30-0.63x	0.18	0.049*
Fungal DNA & the number of pupa and larvae alive	y=1.66+0.07x	0.11	n.s
Fungal DNA & pupa weight	y=14.80-0.045x	0.039	n.s
Fungal DNA & fecundity	y=55.11+1.546x	0.043	n.s
Fungal DNA & sitosterol	y=231.81+1.44x	0.44	0.036*
Fungal DNA & sum of phytosterol	y=304.81+1.98x	0.49	0.023*

(* p≤0.05; ***p≤0.0001; n.s: no significant difference)

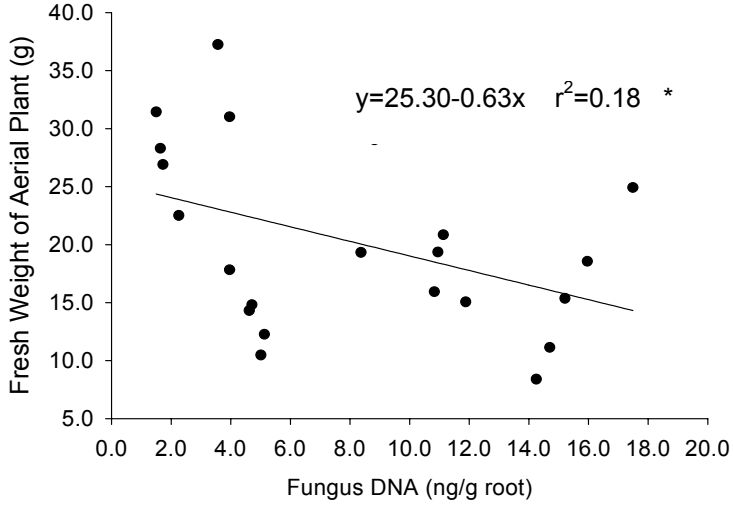

Figure 6 Correlation between DNA amount of *Verticillium longisporum* contained in one gram fresh root of *Brassica napus* and the fresh weight of aerial part of plant

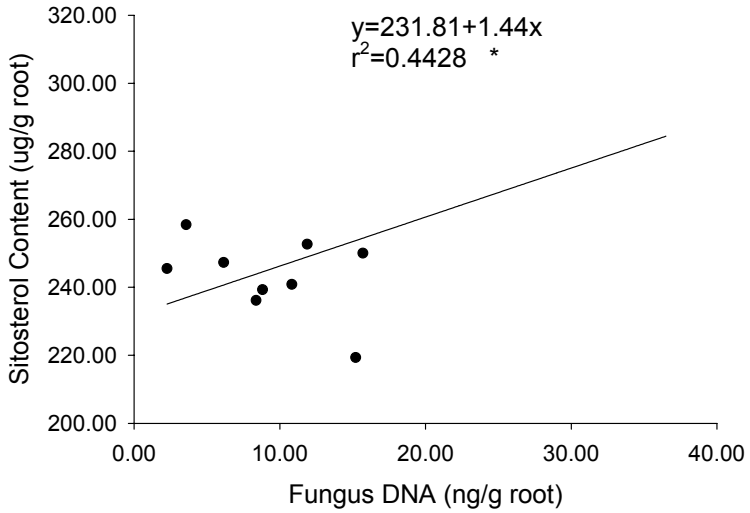

Figure 7 Correlation between DNA amount of *Verticillium longisporum* and Sitosterol content contained in one gram fresh root of *Brassica napus*

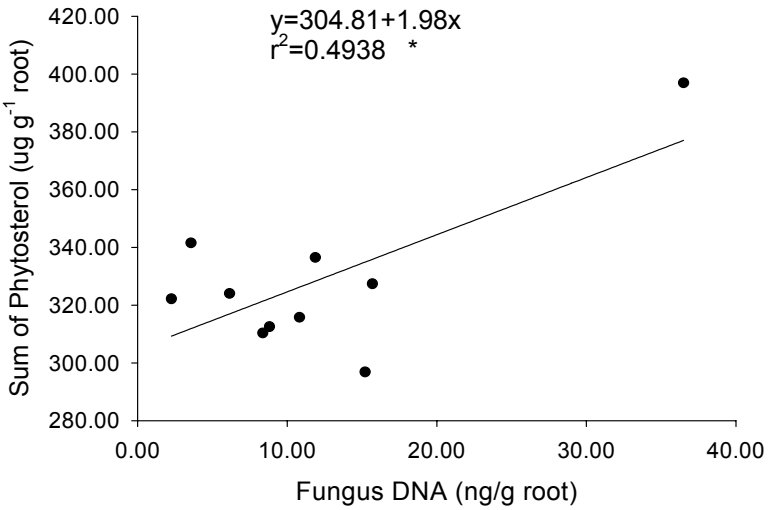

Figure 8 Correlation between DNA amount of *Verticillium longisporum* and the sum of phytosterols contained in one gram fresh root of *Brassica napus*

2.4 Discussion

Influence of fungal pathogen on host preference of CRF larval

In this study cabbage root flies showed an inconsistent orientation behavior to root segments and intact root, and cabbage root fly larvae have not been able to distinguish between a healthy and a pathogenic plant. The ability of cyclorrhaphous larvae, including cabbage root fly larvae, to discriminate between plants has been tested in several papers (Rygg 1972; Rygg & Sömme 1972; Jones& Coaker 1977). Previous studies showed that cabbage root flies had made a preference choice between turnip roots and Swede roots (Rygg, 1972) and the newly hatched cabbage root fly larvae orientated towards typical metabolites of host *Brassicaceae*, however the concentration applied made them either attractive or repellent (Kostal, 1992).
The mechanism for insects locating host plant differs with the species involved. For cabbage root fly larvae, a series of volatiles emanating from the root surface of host plants was found to be a stimulant for larvae´s host plant finding (Kostal, 1992.). For *Drosophila melanogaster*, ethanol was found to be the major orientation substance (Monte *et al*. 1989); for western corn rootworm larvae (*Diabrotica virgifera virgifera* LeConte), several studies demonstrated CO_2 alone or in combination with other volatile chemical cues were critical in host plant locating (Bernklau *et al*. 1998, 2004). However, these experiments were carried out using specific chemical compounds; volatiles from the rhizophere were not sampled. Thus, studies concerning collecting techniques of these compounds is indispensable for future research on host plant selection in belowground insects.

Effect of CRF feeding and Verticillium infection on plant morphology

We showed that a Verticillium infection significantly influenced plant morphology, such as increasing the number of chlorotic leaves, and reducing fresh shoot and root weight. We thus hypothesized, that larval development should be negatively influenced on V+ as compared to control plants.
A significant correlation was found ($y=-0.066+0.23x$ $r^2=0.936$) between fresh shoot weight and fresh root weight of *B. napus* (including the main root and root hairs); this was neither influenced by damage due to cabbage root fly larva feeding nor by an infection by Verticillium (Li, unpublished data). The correlation was maintained even in case some cabbage root fly larvae were feeding on the roots of control and Verticillium infected plants. However the slope of the regression line changed,

indicating the effect of cabbage root fly feeding on main root biomass buildup. The reduction was more obvious on control plants, which have be loaded with the same number of eggs; however more larvae survived to cause more consumptions on control plants. This was proved by obtaining more pupa on control plants.

The Verticillium infection did not significantly decrease pupal weight and the number of pupa, but negatively influenced the number of adults eclosing and shortened the duration time from pupa to adult. A significantly reduced development was observed for cabbage aphids from new-borne nymphs to adult, when feeding on V+ oilseed rape plants (data unpublished). Several papers showed that herbivorous insects suffered from a retarded development from larvae to pupae when feeding on plant tissue infected by a fungus. This has been shown for Diamondback moth larvae feeding on cabbage leaf-discs inoculated with an endophytic fungus (Raps & Vidal, 1998) and for phytophagous leaf beetles on creeping thirstle infected by a plant pathogenic fungus (Kruess 2002). However no data are available so far for the influence of these fungi on the development from pupa to adult. It was shown that the development of aphids was reduced and specimens suffered from stress, when exposed to less optimal nutrition or environmental change.

The mortality from egg to pupa and from pupa to adult on V+ plants increased in comparison to control plants. Some microorganisms have been shown to negatively impact insect development, such as black vine weevil (*Otiorhynchus sulcatus*), fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Heliothis zea*) fed on plants inoculated by mycorrhizae (Rabin & Pacowsky 1985; Gange 1994;); the leaf beetle (*Cassida rubiginosa*) on leaves from plant creeping thirstle *Cirsium arvense* (L.) infected by the necrotrophic pathogen (*Phoma destructive*) (Kruess, 2002); and Diamondback moth and greenhouse whitefly on soil-borne endohytic cabbage leaves (Raps & Vidal, 1998; Vidal, 1996). However, several studies reported negligible effects. For instance, Lappalainen and Helander (1977) found that an endophytic fungi of the mountain birch had no effect on leaf beetle larva performance under natural conditions and aphids (*S. graminum*) did not show a response to the the colonization of plants by mycorrhiza (*Glomus fasciculatum*) on sorghum (Pacovsky *et al*. 1985); the growth of southern armyworm (*Spodoptera eridania* (Cram.)) was not influenced by the pathogen *Verticillium albo-atrum* when feeding on infected alfalfa (Rke et Berth.) (Kingsley *et al*., 1983). In some cases even a positive

influence of a pathogen infection on herbivore development was found (Gange 1999; Mondy & Corio-Costet, 2000).

These inconsistent results let Barbosa to speculate that the consequence of microorganism colonizing plants on plant-associated insect growth could be beneficial, detrimental or negligible depending on the kind of herbivores, plants and insects involved in these interactions (Barbosa 1991). A reduced development of herbivores on infected plant may be perhaps caused by the production of mycrotoxins, such as alkaloids produced by endophytic fungi colonizing grass species (Clay, 1988; Breen, 1994) or a change in the nutritional suitability of the host plant to herbivores, such as changes in the phytosterol content and composition (Dugassa-Gobena *et al*., 1998, 2003); decreased protein content and increased amino acid content (Barbosa, 1991); reduced sucrose content (Smith *et al*., 1985) and an increase in the carbon/nitrogen balance in infected plants (Gange & West, 1994). A severe reduction in water content caused by vascular plant pathogens can also have a significant negative impact on herbivores.

In our study, we found the phytosterol content and the proportions of specific phytosterols to be influenced by the inoculation of the plants with Verticillium. A positive correlation between pupal weight and eclosion duration was established for herbivorous insects fed on control plant, however due to the inoculation with Verticillium, this correlation was found for male on V+ plants only.

Effect of fungal pathogen infection on CRF oviposition

Visual stimuli and volatile stimuli are essential for egg laying in females (Marazzi, 2004; pers. Observation), A group of new compounds, so-called CIFs were found to be more important than glucosinolates as oviposition stimulants (Roessingh *et al*. 1997) and more CIFs were found in roots than leaves of brassica plants (De Jong *et al*. 2000). In this study, kohlrabi blocks were used as the stimulant for females to lay eggs (Finch & Coaker, 1969). Normally on 5 days after eclosion, females started to lay eggs and 30-50 eggs/female was produced for the first batch (unpublished data). A rather low mean fecundity was found for both treatments, particularly for the V- treatment, with 53 eggs laid within 10 days. Although some causal factors could not be excluded, such as minor differences in the size of kohlrabi relating to differences

in volatile emission, it can be speculated that the V*erticillium* infection not only reduced the days from pupa to adult for females but also subsequently reduced the time for females to lay eggs. When V- females were maturing for laying eggs the second time, V+ female did already complete this. Nevertheless these results fit to the result in another experiment where an endophytic fungus was used to infect the Brassica plants. Within 10 days, E+ females laid 98.6±16.6 eggs and E- female only laid 68.3±17.2 eggs, respectively (unpublish data).

Effect of fungal pathogen inoculation on phytosterols

Cholesterol serving as a cell membrane insert and as a precursor of ecdysteroids, molting hormones that regulate growth and reproduction of insect, are indispensable for herbivorous insects, because insects can not synthesize their own sterols *de novo* (Zandee, 1962; Clayton, 1964; Ikekawa, 1993; Nes *et al*., 1997). Most herbivorous insects metabolize phytosterol to cholesterol to meet their sterol requirements, which are obtained from the host plants or the symbionts (Ritter, 1984; Douglas, 1988; Ikekawa, 1993; Nes *et al*., 1997). Plants contain a mixture of different sterols, such as Sitosterol (Δ^5), Campesterol(Δ^5), and Stigmasterol($\Delta^{5,22}$), and Sitosterol is usually most abundant (Nes, 1977). Brassicasterol is typical for the *Brassicaceae* family. Ergosterol is the main sterol produced by majority of higher fungi and the amount of this compound has been used as an index to detect the establishement of ectomycorrhizae in pine roots (Johnson & McGill, 1990) and plant cells can perceive the invasion of fungi by Ergosterol to establish a defense system (Granado *et al*. 1995). In our study, by fungal infections host plant might have produced Ergosterol which is reported to be an unsuitable sterol for larval development (Nes *et al*. 1997). Although our study did not calculate the production of Ergosterol, its existence should not be neglected. Our result revealed that Verticillium infection significantly decreased the production of Campesterol and increased Stigmasterol production respectively. We did not find any changes in the Brassicasterol and Sitosterol content.

Several papers have shown that the changes in the composition of suitable and unsuitable sterols in diet mixtures negatively influence the development of insects (Richter *et al*. 1987; Nes *et al*., 1997; Behmer & Elias, 2000). Sitosterol is regarded

as a suitable sterol for insect development (Clayton, 1964; Nes *et al*. 1997; Behmer & Elias 2000), however in these studies insect development was monitored on artificial diet differing in composition. By measuring the real composition in leaves, Dugassa-Gobena postulated that Sitosterol is an unsuitable sterol because it negatively correlated with larval weight in the Diamondback moth fed on endophytic cabbage plants (Dugassa-Gobena *et al*. 1998). We found a positive correlation between amount of fungal DNA and Sitosterol content in root samples of one gram. We also established a significant change in the composition of Campesterol and Stigmasterol. The role of Stigmasterol in insect development differs with the species involved. Stigmasterol supported the normal development of corn earworm (*Heliothis zea*) larvae (Nes *et al*., 1997), however it impaired five species of grasshoppers because of metabolic constraints (Orthoptera: Acrididae) (Behmer & Elias 2000). Additionally, a positive correlation was found between fungal DNA and aerial fresh weight of plants. Phytosterols are not only indispensable for phytophagous insects but also for fungal growth and reproduction (Hendrix, 1970), causing a competition between the fungi and phytophagous insects colonizing the same plants. However the specific allocation of phytosterols among the two organisms involved is far from being solved. It has been shown that root feeding by rhizophagous insects (*Tipula paludosa*) resulted in an increased colonization of arbuscular mycorrhizal (AM) in host plants (Currie *et al*, 2006). It may thus be speculated that more insects cause should cause more fungal colonization. In our study no association was found for fungal DNA and number of pupa recovered, pupal weight and fecundity. This was also found in another study, where the leafmining activity caused higher endophyte infections, however the increase in infections was not related to an increase in leafminer mortality (Faeth & Hammon, 1997). Because *V. longisporum* is a plant pathogen and not an entomopathogen such as *Verticillium lecanii* Viegas, the direct influence (virulence) on the development of the insect could be expected not to correlate with its pathogenicity (Korolev & Gindin 1999). Raps and Vidal (1998) proposed the “spatial disjunction hypothesis” and hypothesized that the performance of organisms feeding or living close in space on a shared host plan should be less effected by fungal infections as compared to those herbivores and pathogens which a widely apart from each other (Raps & Vidal, 1998).

Prospect and Suggestions

The dual-choice equipment using intact root systems did not destructively disturb the soil and kept roots intact for a natural volatile emission. The detachable tube segment made it feasible to measure larval movement even when adding some opaque medium such as soil, and the gauze at the tube end prevented larvae from entering into the pots. Given these advantages, some improvements would be necessary to optimize the design. We recommend that column tubes and the respective media (soil or vermiculite) should be tightly filled, because the smooth and lubricious surface retarded the locomotion of the larvae to odour sources (pers. observation).

Given the difficulties to monitor the behavior of soil-borne insects within the soil matrix, some novel techniques should be deployed, such as X-ray microtomography and acoustic sensors (Zhang *et al*., 2003; Johnson *et. al*, 2004).

For controlling the destructive Verticillium wilt, biological control methods may be used, such as the use of wood chip-polyacrylamide medium to favor the growth of biocontrol microorganism (biocontrol bacteria) suppressing the growth of Verticillium (Entry *et al*. 2000) and the root endophyte *Phialocephala fortinii* which was found to suppress *Verticillium* wilt in eggplants (Narisawa *et al*. 2002).

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Chapter 3

Preference-performance of the Diamondback moth (*Plutella xylostella* L.) feeding on winter oilseed rape (*Brassica napus* L.) inoculated with a soil-borne fungus (*Verticillium longisporum*)

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Abstract

When two antagonists of plants simultaneously colonize the same host plant species, direct and indirect interactions between the pathogen and the herbivorous insects will be possible. In this study the effect of vascular wilt fungus *Verticillium longisporum* on the preference-performance relationship of the Diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) was investigated on oilseed rape, *Brassica napus*. An infection rate of 84% was found in inoculated plants by real-time PCR (polymerase chain reaction). Results of dual-choice bioassays showed that Diamondback moth females preferred to deposit more eggs on Verticillium-free (V-) as compared to Verticillium-infected (V+) plants. The same pattern was found when testing the food choice preference in Diamondback moth larvae.

We found that feeding of larvae on *B. napus* plants inoculated with *Verticillium longisporum* increased larva mortality, reduced developmental time of from pupa to adult and reduced potential fecundity predicted by a shorter hind tibia. However pupal weight, adult weight and fecundity were negligibly influenced by pathogen infection. The effect of *V. longisporum* on insect performance was influenced by the bioassay design. Among the volatiles emitted from aerial parts of V+/V- host plants, eight constitutive volatile compounds were identified in both treated and untreated plants, however differing in quantities and relative proportions. Significantly more 1,2,4-trimethyl- benzene was found in V+ plants eliciting a strong response in Diamondback moth females. The profiles of phytosterols in *V. longisporum* inoculated (V+) and uninoculated (V-) plant root were quantified with regard to the development of the larvae mechanisms. Verticillium infections significantly enhanced the sum of phytosterols in the leaves, in particular the amount of Sitosterol, the most abundant sterol in both treatments, followed by Brassicasterol, Campesterol and Stigmasterol. However, no significant difference with regard to the overall phytosterol composition was found.

The results are discussed with regard to general pattern of herbivorous insect preference and performance hypothesized to follow when developing on plants infected with plant pathogens.

Key words: *Plutella xylostella*; oilseed rape; *Verticillium longisporum*; preference; performance; volatiles; phytosterols;

3.1 Introduction

Under natural conditions herbivores and plant pathogenic fungi frequently share the same host plants (Hawksworth, 1991; Hatcher, 1995; Gang & Bower, 1997). Interactions between herbivores and pathogen are thus likely to happen. These interactions may be regarded direct, in case of mycophagous insects consuming mycelia of fungi (Sabatini & Innocenti, 2000) or pathogens vectored by insect vectors (Hodgson, 1981; Palermo *et al*., 2001). They may be regarded indirect, for example by the reciprocal impact caused by plant antagonists on the other organisms via the shared host plant (Hatcher & Ayres, 1997). The consequences of herbivorous insect feeding on plants infected by pathogens could be regarded beneficial, detrimental, or negligible to one or both sides (see review by Jones 1991).

Few studies so far have investigated the effect of a pathogen infection on herbivorous insect performance. Among them aphids were studied frequently and in most cases, their development indices increased by pathogen infections including higher weight and enhanced reproduction (Moran, 1998; Gange *et al*., 1999; Johnson *et al*., 2003). However, other papers have documented the detrimental effects caused by a pathogen infection on plant-associated insect performance, including an enhanced mortality of larvae, retarded development of larvae and pupa, and poor weight of larvae and pupa. These effects have been documented in the leaf beetle *Cassida rubiginosa* feeding on thistles inoculated with the fungus *Phoma destructive* (Plowr.) (Kruess, 2002), in *Helicoverpa armigera* and the greenhouse whitefly *Trialeurodes vaporariorum* (Westw.) reared on tomato plants (*Lycopersicon esculentum* Mill.) infected by soil-borne endophtic fungi, *Acremonium alternatum* or *A. stritum* (Vidal, 1996; Jallow *et al*. 2004, 2008) and the spider mite *Tetranychus urticae* feeding on cotton plants infected with *Verticillium dabliae* (Karban *et al*., 1987). Negligible or no effects on herbivorous insects have been found when a pathogen infection of *Verticillium albo-atrum* was tested with regard to the southern armyworm *Spodoptera eridania* (Cram.) on alfalfa (Kingsley *et al*., 1983) and when the mycorrhizal fungus *Glomus fasciculatum* was tested with regard to the aphid *S. graminum* on sorghum (Pacovsky *et al*., 1985). Given these results, Gange & West (1994) hypothesized that insect feeding pattern might be an important parameter

explaining these different outcomes, with chewing insects performing worse as compared to phloem-sucking insects on plants infected with mycorrhizae.

The mechanisms resulting in these effects on herbivorous insects are regarded physiological changes in the host plants. Rapid death of cells surrounding infection sites (typical for hypersensitive responses) have been supposed to induce resistance in willow tree varieties (*Salix viminalis*) to the gall midge *Dasineura marginemtorquens*, resulting in increased larval mortality (Heath, 2000; Höglund *et al*., 2005). On the other hand, changes in the composition of primary metabolites have been found in infected plants, affecting insect herbivores feeding on these plant tissues, such as carbohydrate (Farrar, 1992; Costa Pinto *et al*., 2000), proteins (Barbosa, 1991), amino acids (Johnson *et al*., 2003), sucroses (Smith *et al*., 1985) and the carbon-nitrogen balance (Hare & Dodds, 1987; Jones & Last, 1991; Gange & West, 1994; Gange, 1996; Faeth & Fagan, 2002). Moreover, The infection of plants by plant pathogens resulted in the induction of a diverse array of secondary metabolites, among which antimicrobial substances for improving plant disease resistance (Moran, 1998; Dixon, 2001) and induction of defense compounds such as flavonoids and glucosinolates have been documented (Doughty *et al*., 1991; Ponce *et al*. 2004; Treutter, 2006). Alkaloids, produced by endophytic fungi infecting grasses explained the negative impact on insects feeding on these plants (Clay, 1988; Dahlman *et al*., 1991; Breen, 1994; Bush *et a*l, 1997; Gimenez *et al*., 2007). In recent years, changes in the phytosterols content and composition have been used to explain the underlying mechanisms of microorganism mediation on insect performance via host plants (Bernays, 1993; Dugassa-Gobena *et al*., 1998, 2003).

Phytosterols are needed by herbivorous insects as precursors of steroid hormones and as regulators of developmental processes; however although important insects are unable to synthesize sterols, and sterols in the diet are the unique resources (Ritter, 1984; Douglas, 1988; Ikekawa, 1993; Mondy & Corio-Costet, 2000; Behmer & Nes, 2003). Several studies have documented the negative impact of changes in the phytosterol composition on insect development (Nes, *et al*., 1997; Richter *et al*. 1987; Bodnaryk *et al*., 1997; Dugassa-Gobena *et al*., 2003).

Finally, pathogen-induced quantitative and qualitative variation in volatile and non-volatile secondary metabolisms has been documented in several studies (Doughty *et al*., 1991; Huang, *et al*., 2003), resulting in changes of insect preference during host-finding (Jallow *et al*., 2008).

The Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a highly mobile, destructive oligophagous pest of crucifers world-wide. It attacks host plants from seedling stage to harvest and causes serious losses to cruciferous crops the whole year (Yang *et al*., 1993; Talekar & Shelton, 1993). The long-term extensive use of broad-spectrum insecticides resulted in resistance of the Diamondback moth to synthetic, bacterial, and biological insecticides (a review by Talekar & Shelton, 1993). This prompted the research on microorganisms which could be potentially used as biocontrol agents against the Diamondback moth. Currently some entomopathogenic agents, including bacteria (Blackburn *et al*., 2006; Abdel-Razek, 2003), fungi (Pell *et al*., 1993; Vandenberg & Ramos, 1997) and nematodes (Baur *et al*., 1995) have been tested as an alternative control strategy in IPM.

Vascular wilt is an increasing problem on Brassica oilcrops in northern European countries (Svensson & Lerenius, 1987; Zielinski & Sadowski, 1995). On oilseed rape (*Brassica napus* L. spp. *oleifera*) (**OSR**) this disease is caused by *Verticillium longisporum*, a host-specific vascular wilt pathogen (Karapapa *et al*., 1997; Zeise & von Tiedemann, 2001). Verticillium fungi are soil-borne pathogens and mainly spread via infested soil from field to field (Xiao & Subbarao, 1998). The typical symptoms in plants infected by *V. longisporum* include necrosis and chlorosis of leaves, stunting, ripening and premature senescence (Bhat & Subbarao, 1999; Zhou *et al*., 2006; Steventon, 2002; Babadoost *et al*., 2004). Presently the relative intensive cultivation and continuous crop rotation of OSR has rendered this disease spreading quickly and resulted in dramatic economic losses (Svenson & Lerenius, 1987).

In this study, the preference of the Diamondback moth larva and females were tested in dual-choice bioassays on Verticillium infected (V+) and uninfected (V-) oilseed rape *Brassica napus.* The subsequent development of larvae, pupa and adults was monitored. The secondary metabolism, including the constitutive volatile organic compounds (VOCs), and inducible volatiles (IVOCs) were collected and identified and the corresponding response of Diamondback moth was depicted in GC-MS/EAD to understand the host preference of insect larvae and adults. The quantity and relative proportions of the four main phytosterols (i.e. Sitosterol, Campesterol, Stigmasterol and Brassicasterol) in the root tissues of V+/V- oilseed rape plants were quantified to understand the corresponding insect performance.

3.2 Material and Method

Host plant

In this study we used the winter oilseed rape variety 'Falcon', (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG). In the dual-choice experiments we used three weeks old plants which had been inoculated the previous week to avoid the symptoms of Verticillium infection becoming visible.

Brassica napus seeds were sterilized using 70% alcohol for 2mins, then rinsed 3 times using autoclaved tap water and were grown in quartz sand for 7 days. Seedlings were extracted and washed using tap water thoroughly and the roots of seedlings were dipped into a spore suspension (10^6 conidia ml^{-1}) for 40 min according to the method described by Bhat and Subbarao (Bhat & Subbarao, 1999), however, without trimming the roots. Roots serving as the controls were dipped into tap water for 40 min. Thereafter seedlings were replanted in a substrate containing sand and soil (ökohum®-Rindentorfsubstrate, Type RTS; Ratio: 1:2) in plastic pots (Ø11cm) and were grown in the greenhouse at 24±3°C, 80% relative humidity (r.h.) and at a 16:8 h LD photoperiod for three weeks. Plants were arranged in a randomized block design on greenhouse benches and were watered every day and fertilized at weekly intervals with Wuxal® Super (10-10-8 w/v).

Fungus

Verticillium longisporum, isolate VL 43, originated from field soil with *B. napus* in the north of Germany. The isolation method and fungus cultivation has been described in detail by Zeise and von Tiedemann (2001, 2002a, b). To maintain the pathogenicity, a spore suspension (10^6-10^7 conidia mL^{-1}) was stored long-term with Czapek Dox medium supplemented with 25% glycerol in 1.5 ml Eppendorf cups stored at -20°C. For propagation, droplets of the suspension were plated onto potato dextrose agar (PDA) and incubated for three weeks at 21°C in darkness. The spore suspension was obtained by gently adding a small amount of distilled water and harvested using hair-brush gently. Inocula for the root dipping inoculation was produced by adding 500 uL of the spore stock suspension to 250 ml potato dextrose

broth (PDB) and kept for three weeks in darkness at 23°C on a rotary shaker at 100rpm. The concentration of conidia suspension was calculated using a Thoma counting chamber (Marienfeld, Germany) (depth 0.1mm, square width: 0.05mm) under a microscope.

Insect

Adults and larvae of *Plutella xylostella* were derived from a permanent culture at the institute on cabbage (*Brassica oleracea var. viridis*) in a climate controlled culture room (21±1°C, 70±5% r.h. and LD 16h : 8 h photoperiod).

Larvae preference experiment

In order to get synchronized larval instars, 15 pairs of females and males were released into one cage with cabbage plants inside. After 24 hours, plants loaded with eggs were placed in another insect rearing cage (BugDorm-2 Insect Rearing Cage, MEGA VIEW, Thaichung, Taiwan). I-II instar larvae were randomly selected for the host plant preference experiments. The remaining larvae were further reared for use in adult oviposition preference experiments. Three weeks old *Brassica napus,* inoculated with *V. longisporum* for three weeks or control plants were used in this experiment.

A special board was designed as dual-choice platform for larvae. This board (length: 25cm, whole width: 10cm) was divided into two identical parts with two holes (Ø1.5 cm) at the middle line with 3cm interval distance for keeping the plant shoots. For protecting the plant shoots, a small piece of sponge was fixed at the sides of the hole. One pair of V+/V- host plants was held by the special board in the rearing cage (BugDorm-2 Insect Rearing Cage, MEGA VIEW). The experiment was conducted in an environment-controlled room (21±1°C, 70±5% r.h.). If one larva moved away from the middle point on the edge of the board and reached the host plant within 15 min., it was recorded one effective choice, otherwise it was regarded no choice. In this case, a new board and a new larva were introduced to repeat the test with the same host plant. The test was replicated 28 times.

Adult oviposition preference and offspring development experiment

One pair of V+/V- host plants (4-5 weeks dpi), already used in the larva preference test was arranged with approximately 25cm distance in each of ten insect rearing cages (BugDorm-2 Insect Rearing Cage, MEGA VIEW, Thaichung, Taiwan). For controlling the age of the adults, all these adults originated from the stock rearing as described in the larvae preference test. Five pairs of newly eclosed females and males were released. The moths were allowed to mate and oviposite for 72 hours, after that the adults were removed and the number of eggs on each plant was counted. This experiment was conducted in an environment controlled room and the light was provided by a lamp (500W) above each cage (21 ±1°C, 70% r.h., LD 16h : 8 h). Every 24 hours, the position of plant was changed 90° clockwise to eliminate conflicting light conditions on egg laying.

Seven V+/V- plants containing eggs were maintained in separate cylinders (diameter: 20cm, height: 25 cm) until egg hatching. Fifteen first instar larvae from each cylinder were transferred to fresh plants (15 larvae per plant, 6 weeks dpi) to complete development. Pupae were collected using a fine tweezer and weighted, and were kept separately in glass tubes (diameter: 2cm), sealed with Parafilm. The pupae were transferred to a climatic chamber and incubated at a constant environment (21±1°C; 65% r.h. under LD 16h:8h photoperiod). When the adults eclosed, they were weighted within 12 hr, then frozen in a fridge (-20°C). The length of the hind tibia of adults was measured using a binocular with a microruler to report the body size.

Larva weight gain and food consumption experiment

In this experiment, larval weight gain and food consumption was compared for larvae feeding on V+/V- host plants for 72h. Leaf-discs (Ø 2cm), cut from V+/V- plants, were dipped in tap water for 1-2 seconds and thereafter placed on a wet filter paper (Ø50mm) in Petri-dishes (Ø 52mm). These leaf-discs were divided into two subgroups. One group, including ten leaf-discs from infected or uninfected plants, was used as a reference to calculate the food consumption of larvae. The other leaf-discs group was used for larval feeding. Fifteen I-II instar larvae of *Plutella xylostella* obtained from the same female were weighted (W_1) individually (weight of larva on

V+ leaf disc: 0.65 ± 0.31mg , weight of larva on V- leaf disc: 0.51 ± 0.19mg) then one larva was transferred to each Petri-dish using a fine tweezer. Fifteen replications were established. This experiment was run in a climatic chamber (16l : 8d, 25°C, 75r.h.). To reduce water loss of leaf-discs, six drops of distilled water were added after 8hr. The leaf-discs were replaced every 24hr. This experiment was performed for 72hr., therefore 3 leaf-discs were consumed. Larvae were weighted again (W_2). The debris of the leaf-discs and the reference leaf-discs were transferred to paper bags separately and dried at 60°C for one week. The tested larvae were continually reared using either V+/V- plant leaves until pupation. Then the number of pupa and their weight and the subsequent adult numbers were recorded.
Weight gain of larvae was calculated as follows:

$$w = W_2 - W_1$$

w: weight gain of larva

W_2: larval weight after 72hr. feeding

W_1: initial larval weight

Volatiles profiles in V+/V- *B. napus*

Volatile Collection

Volatile collections were done using the method described in Jallow *et al*. (2008) with some modifications. Volatiles were collected for 22 hr from plants inoculated for 6 weeks. The aerial part of individual plants was covered with an ethylen-tetraflorethylen (ETFE) bag (32 cm width × 43 cm height) that was mounted on a laboratory pedestal with the bag opening turned down to cover totally the aerial part of the plant. The opening of the bag was closed around the stem basis by mounting its opposite edges between aluminium splints, and fixed with clips. Volatile collection started 1h after the air in the bag was homogenised. The bag had a hole in the upper side, which was fitted with an ETFE stopper (20mm diameter). The ETFE stopper contained two openings (0.67mm diameter). Volatiles were extracted through one of the openings from the interior bag by a miniature pump (DC12/16NK Fa. Fürgut, Germany), fixed to an adsorbent volatile trapping device (Prec. Charcoal filter (1.5mg)

(Fa._Brechbuehler AG, Switzerland) mounted between the bag and the pump at the inlet part of the pump. The outlet of the pump was connected with the inside of the bags by the alternative opening to maintain a constant air circulation. The air circulation rate of the pump was kept constantly at 9 volt by adjusting its power supplier (PS-302A, Fa. Conrad Electronic, Germany). Volatile were collected from ten V+/V- plants used in larva host preference experiment. At the end of each trapping period, the enclosed plant parts within the bags were harvested and their wet weight was determined for computing the volatile amounts. The plant root was washed carefully and deeply frozen in liquid nitrogen and stored at -20°C until further Real time-PCR processing.

GC-MS/EAD

Plant volatiles were eluted from the absorbent filters with 145 ul of dichloromethane and methanol (2:1; v/v); 2 ul (0.004 mM) of camphor was added as internal standard. Aliquots of the samples were analysed with GC-MS/EAD (6890N gas chromatograph, 5973N quadrupole mass spectrometer).The process was depicted by Weissbecker (Weissbecker *et al*., 2004). The GC was equipped with a type 7163 autosampler and a split/splitless injector. A J&W Scientific HP-5MS column (Agilent) was used (30 m×0.25 mm i.d., film thickness 0.25 um). The effluent from the column was splitted into two pieces of deactivated capillary using a Graphpack 3D/2 flow splitter (Gerstel, Mülheim, Germany). One capillary (1 m× 0.1 mm i.d.) led to the mass spectrometer, the other (1 m× 0.15 mm i.d.) to an “olfactory detector port” (ODP-2, Gerstel).

One micro liter of sample was injected into the pulsed splitless mode at a temperature of 250°C. The column temperature of GC was first held at 50°C for 1.5 min. Thereafter, the temperature was programmed to increase from 50°C to 200°C at 6°C min^{-1} holding for 5 min. Helium (purity 99.99%) flowing at 1 ml min^{-1} was used as carrier gas. Electron ionization (EI) at 70 eV was used in mass spectrometer and the scan speed was 2.78 scans per second with a scan mode from 35 to 300 mass units. For a tentatively peak identification the National Institute of Standards and Technology mass spectral library (NIST, Gaithersburg, USA) was used.

Insect and EAD test

All insects used in the EAD analyses were mated females from a separate rearing with approximately the same age. Excised antennae of females were placed in an antenna holder (Koch, Kaiserslautern, Germany). Within this holder the ends of the antennae were in touch with an electrode solution providing electrical contact to a pair of Ag/AgCl electrodes. In this holder, the antennae were freely accessible to the air flow from the EAD interface.

DNA extraction and real-time PCR analysis

All twenty five plants (6 weeks dpi) used in larval host plant preference experiment, adult oviposition preference and volatile collection experiments were harvested and the roots were washed carefully and deeply frozen in liquid nitrogen and stored at -20°C. To extract DNA, the whole root (main root and root hairs) were ground to fine powder under liquid nitrogen using a mortar. DNA extraction was conducted using the DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). 100mg root powder was weighted and dispersed in 1ml CTAB buffer (20 mM EDTA, 10 mM Tris, 0.13 M sorbitol, 0.03 M N-laurylsarcosine, 0.02 M hexadecyltrimethylammonium bromide, 0.8 M sodium chloride, 1% (w/v) polyvinylpolypyrolidone, pH set to 8.0 with HCl) containing 2µl mercaptoethanol and 1µl proteinase K. The mixture was completely mixed using Vortex for 30 seconds followed by incubation in water bath for 20 min at 40°C and 20 min at 60°C. Cooled down to room temperature and extracted with 750 ul chloroform-isoamyl alcohol (24:1). Phases were separated by centrifuging at 8.000 rpm for 20 min. The supernatant was transferred to a 1.5-ml Eppendorf cup and added 500µl isopropanol with slight shaking for 15 sec. After incubation at room temperature for 15min, DNA was deposited by centrifuging at 11.000 rpm for 20 min. The pellet was washed with 500 µl 70% (v/v) ethanol and dried in SpeedVac at 30°C for 10 min. Then dissolved in 30µl TE buffer (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl) and incubated over night at 4°C to ensure that the DNA was dissolved completely. The quality and quantity of DNA were assessed by electrophoresis in 0.8% (w/v) agarose gels (Biozym LE Agarose, Biozym Scientific GmbH, Oldendorf, Germany) prepared in TAE buffer (40 mM Tris, 1 mM EDTA, pH set to 8.5 with acetic acid) with 25ng Lambda Phage DNA as a standard. The electrophoresis was run at 65 Volt for 80 min. Double-stranded DNA was stained with ethidium bromide

(ethidium bromide, 2 mg L^{-1}) for 15 min and washed with H_2O for 10 min. The gels were documented with the help of a digital imaging system (Vilber Lourmat, Marne-La-Vallée Cedex1, France). If the density of DNA was too high, a 1:10-dilution of the DNA solution or higher folder was used for homogenizing DNA in the following Real-time PCR amplification to determine fungal biomass.

Real-time PCR Amplification

Samples stored in a fridge at -20°C were melted in water-bath at 42°C for 2 hours. The real-time PCR was carried out in accordance with the method used by Eynck (Eynck *et al*., 2007). The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *Verticillium longisporum* DNA. Forward Primers OLG70 (CAGCGAAACGCGATATGTAG) and backward primer OLG71 (GGCTTGTAGGGGGTTTAGA) (P. Karlovsky, unpublished) were used to multiply a fragment specific for *Verticillium longisporum*. The reaction mixture consisted of NH_4-reaction buffer (16mM$(NH_4)_2$ SO_4, 67mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Luckenwalde, Germany), 3 mM $MgCl_2$ (Bioline, Luckenwalde, Germany), 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of every primer, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM fluorescein (BioRad, Hercules, CA, USA), 100,000 dilution folders SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 2 µl of template DNA and doubly distilled water (ddH_2O) filled to a total volume of 25 µl. The detection of amplification products, based on the fluorescence of SYBR Green I, was implemented with filters set at 490±10 nm for excitation and 530±15 nm for emission. The PCR amplification was run following cycling process. Initial denaturation for 2 min at 94°C, followed by 36 cycles of denaturation step at 94°C for 20 s., then an annealing steps at 59°C for 30 s and at 72°C for 40 s. The final elongation last for 5 min at 72°C. With PCR going-on, the detection of fluorescence was performed in the annealing step of each cycle. To verify amplification of the specific target DNA, a melting curve analysis was included. The melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for 1 min and then slowly increasing the temperature from 65°C to 95°C at the rate of 0.5°C s^{-1} with a continuous measurement of the fluorescence.

V. longisporum DNA quantification

The amount of DNA of *V. longisporum* was assessed from a calibration curve using increasing amounts of genomic *V. longisporum* DNA from 0.5 to 64.0 pg. The final DNA amount (µg DNA g^{-1} root) in plant sample was calculated following the equation:

$$M = 150\, fd$$

M :final DNA amount in one gram plant material

f : DNA diluted folder

d : DNA amount in real-time PCR

Phytosterol profile

Because of the intensive feeding on leaves and stems in the larval performance experiment, the remaining plant material was not suitable for phytosterol analysis. Therefore new plants were cultivated and inoculated using the root-dipping method for 7 weeks for phytosterol analyses. 10 V+/V- host plants were used. All leaves were excised and pulverized into fine powder in liquid nitrogen using a mortar and a pestle. Only 0.5-0.6 g of plant material was transferred to a 40 ml glass bottle glass bottle with screw-cup. The phytosterols were extracting following the procedure described by Newton (1989) with modification as follows: 20ml mixed solvent (5 ml 10 M KOH, 15 ml 96% ethanol and 0.06 g pyrogallol was added to each bottle containing plant material. An ultrasonic homogenizer (Model: Sonoplus HG 2200 / UW 2200, 200 W, 20 kHz, Bandelin GmbH, Germany) was used for 30 s for cracking cell completely and degassing and then bottles were placed in a shaker water bath at 80°C for 2.5 h. After cooling the samples to room temperature, 40 µl cholesterol was added as an internal standard (Cholesterol 5 ug / ul chloroform, Merck GmbH, Germany). Phytosterols were extracted with 10 ml hexane for 3 times. After thorough shaking for 20 s, deposited for 5-10 min to separate phases, then the supernatant was transferred to rotary glass. One ml distilled water was added to wash fractions, the separated lower phase was sucked out. Samples were distilled with rotary-evaporator at 42°C. The concentrated sterols were resolved in 1.5ml hexane and

transferred to 2 ml Eppendorf cups. After centrifuging with 10,000 rpm for 10 min, the supernatant was transferred to GC-vial and the hexane was evaporated overnight at 50°C in thermal block. The concentrated sterols was resolved in 240 µl hexane and 80 µl N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)(Fluka / Riedel-deHaen GmbH,Germany) and incubated in the thermal block at 70°C for 20 min. After cooling down to room temperature, transferred 100-150 ul of the extract to GC-vial equipped with 300 ul inserts.

Phytosterol identification and quantification was based on the internal standard method (peak area and retention time). 1 µl of each sample with a split of 1:50 of extracted sterols were analyzed using a gas chromatograph (Shimadzu GmbH, Model: GC14/15A) with a flame ionization detector. The samples were run on a fused silica column (SPB-1; 1.3m x 0.32mm, 0.25µm film thickness, Supelco Inc.; Sigma-Aldrich, Germany). Helium was used as a carrier gas, the make-up gas was synthetic air with a linear velocity of 35cm/s. The temperature program: 180°C for 3 min, then increased to 290°C at a heating rate of 10°C/min and held for 36 min. the total analyzing time is 50min. The detector temperature was 300°C. Peak areas were calculated using an integrator and the internal standard. A set of typical peaks indicating phytosterols, such as brassicasterol, campesterol, stigmasterol and sitosterol individually had been identified beforehand using GC-MS with synthetic sterols as referent. The phytosterol content in one gram plant material was calculated following this equator:

$$G = \frac{200 \times p_2}{p_1 \times g}$$

G: weight of unknown phytosterol in one gram plant material p_1: peak area of cholesterol, p_2: peak area of unknown phytosterol, g: weight of plant material

Total phytosterol in one sample was calculated by summing up all the four phytosterols.

Statistics

SPSS 10.0 for Windows (SPSS inc. 2000) was used for the statistical analyses. A binomial test was used for the larval dual-choice experiment. A paired-samples T-Test was used for the oviposition preference of adults. For hind tibia of male, data was transformed firstly by lg10, then by SQRT for normal distribution. Homogeneity of variance and independence of samples were tested, than one-way ANOVA (analysis of variance) was conducted.

The quantity of volatiles was analyzed by ANOVA, in case the data was normally distributed; otherwise a Man-Whitney U-test was used. Phytosterol contents, such as sitosterol and the sum of phytosterols were analysed by ANOVA, while other phytosterol content and relative content were analysed by Man-Whitney test.

3.3 Result

3.3.1 Real-time PCR and host preference of *P. xylostella* larvae and adults

Using 25 plants, randomly chosen for the realtime-PCR analysis, 84% were infected successfully and the average fungus amount was 9.78±8.66 ng DNA g^{-1} fresh root tissue of *Brassica napus* at 6 weeks dpi. The result of gel electrophoresis was displayed (Figure 1).

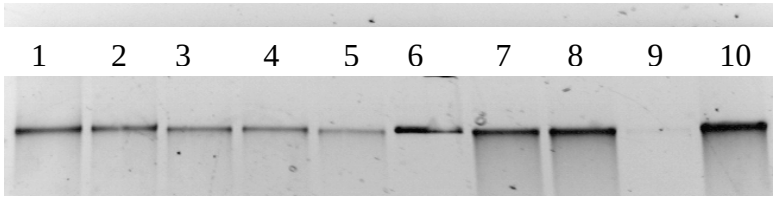

Figure 1: Yield and quality of DNA extracted from fresh root of *Brassica napus* either inoculated with *Verticillium longisporum* or control (lane 1-5: with Verticillium; lane 6: 25ng λ-DNA; lane7-10: control)

Three plants were discarded because of a light mildew infection on the plant leaves. Following the realtime-PCR test, 7 data sets of the larval preference from the 28 replications were excluded because of non-successful inoculation by *V. longisporum* or occasional infection by mildew. 7 larvae chose V+ plants and 14 larvae chose V- plants. No significant statistical difference was found (p=0.189, Binomial test) in this

experiment. *P. xylostella* females preferred to lay eggs on non-infected host plants (directly on the host plants and on the pots) as compared with those on infected host plants (mean number of eggs on V+ plants: 78.7 ± 41.43; on V- plants: 122.9 ± 66.02, paired t-test T=-2.639, df=9, p=0.027<0.05*).

3.3.2 Performance of Diamondback moth feeding on V+/V- *B. napus*

Results from the leaf-disc experiment showed that weight gain of larvae did not significantly decrease when feeding on leaf-discs from plants infected by *V. longisporum* for 72 h. Consecutive rearing on V+/V- plant leaf-discs did not result in a significant difference with regard to pupal weight. However a higher mortality of larvae feeding on V+ plants was found in comparison with those feeding on V-plants (Chi-square test, χ^2=27.272, df=1, p=0.000<0.01***) (Table1). No significant difference was found with regard to pupal mortality and development time from pupa to adult.

Table 1 Development of *Plutella xylostella* larvae feeding on *Verticillium longisporum* infected/uninfected *Brassica napus* for 72 hours (means ± SD)

Treatment	V+	V-	F or χ^2	p
larva weight (mg)	0.65±0.31 (11)	0.51±0.19 (10)	1.413[b]	n.s
biomass gain of larva (mg)	2.34±1.58 (11)	3.10±1.30(10)	1.388[b]	n.s
pupa weight (mg)	4.10±0.58 (4)	4.47±0.95 (10)	0.523[b]	n.s
DW of referent leaf-disc(mg)	18.17±1.88 (9)	18.11±1.71(8)	0.005[b]	n.s
DW of 1st leaf-disc debris (mg)	17.76±3.17 (11)	17.73±2.61 (12)	0.001[b]	n.s
DW of 2nd leaf-disc debris (mg)	16.73±5.15 (11)	13.96±4.96 (11)	1.655[b]	n.s
DW of 3rd leaf-disc debris (mg)	12.59±3.07 (11)	11.31±2.91 (10)	0.946[b]	n.s
mean DW of three leaf-disc debris (mg)	15.69±4.42 (33)	14.53±4.43 (33)	1.145[b]	n.s
days from pupa to adult (d)	4.33±0.58 (3)	3.88±0.84 (8)	0.744[b]	n.s
Mortality of larvae (%)	64% (11L-4P)	17% (12L-10P)	27.272[a]	***
Mortality of pupa (%)	25% (4P-3A)	20% (10P-8A)	0.556[a]	n.s

the number in parenthesis indicated the samples size; a: χ^2 value, b: F value;
DW: dry weight; L: larvae, P: pupa, A: adult. amount of larvae, pupa and adult.

With regard to the experiment using whole plants, *Verticillium* infection did not cause more deformed pupae (V+: 13, V-:7, Chi-square test, p=0.18, n.s). Larval mortality significantly increased on V+ plants (V+: 10.5%, V-:19.1%). No significant difference for pupal and adult weight was found feeding when on V+/V- *B.napus*. However,

eclosion days (from pupae to adult) were significantly shorter for females feeding on V+ host plants than on V- host plants (Man-Whitney U test, p=0.011). No significant difference of eclosion days was found for females and males (females: 5.01±1.65 (d), males: 4.93±1.58 (d); Man-Whitney test, p=0.822). A significant difference was found for the length of the hind tibia of females and males feeding on V+/V- *B. napus* (♀: $F_{(1, 72)}$=5.054, P=0.028<0.05*; ♂: $F_{(1, 83)}$=10.685, P=0.002<0.01**) (Table 2). Body sizes of females feeding on V+/V- *B. napus* was significantly larger than in males (V+ plants: pupal weight between ♀ and ♂, $F_{(1,79)}$= 3.966, P=0.05≤0.05; adult weight between ♀ and ♂, $F_{(1,79)}$=69.833, p=0.000<0.01; hind tibia length between ♀ and ♂,: $F_{(1,79)}$=6.520, p=0.013<0.05; V- plant: pupa weight between ♀ and ♂: $F_{(1,76)}$= 5.744, P=0.019≤0.05; adult weight between ♀ and ♂: $F_{(1,76)}$=41.43, p=0.000<0.01; hind tibia length between ♀ and ♂: $F_{(1,79)}$=5.657, p=0.02<0.05). A significant correlation was found between pupal weight, adult weight and the length of hind tibiae (Figure 2 and 3). Verticillium infection did not change the correlation, however according to the established equation, when *P. xylostella* larvae feeding on different treated host plant (i.e. Verticillium infected or uninfected plants), even in case the pupal weight displayed no difference, the subsequent adult weight and hind tibia were higher on V- host plant than on V+ host plants. Pupal weight was significantly higher when feeding on whole V+/V- host plants as compared to the leaf-discs (weight of pupa on V+ plant: 4.10±0.58 (on leaf-disc), 6.65±1.03 (natural feeding), Man-Whitney U test p=0.000; weight of pupa on V- plant: 4.47±0.95 (on leaf-disc), 6.63±0.86 (natural feeding), Man-Whitney U test p=0.000;).

Table 2 Performance of the Diamondback moth developing on *V. longisporum* infected / uninfected *B. napus* plants

treatment		V+	V-	F value	p
pupa weight (mg)	♀	6.89±1.08 (38)	6.89±0.87(36)	0.00[b]	n.s
	♂	6.44±0.94 (43)	6.44±0.77(42)	0.00[b]	n.s
adult weight (mg)	♀	4.79±0.88(38)	4.83±1.06(36)	0.05[b]	n.s
	♂	3.27±0.74(43)	3.53±0.72(42)	2.58[b]	n.s
eclosion days (d)	♀	4.58±1.54(38)	5.47±1.66(36)	-2.53[a]	*
	♂	5.10±1.74(43)	4.75±1.39(42)	-0.85[a]	n.s
length of hind tibia (mm)	♀	2.34±0.16(38)	2.42±0.16(36)	5.05[b]	*
	♂	2.24±0.18(43)	2.34±0.13(42)	10.7[b]	**

Numbers in parentheses give samples sizes; for hind tibia of female, one-way ANOVA was used while for hind tibia of males, data were transformed by lg10, and SQRT for normal distribution. Eclosion day was analysed with the Man-Whitney U test. a: Man-Whitney U test, b: F value in one way ANOVA (p>0.05 n.s: no significant difference; 0.01<p<0.05. significant difference*; p<0.01, high significant difference)

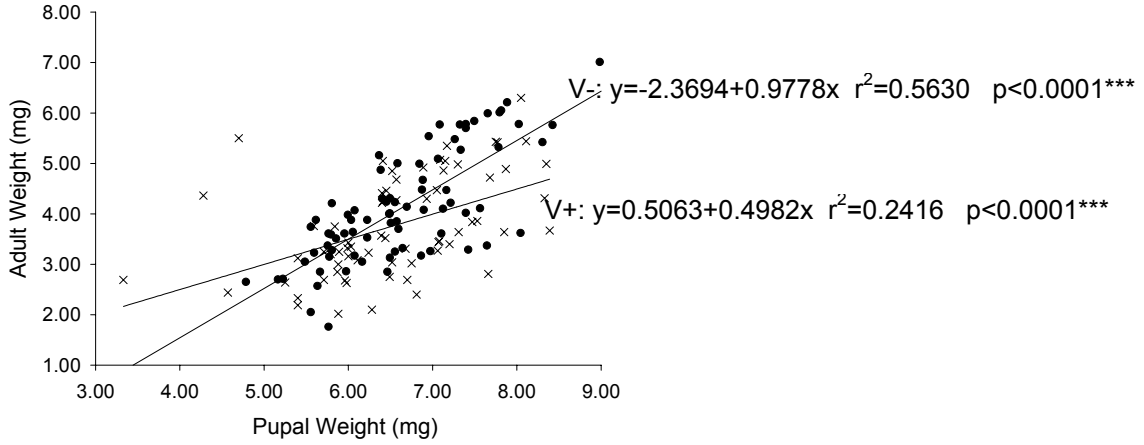

Figure 2 Correlation of pupal and adult weight of *Plutella xylostella* fed on *Verticillium longisporum* infected (x) or control *Brassica napus* (dots) plants during the larval stage. Verticillium infections did not influence the significant correlation between pupal and adult weight (V+: y=0.5063+0.4982x r^2=0.2416 p<0.0001; V-: y=-2.3694+0.9778x r^2=0.5630 p<0.0001). X: Verticillum infection; Dots: Controls.

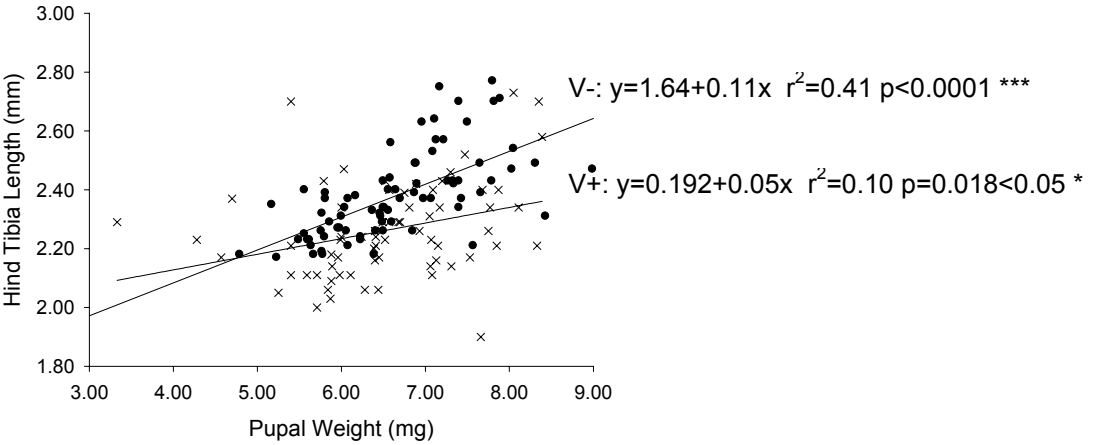

Figure 3 Correlation between pupal weight and the length of the hind tibia of *P. xylostella* feeding on *V. longisporum* infected or control *B. napus* plants during larval stage. Verticillium infection did not influence the significant correlation between pupal weight and hind tibia length in adults (V+: y=0.192+0.05x r^2=0.10 p=0.018; V-: y=1.64+0.11x r^2=0.41 p<0.0001). X: Verticillum infection; Dots: Controls.

3.3.3 GC-MS/EAD

No significant differences with regard to IVOCs were detected. Eight dominant constitutive volatile compounds were quantified and identified emanating from the aerial parts of V+/V- oilseed rape plants. More volatiles were emitted by Verticillium infected oilseed rape, especially 1,2,4-trimethyl- benzene and 3,5-Dimethyl-[1,2]dithiolane 1,1-dioxide. Diamondback moth obviously responsed to seven substances identified, specifically 3,5-Dimethyl-[1,2]dithiolane 1,1-dioxide. At the retention time at 21-22 min, females obviously responded to this compound, however, no corresponding compound was detected in the samples with regard to the same treatments (Table 3 and Figure 4).

Table 3 Quantity of volatile compounds emitted by *Verticillium longisporum* inoculated and control oilseed rape plants and responses of Diamondback moth adults (*Plutella xylostella*) (N=9 plants/treatment; mean ± SE, (umol/g/h))

Peak No.	Retention Time (min.)	VOCs	infected plant	control plant	$F_{(1,16)}$ or χ^2	p	EAD
1	9.30	Butanoic acid, 3-methyl-2-[(phenylmethoxy)imino]-, trimethylsilyl ester	14.27±2.05	9.91±1.35	3.13[a]	n.s	+
2	10.16	1,2,4- Benzene trimethyl-	29.63±8.55	9.24±2.25	18.0[b]	0.047*	+
3	12.0	Tetradecane/nonanal	4.20±1.69	2.87±1.22	33.0[b]	n.s	+
4	13.5	2-ethyl-1-Hexanol	13.25±2.99	7.03+1.84	3.12[a]	n.s	+
5	13.8	Pentadecane or Decanol	4.86±1.70	3.98±1.44	33.0[b]	n.s	+
6	16.4	Acetophenone	2.76±0.64	1.57±0.50	2.15[a]	n.s	+
7	17.8	Naphthalene	4.47±0.87	3.03±0.80	1.46[a]	n.s	+
8	22.6	3,5-Dimethyl-[1,2] dithiolane 1,1-dioxide	6.70±1.79	2.30±0.93	4.76[a]	0.044*	-

a: ANOVA test, b: Man-Whitney U test; Peak numbers correspond with assignments in Fig. 3;
VOCs : volatile organic compounds (Mean±SE); A "+" in the last column indicates that the respective compound elicits a response in the EAD signal. A"-" in the last column indicates that the respective compound does not elicits a response in the EAD signal.

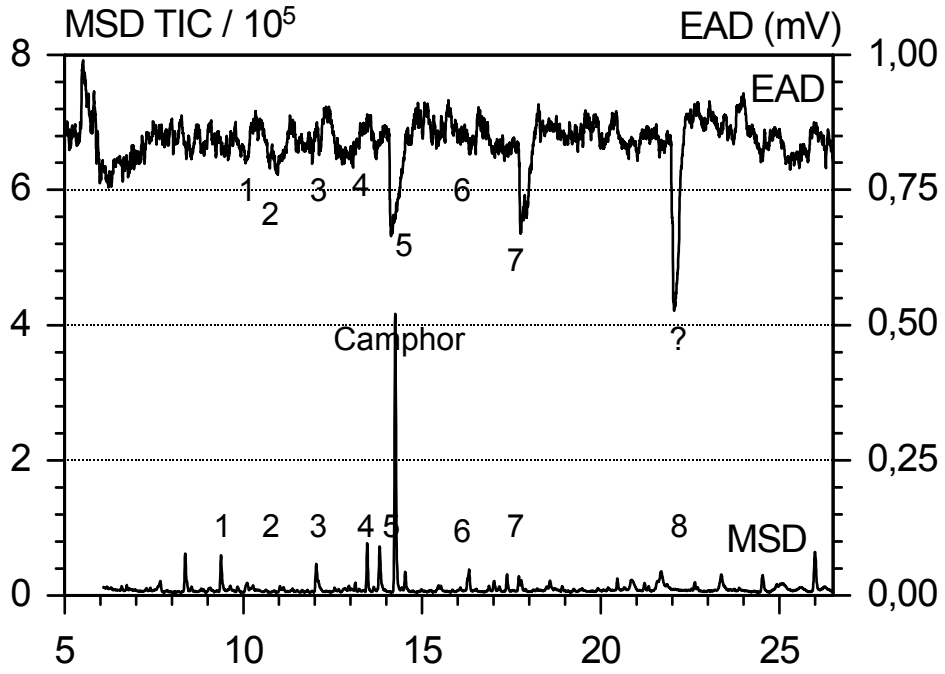

Figure 4 GC-MS/EAD chromatogram of volatiles sampled from *Verticillium* infected *Brassica napus*. The upper line represents the EAD signal of a female diamondback moth, and the lower line depicts the MS-TIC (total ion current). Numbers assign the peak numbers as listed in Table 3.

3.3.4 Phytosterol profiles

Four major phytosterols were analysed and summed up to obtain the total phytosterols content: Brassicasterol, Campesterol, Stigmasterol and Sitosterol.

Verticillium infection significantly enhanced the sum of phytosterols in the leaves, in particular the amount of Sitosterol, the most common sterol in both treatments, followed by Brassicasterol, Campesterol and Stigmasterol. Regarding the relative sterol content, in leaves of Verticillium-infected plants, the proportion was 64.7% Sitosterol, 16.0% Brassicasterol, 13.0% Campesterol and 6.3% Stigmasterol; in control plants, the composition was 63.3% Sitosterol, 15.8% Brassicasterol, 14.9% Campesterol and 6.0% Stigmasterol; Npar Man-Whitney was used for analyzing the relative content of phytosterols; no significant differences were found.

V+ leaves contained significantly more Sitosterol and the sum of phytosterols was significantly enhanced as compared to V- leaves of *B. napus*. However, no significant differences for the phytosterol composition were detected (Table 4)

Table 4 Phytosterols content (ug g^{-1} leaf) and relative composition in the leaves of *Brassica napus* inoculated/uninoculated with *Verticillium longisporum.* Mean content of Sitosterol and sum of phytosterols were analyzed using one-way ANOVA (Sitosterol: $F_{(1,18)}$=6.913, p=0.017<0.05*; sum of phytosterol, $F_{(1,18)}$=9.068, p=0.007<0.01**; ns: not significant). The content of Brassicasterol, Campesterol, Stigmasterol and relative content were analyzed using the Man-Whitney U test.

phytosterol in *Brassica napus* leaf	**V+**	**V-**	***P* value**
mean of brassicasterol	53.18 ± 7.78	45.84 ± 16.29	n.s
mean of campesterol	43.35 ± 6.84	43.68 ± 11.66	n.s
mean of stigmasterol	20.87 ± 7.22	17.18 ± 4.93	n.s
mean of sitosterol	215.92 ± 25.18	185.67 ± 26.26	*
Sum of phytosterol	333.32 ±31.64	292.37 ± 29.13	**
relative content of brassicasterol	0.16 ± 0.020	0.158 ± 0.055	n.s
relative content of campesterol	0.13 ± 0.014	0.149 ± 0.032	0.075
relative content of stigmasterol	0.063 ± 0.025	0.060 ± 0.020	n.s
relative content of sitosterol	0.647 ± 0.028	0.633 ± 0.041	n.s

3.4 Discussion

Preference of larvae and adults for V+/V- *B.napus*

Pathogen-induced variation, quantitatively and qualitatively, has been documented for volatiles and non-volatiles in the secondary metabolism in some studies (Huang *et al*., 2003). Recent findings demonstrated that the content of glucosinolates in the leaves of oilseed rape (*Brassica napus*), infected by the dark leaf spot pathogen (*Alternaria brassicae*) was altered (Doughty *et al*., 1991) and the composition of flavonoid mixtures extracted from white clover (*Trifolium repens*) inoculated with the mycorrhizal fungus *Glomus intraradices* were changed as well (Ponce *et al*. 2004; Treutter, 2006). Within a certain concentration range, the isothiocyanates (hydrolysis products of nonvolatile glucosinolates) and flavonoids acted as the active stimulants for Diamondback moth feeding and oviposition (Nayar & Thorsteinson, 1963; Reed *et al*., 1989; Renwick *et al*., 1992; Pivnick *et al*., 1994; Fahey *et al*., 2001; van Loon, *et al*., 2002). Given this theoretical basis, it was hypothesized that the preference of Diamondback moth larvae and females should be influenced by a *V. longisporum* infection in oilseed rape plants. The results show that more eggs were deposited on

uninfected oilseed rape as compared to *V. longisporum* infected plants, and the larvae displayed the same preference pattern.

Visual and olfactory cues (volatile and/or leaf-surface chemicals) are the two main crucial factors for herbivorous insects to land and oviposition (Renwick *et al*., 1992; Renwick & Chew, 1994; Bernays & Chapman, 1994). In this study no significant morphological differences between V+/ V- oilseed rape plants were found, thus visual factors may be excluded (Li, unpublished data). Eight similar constitutive volatile organic compounds (VOCs) emanating from V+/V- oilseed rape were identified, with detectable differences in quantity and relative proportions. More volatile compounds associated with plant resistance were emitted by *V. longisporum* infected plants, such as Acetophenone, Butanoic acid, 2-ethyl-1-Hexanol which inhibited the spread of the pathogen into the plant tissue (Nonomura *et al*., 2001; Holopainen, 2004; Baker *et al*., 2005; Fernandoa *et al*., 2005). Insects are able to distinguish between volatiles both qualitatively and quantitatively (Visser, 1986). The only compound with a significant quantitative difference was Benzene, 1,2,4-trimethyl- which induced responses of the herbivore in this study. It was documented that a certain class of substituted benzene compounds are effective repellents for many insects, including some species of in Lepidopetra (Inazuka & Tsuchiya, 1980). The considerable amount of benzene released by V+ oilseed rape plants might explain why the Diamondback moth larvae and adults avoided the infected plants. Although there was a significant difference of the volatile content of 3,5-Dimethyl-[1,2] dithiolane 1,1-dioxide, no corresponding response in Diamondback moth females was found. Avoidance of plants infected by pathogens was found in many instances including specific insects, plants and pathogens (Kanda *et al*. 1994; Kuress, 2002). However, the reverse, a feeding and oviposition preference for infected plants, was reported as well, for example in the beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), which preferred to oviposite on peanuts (*Arachis hypogaea* L.) infected by the white mold fungus *Sclerotium rolfsii* Saccodes, mediated by secondary metabolisms induced by fungal infection (Cardoza *et al*., 2003).

Performance of insect on V+/V- *B.napus*

By means of real-time PCR we have been able to demonstrate a successful infection of the plant roots by the pathogen. A higher amount of fungal DNA of *V. longisporum* was also detected in leaves of *B. napus* at 35 dpi (days post-inoculation) (Eynck *et al*., 2007). However infected plants might contain not yet infected plant tissues, as has been found for young leaves in *Rumex obtusifolius* infected by the rust fungus *Uromyces rumicis* (Hatcher *et al*., 1997). This points to two potential interactions between the vascular wilt fungus and the Diamondback moth larvae, including a direct contact by ingesting fungal DNA during feeding on infected leaves from inoculated plant and an indirect contact by feeding on leaves without fungal DNA from inoculated plants.

Given either a direct or indirect contact of the larvae with the fungus, we show a negative effect of the *V. longisporum* infection on the Diamondback moth, such as a high mortality of larvae and a short hind tibia length of adults, potentially relating to a low fertility. Simultaneously, some developmental indices were not significantly impacted by the *V. longisporum* infection, such as the weight gain of larvae feeding on leaf-discs from inoculated plant within 72 h, the pupal or adult weight. In terms of the remarkable effect (i.e. high mortality) during the larval stage, we speculate that a *V. longisporum* infection of the plants on Diamondback moth performance is particularly effective during a specific developmental stage of the insect. This argument is corroborated by the results of a related experiment, in which during the first 10 days Diamondback moth larvae suffered from increased mortality when feeding on leaf-discs from cabbage inoculated with a soil-borne endophyte (*Acremonium alternatum*), however pupal or adult weight was not significantly different (Raps & Vidal, 1998). A higher larval mortality when feeding on host plants infected by soil-borne fungi than on control has been documented in other studies as well, for instance the cotton bollworm *Helicoverpa armigera* and greenhouse whitefly nymphs *Trialeurodes vaporariorum* (Westw.) feeding on tomato plants *Lycopersicon esculentum* Mill. infected by *Acremonium alternatum* or *A. strictum* (Vidal, 1996; Jallow *et al*., 2004, 2008;) and the black vine weevil larvae *Otiorhynchus sulcatus* (Fab.), the fall armyworm (*Spodoptera frugiperda*) and the corn earworm (*Heliothis zea*) feeding on host plants infected by arbuscular mycorrhizal (*Glomus mosseae* and *Glomus fasciculatum*) (Pacovsky *et al*., 1985; Gange *et al*., 1994; Gange, 2001). To date few studies have investigated the impact of vascular wilt on insect performance, among which the growth of the spider mite *Tetranychus urticae*

displayed retarded feeding on cotton seedlings inoculated with *Verticillium dahliae* (Karban *et al*., 1987). However the pathogen *Verticillium albo-atrum* (Rke et Berth.) had negligible effects on the growth of the southern armyworm *Spodoptera eridania* (Cram.) reared on alfalfa (Kingsley *et al*., 1983).

In summary, all organisms involved in the interaction of phytophagous insects, host plants and microorganisms contributed to the net effect of the microorganism's on insect performance mediated via their host plant (Barbosa 1991). In our experimental systems, two reciprocal bioassays of Diamondback moth performance resulted in different results with regard to larval mortality, accordingly the practicability of the assays used should been taken into account when discussion the results.

Feeding on V+ host plants significantly reduced the time from pupal to female adults. This finding is in agreement with the observations on cabbage root flies (*Delia radicum)* or cabbage aphids (*Brevicoryne brassicae)* reared on *V. longisporum* infected oilseed rape plants (Li, unpublished data). The underpinning mechanism is regarded to be nutritional stress for larvae feeding on inoculated plants since the pupae and eclosing adults are smaller in size and lighter in weight than on control plants.

Phytosterols

Development of herbivorous insects depends not only on the qualitative but also on the quantitative composition of nutritional compounds, and any departure from the normal composition might cause pests to suffer from impaired metabolic and other nutritional troubles (House, 1969). Cholesterols are essential compounds used by insects to build up membranes and for the production of ecdysteroids, the molting hormones (Zandee, 1962; Clayton, 1964; Svoboda & Thompson, 1985). Unlike vertebrates, however insects can not biosynthesize their sterols *de novo;* the only source comes from the diet or from symbionts (Hobson, 1935a, b; Ritter, 1984; Douglas, 1988; Ba *et al*., 1995). By rearing insects on different phytosterols singly and in an array of different composition, phytosterols can be classified as suitable or unsuitable (utilizable/nonutilizable) (Nes *et al*., 1997; Behmer & Elias, 1999a, 2000).

In this study, three ubiquitous phytosterols (i.e. Sitosterol, Campesterol, and Stigmasterol) typical for flowering plants (Nes, 1977; Behmer & Nes, 2003) and one

specific phytosterol in the *Brassicaceae* family (i.e. Brassicasterol) (Amar, 2007) were found and the quantity and composition in infected and uninfected plant leaves were measured. We showed that Sitosterol was the most dominant phytosterol in quantity, followed by Brassicasterol, Campesterol and Stigmasterol in leaves of both treatments. Remarkably, the content of Sitosterol and the sum of all phytosterols measure were significantly higher in V+ than in V- leaves of *B. napus*. Moreover, the quantity and relative proportion of Stigmasterol was slightly higher in Verticillium infected leaves of oilseed rape plants than the control.

To date many studies have documented the positive effects of Sitosterol for supporting the growth and reproduction of many species of insects, such as the corn earworm *Heliothis zea* (Lepidoptera: Noctuidae) reared on an artificial diet (Nes *et al*, 1997, Behmer & Elias 2000). It has been demonstrated for the Diamondback moth that survival of larvae was highest on an artificial diet containing Sitosterol as the primary phytosterol, whereas the larvae survival decreased, larvae development and pupation retarded followed by a low fecundity of adult when feeding on a diet containing Stigmasterol (Behmer & Grebenok, 1998). These previous finding supported that Sitosterol can be regarded as a non-utilizable sterol whereas Stigmasterol as a utilizable sterol. The former could not be metabolized efficiently to cholesterol because of constraints with regard to the configuration (Dadd, 1960; Ritter, 1984; Behmer & Elias, 1999b, 2000). By containing more Stigmasterol in Verticillium infected leaves, impaired insect performance feeding on infected leaves could be partially explained by this finding. However the finding of more sitosterol in infected leaves is confusing. A potential explanation may come from another previous study in which it has been demonstrated that the content of Brassicasterol in leaves of Brussels sprouts inoculated with the soil-borne endophyte *Acremonium alternatum* correlated positively and the content of Sitosterol negatively with larval weight of Diamondback moth. The authors proposed to classify Brassicasterol as an utilizable and Sitosterol as a non-utilizable sterol (Dugassa-Gobena *et al*., 1998). This finding may explain the negative effect on Diamondback moth larvae feeding on infected plants containing a higher amount of Sitosterol.

Several studies have also documented the negative impact of the phytosterol composition on insect development (Richter *et al*. 1987; Bodnaryk *et al*., 1997; Nes, *et al*., 1997). However in our study no significant differences with regard to the phytosterol composition was detected in leaves from inoculated plants, which to

some extent accounted for the small differences in pupal or adult weight. However we are unable to explain the significant differences in adult size on the treated plants.

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Chapter 4

Preference-performance of the cabbage aphid (*Brevicoryne brassicae* L.) feeding on oilseed rape inoculated with soil-borne fungi (*Acremonium alternatum* or *Verticillium longisporum*)

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Abstract

Recently the role of micro-organisms as mediators of plant-herbivorous insects interactions has been increasingly acknowledged in ecological research. We investigated the interactions between two soil-borne fungi, i.e. the unspecialized root fungal endophyte *Acremonium alternatum* and the vascular fungal pathogen *Verticillium longisporum* and the cabbage aphid *Brevicoryne brassicae* L. (Homoptera: Aphididae) in greenhouse bioassays and under field conditions. Specifically, we examined the systemic effects of the endophytic fungus and the vascular pathogen on the host selection of cabbage aphids nymphs and the subsequent insect performance within three consecutive generations (F_1-F_3) on two oilseed rape varieties (*Brassica napus*, *B. napus* var.). The phytosterols profiles in the leaves of the host plants were determined qualitatively and quantitatively to understand the mechanisms of insect performance. A field experiment was established to test host-choice and performance of cabbage aphids to cabbage plants treated by the endophyte (E+) or Iprodione (control-CoI) or left untreated (control - CoII). In the laboratory, cabbage aphids nymphs significantly preferred to feed on leaf-discs cut from endophyte-free (E-) or pathogen-free (V-) host plants as compared to leaf-discs from plants inoculated with *A. alternatum* (E+) or *V. longisporum* (V+). However, cabbage aphids were incapable of distinguishing E+/E- or V+/V- treated intact host plants, a result in agreement with field results. No significant difference in the number of nymphs produced by cabbage aphids feeding on E+/E- host plants and the subsequent development time from nymph to adult were found throughout F_1-F_3 generations. No significant differences in the carbon/nitrogen-ratio were detected in the young leaves of E+/E- cabbage plant in the field experiment. Significantly lower relative proportions of brassicasterol in E+ *B. napus* plants were detected relating to the slightly retarded development of the associated aphids from nymphs to adults. Although a significantly shorter development time from nymph to adult was found in V+ *B. napus* var., the number of nymphs produced by adults feeding on the V+/V- host plant was not significantly different. Based on these findings, we conclude that the ability of the cabbage aphids to select suitable inoculated/non-inoculated host plants depended on the pattern of host plants provided. The soil-borne fungi (*A.alternatum* or *V.longisporum*) had negligible impact on the cabbage aphids' fecundity, however *V. longisporum*

infections significantly accelerated the development of cabbage aphids from nymph to adults.

Key words: *Brevicoryne brassicae* L.; soil-borne fungi; preference/performance; phytosterols;

4.1 Introduction

It is commonly accepted that herbivorous insect performance depends on plant nutrient supply (Harvey *et al*., 2003; Wittstock *et al*., 2004; Johnson *et al*., 2006). However, there is now increasing evidence that metabolites produced by the host plants may be altered when infected by fungi (Bowden *et al*., 1990; Costa Pinto *et al*., 2000; Arnold & Engelbrecht 2007). Under field conditions most plant species are regularly infested with these organisms resulting in more complex multispecies interactions such as herbivore-host plant-fungi-interactions, rather than simple bilateral interactions typically investigated in the majority of papers (Hammon & Faeth, 1992; Hatcher, 1995a; Gang & Bower, 1997; Hatcher *et al*., 1997; Azevedo *et al*., 2000).

Endophytic fungi are species occurring within living tissues of plants, without causing visible disease symptoms at a particular time (Stone *et al*., 2000). To date, fungal endophytes were found in almost all plant species worldwide, with an extremely high diversity and abundance in the tropical trees and herbaceous plants (Arnold *et al*., 2001; Seena & Sridhar, 2004; Gange *et al*., 2007; Li *et al*., 2007). The ecological role of endophytes colonizing grasses has bee studied in detail, and, to a less extent, in trees (Gaylord *et al*., 1996; Saikkonen *et al*., 1996, 1998; Wilson & Carroll, 1997; Arnold *et al*., 2003). Many studies argued for a mutualistic role of these grass endophytes by receiving shelter and nutrients from their host plants and in return conferring host plant resistance to herbivores and tolerance to abiotic stresses (West *et al*., 1990; Bacon, 1993; Breen, 1994; Saikkonen *et al*., 1996; Clement *et al*., 1997). However, this mutualism is not regarded universal due to some antagonistic roles of endophytes in host plants (Saikkonen *et al*., 2004). Grass endophytes are specific to their host plants in being vertically transmitted via plant seeds and in being able to produce toxic substances (e.g. alkaloids) which contribute to the herbivore resistance of the infected plants (Dahlman *et al*., 1991; Faeth, 2002; Gimenez *et al*., 2007). In contrast to these intensively studied grass endophytes, unspecialized endophytes in forests and crops received less attention, among which *Acremonium spp*. are of particular interest because of their frequent occurrence in endophyte assemblage surveys (Collado *et al*., 1999; Kumaresan & Suryanarayanan, 2001; Grunewaldt-Stöcker & von Alten, 2003). Some *Acremonium spp*. strains are known

to induce resistance to pathogen infections in their host plants (Wicklow *et al*., 2005). The *Acremonium spp*. are unspecialized fungi, horizontally transmitted via spores and do not produce toxic compounds (Clay, 1988; Raps & Vidal, 1998). In this study, the soil-borne fungus *Acremonium alternatum* was used. Due to inhabiting the intercellular spaces of host plants, the associations between endophytes and their host plants are less intimate as compared to pathogenic fungi (Carlile *et al*., 2001).

Verticillium wilt is an increasing problem on Brassica oilcrops in northern European countries (Svensson & Lerenius, 1987; Zielinski & Sadowski, 1995). On oilseed rape (*Brassica napus* L. spp. *oleifera*) (**OSR**) this disease is triggered by *Verticillium longisporum*, a host-specific vascular fungal pathogen (Karapapa et al., 1997; Zeise & von Tiedemann, 2001). The typical symptoms induced by *V. longisporum* on host plants include necrosis and chlorosis of the leaves, stunting of plant growth, ripening and premature senescence (Bhat & Subbarao, 1999; Steventon *et al*., 2002; Veronese *et al*., 2003; Babadoost *et al*., 2004; Zhou *et al*., 2006). Morphological and/or physiological changes in the host plants induced by the pathogen infection may influence host-choice of herbivorous insects (Pfunder & Roy, 2000; Kellogg *et al*., 2005). For example, when fed on these fungal infected plants, aphids underwent an induced cascading effect resulting in being beneficial (Moran, 1998; Gange *et al*., 1999; Johnson *et al*., 2003), detrimental (Hatcher *et al*., 1994, 1997; Kruess, 2002) or neutral performance (Kingsley *et al*., 1983) with regard to the performance of insect specific species involved in these interactions (Barbosa, 1991; Gange, 2001). However, only few studies evaluated the impact of *Verticillium* spp. on insect performance. One study found negligible effects of a *Verticillium albo-atrun* infection on the southern armyworm *Spodoptera eridania* (Cram.) on alfafa (Kingsley *et al*., 1983). Another study showed a negative effect on the spider mite *Tetranychus urticae* (e.g. the delayed growth) feeding on *Verticillium dahliae* inoculated cotton seedlings (Karban *et al.*, 1987).

The cabbage aphid *Brevicoryne brassicae* (L.) (Homoptera: Aphididae) is an important specialist pests on plants of the family Brassicaceae (=Cruciferae) grown in temperate and warm climate zones (Minks & Harrewijn, 1988; Cole & Lynn, 1996; Gabrys *et al*., 1997; zhang & Hassan, 2003). They prefer to feed on newly emerged plant tissues causing deformations of the heads and flowers and resulting in yield losses (Bakhetia, 1983; Costello & Altieri, 1995; Kelm, & Godomski, 1995; Gabrys *et al*., 1998). Aphids are not only able to damage plants, but also cause plant diseases

by transmitting plant viruses (Hodgson, 1981; Masterman, *et al*., 1994; Nebreda *et al*., 2004).

It has been demonstrated, that the honeydew produced by aphids can be easily consumed by epiphytic microorganism on leaves of forest trees, promoting the growth of microbes (Stadler & Müller, 2000). These findings highlight the role of aphids for microorganism. Other studies have shown the positive effects of a pathogen infection on aphid performance, resulting in enhanced weight and reproduction (Moran, 1998; Gange, 1996; Gange *et al*., 1999; Johnson *et al*., 2003). However, the development of the aphid *S. graminum* was not influenced by a mycorrhizal fungi (*Glomus fasciculatum*) on sorghum (Pacovsky *et al*., 1985), pointing to some evidence that the effect of mycorrhizae on insect performance is related to the feeding pattern of the insects: chewing insects performed worse as compared to phloem-sucking insects on plants infected by mycorrhizae (Gange & West, 1994; Gange *et al*., 1994). In contrast to these reports, an inoculation of the plants with *A. strictum* resulted in changes in the preference and performance of the greenhouse whitefly *Trialeurodes vaporariorum* (Westw.), e.g. a higher mortality and retarded development o the larvae (Vidal, 1996). A significant negative impact of an *A. alternatum* or *A. stritum* inoculation on insect performance was also documented in Lepidopteran insects, such as the Diamondback moth *Plutella xylostella* L. and a polyphagous moth (*Helicoverpa armigera*) (Raps & Vidal, 1998; Dugassa-Gobena *et al*., 1998, 2003; Jallow *et al*.,2004, 2008;).

We hypothesized that the preference and performance of the cabbage aphids is mediated by an inoculation with either the soil-borne endophyte, *A. alternatum* or the plant pathogen *V. longisporum*. Because the effect of a microorganisms infection may be transmitted vertically to next generations as observed in the cabbage root fly (Li, unpublished data), we followed insect performance (i.e. the fecundity and the development time from nymph to adult) for three consecutive generations.

So far only few studies integrated laboratory and field experiments (Gange & West, 1994; Gange, 1996; Moran, 1998). We therefore set up a field experiment in order to compare the results obtained in the laboratory with field data.

Volatile profiles emitted from host plants may be altered by pathogen inoculations (Huang *et al*., 2003), resulting in different cues for herbivores to find their host plants (Cardoza *et al*., 2003; Jallow *et al*., 2008). We used a dual-choice bioassay for cabbage aphid nymphs to test for the effects of mechanically damaged leaf-discs

using either pathogen or endopyhte infected (V+/E+) and uninfected (V-/E-) oilseed rape leaves on host plant choice.

In addition to changes in the secondary metabolism, changes in the primary metabolism of host plants due to fungal infections render these plants less nutritionally suitable for herbivores. Fungal infections resulted in changes in the carbon/nitrogen balance and carbohydrate levels (Jones & Last, 1991; Hatcher, 1995b; Costa Pinto *et al*., 2000) and the content and relative proportion of phytosterols (Bernays, 1993; Dugassa-Gobena *et al*., 1998, 2003). Cholesterol is a essential substance needed by insects for membrane molting hormone production (Clayton, 1964; Svoboda & Thompson, 1985). Insects are not able to biosynthesize sterols *de novo*; they depend on either plants or symbionts (Ritter, 1984; Douglas, 1988). Several studies documented the negative impact of changes in the phytosterol composition on insect development (Richter *et al*. 1987; Bodnaryk *et al*., 1997; Nes, *et al*., 1997). We therefore hypothesized that phytosterol profiles are influenced in infected / uninfected host plants. We analyzed the quantity and composition of three dominant phytosterols in flowering plants (i.e. sitosterol, campesterol and stigmasterol) (Nes, 1977; Behmer & Nes, 2003) and one specific phytosterol of the *Brassicaceae* family (i.e. brassicasterol) (Amar, 2007) in infected and uninfected plant to elucidate the underlying mechanism of the impact caused by soil-borne fungi on insect performance.

4.2 Material and Method

Laboratory experiment

Plants

Oilseed rape plants (*Brassica napus* L. (cv Falcon) and *Brassica napus var*.(cv Licosmos) (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG) were used in this study. Seeds were sowed and grown in plastic pots (diameter: 11 cm) in the greenhouse [24±3°C, 80% relative humidity (r.h.) under 16L : 8D photoperoid] for one week. The young seedlings were transplanted to same sized plastic pots with a substrate comprising soil and sand (ökohum®-Rindentorfsubstrate, Type RTS; Ratio:

2:1) and were grown for another 3 weeks in the greenhouse. Water was applied daily and fertilizer was applied weekly (Wuxal® Super (10-10-8 w/v))

Fungus

The root endophyte, a strain of *Acremonium alternatum* (CBS 831.97) from the Netherlands was maintained on potato dextrose agar (PDA) in the laboratory. For production of a spore suspension, the autoclaved potato dextrose broth (PDB) was inoculated with two pieces of PDA agar containing the fungal mycelia. The flask was kept at 23°C on a rotary shaker at 100rpm for approximately three weeks to ensure fungal growth and sporulation. The spore suspension was filtrated under vacuum and the concentration of spore suspension was adjusted using a Thoma counting chamber (Marienfeld, Germany) (depth 0.1mm, square width: 0.05mm) under a microscope. The spore suspension was finally adjusted by adding tap water to 10^6 conidia mL^{-1}.

Verticillium longisporum, isolate VL 43, originated from field soil with *B. napus* in the north of Germany. The isolation method and fungus cultivation are described in detail by Zeise and von Tiedemann (2001, 2002 a,b). To maintain the pathogenicity, the spore suspension (10^6-10^7 conidia mL^{-1}) was divided into 1.5 ml Eppendorf cups with Czapek Dox medium supplemented with 25% glycerol at -20°C. For propagation, droplets of these suspensions were plated onto potato dextrose agar (PDA) and incubated for three weeks at 21°C in darkness. Spore suspensions were obtained by gently adding some distilled water to the plates, harvested using a hair-brush. Inoculum for root dipping inoculations was produced by adding 500 µL of the spore stock suspension to 250 mL potato dextrose broth (PDB), grown for three weeks in darkness at 23°C on a rotary shaker at 100rpm. The concentration of the conidia suspension was adjusted using a Thoma counting chamber (Marienfeld, Germany) (depth: 0.1mm, square width: 0.05mm) under a microscope.

Aphids

Apterous cabbage aphids *Brevicoryne brassicae* were collected in a private garden in Goettingen in 2005 and mass reared on cabbage plants in a climate controlled insectary (temperature: 21°C±1°C, 70±5% r.h. with a photoperiod: 16h light: 8 h dark). The cabbage plants were replaced every three days.

Protocol for the dual-choice experiment using cabbage aphid and E+/E- V+/V- leaf-discs of the host plants

Three weeks old plants (*Brassica napus/Brassica napus var.*) were watered with 70ml of a conidial suspension (10^6 conidia ml^{-1}) of *Acremonium alternatum* or *Verticillium longisporum*; the same amount of sterilized culture filtrate was used for control plants. Two weeks after inoculation the plants were used for the experiments. The experiment was set-up using Petri-dishes (Ø: 14 cm) with ventilated gauze (Ø: 6 cm) at the center. Infected ivided into infecte Control +), a control (V- or E-) and a searching area, respectively. Considering that the distribution and concentration of mycelia might not be homogeneous within and among leaves (Keogh *et al*., 1996) in this experiment, ten pieces of leaf-discs (Ø: 2 cm) cut from the third and forth leaf were placed in each part of the Petri-dishes. Ten aphid nymphs (II-III stadium) produced by three maternal aphids and experiencing no-food for 3 hours in isolated Petri-dishes were introduced through a small tip cup (2ml) at the centre of the Petri-dish (Figure 1). Aphids entering either the infected or the uninfected area were recorded as experiencing a positive choice; otherwise we regarded their behavior as no choice. This experiment last 180min. The Petri-dishes were turned clockwise 90° every 15 min to correct for daylight influences. The experiment was replicated 10 times in an environment-controlled room (temperature: 21°C±1°C, 70±5% r.h.).

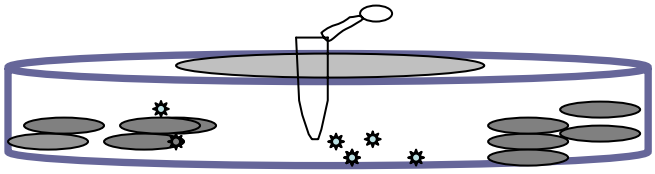

Figure1 Dual-choice experimental set up for cabbage aphid (*Brevicoryne brassicae* L.) nymphs to test for discrimination between leaf-discs either infected by *Acremonium alternatum*/*Vertillium longisporum* or controls on *Brassica* spp. leaves

Protocol for the dual-choice experiment using cabbage aphid and E+/E- V+/V- intact host plants

Three weeks old plants (*Brassica napus/Brassica napus var.*) were watered with 70ml of a conidial suspension (10^6 conidia ml^{-1}) of *Acremonium alternatum* or

Verticillium longisporum; the same amount of sterilized culture filtrate was used for control plants. Two weeks after inoculation the plants were used for the experiments. The experiment was set-up using Petri-dishes (Ø: 9 cm) with ventilated gauze (Ø: 4 cm) at the center and the edge of lid was attached with a layer of sponge. The arena was divided into infected area (V+ or E+), a control (V- or E-) and a searching area along the edge of gauze, respectively. The third leaf from infected (E+/V+) or uninfected plants (E-/V-) was clasped between the Petri-dishes on a transparent prexiglass cylinder. The two leaves did not have contact to each other, and it was taken care that the area included in the Petri-dishes was the same; the leaves were kept near to the bottom of the Petri-dishes. Ten aphid nymphs (II-III stadium) produced by 3 maternal aphids and experiencing no-food for 3 hours in isolated Petri-dishes were introduced through a small tip cup (2ml) at the centre of the Petri-dish (Figure 2). Aphids entering either the infected or the uninfected area were recorded as a positive choice; otherwise we regarded their behavior as no choice. This experiment last 180min. The Petri-dishes were turned clockwise 90° every 15 min to avoid a conflicting impact of light conditions. The experiment was run in an environment-controlled room (temperature: 21°C±1°C, 70±5% r.h.). Ten replications were carried out.

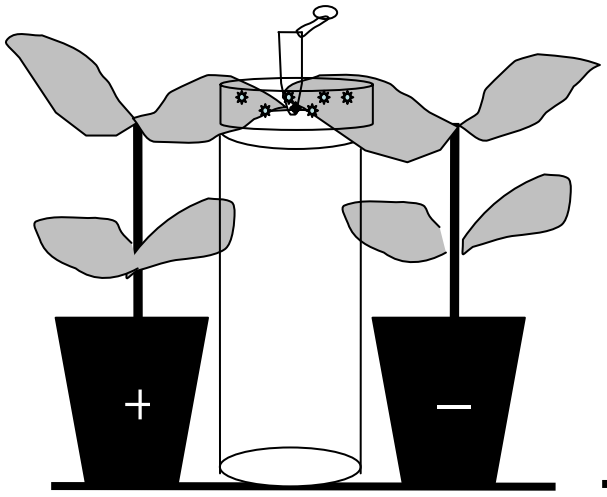

Figure 2

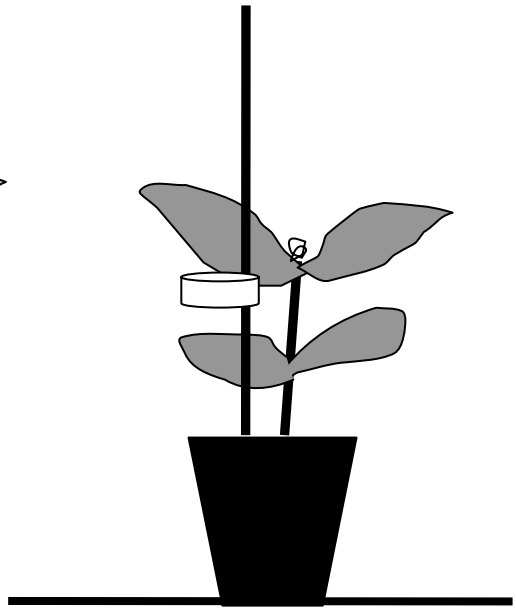

Figure 3

Figure 2 Set-up of the dual-choice experiment for cabbage aphids (*Brevicoryne brassicae* L.) nymphs to test for the discrimination between intact plants either infected by *Acremonium alternatum*/*Vertillium longisporum* or controls of *Brassica* spp.

Figure 3 Performance experimental set up for cabbage aphid (*Brevicoryne brassicae* L.) feeding on plant either infected by *Acremonium alternatum*/*Vertillium longisporum* or controls of *Brassica* spp.

Experimental protocol for development of cabbage aphid feeding on E+/E- *B.napus*/*B.napus* var.

Three weeks old plants (*Brassica napus/Brassica napus var.*) were watered with 70ml of a conidial suspension (10^6 conidia ml^{-1}) of *Acremonium alternatum* or *Verticillium longisporum*; the same amount of sterilized culture filtrate was used for control plants. One week after inoculation the plants were used for the experiments. For investigating the effect of inoculation time on aphid performance, host plants, inoculated for four weeks, were used in another bioassay with the same experimental design.

One aphid (I-II instar), produced by four wingless aphids, was introduced into one clip cage (3.5 cm × 1.5 cm). One leaf from a plant was clasped between the clip cages. Two replications were established on one plant. Aphid progeny of the first generation (F_1) were removed from the cage and adults were left in the cage to record the number of nymphs produced within the 72 h (n_1). Only one nymph of the 1-2 instar of F_1 was left in cage and given time to develop to produce the second generation (F_2). The development days from the first generation to the second generation were recorded as d_1. Afterwards the young nymphs were removed from the cage and only the adults were left in the cage to record the nymphs laid in the next 96 h (n_2). Again only one nymph of the 1-2 instar of F_2 was left in cage and to allow development into the adult stage to produce the third generation (F_3). The development days from F_2 to F_3 were recorded as in d_2. Afterwards the young nymphs were removed from the cage and only one adult was left in the cage to record the nymphs laid in the next 96 h (n_3) (Figure 3). Leaves for aphid feeding were changed after one generation to minimize the damage to leaves and guarantee nutrients supply. The experiment was set up in an environment-controlled room (temperature: 21°C±1°C, 70±5% r.h. under photoperiod: 16 L:8 D). Thirty plants were used for each treatment.

Experimental protocol for development of cabbage aphid feeding on V+/V- *B.napus* var.

Three weeks old plants (*Brassica napus var.*) were watered with 70ml of a conidial suspension (10^6 conidia ml^{-1}) of *Verticillium longisporum*; the same amount of sterilized culture filtrate was used for control plants. Three weeks after inoculation the plants were used for the experiments.

One aphid (I-II instar) was introduced into one clip cage (Ø: 3.5cm), thereafter this clip cage was clasped onto the third or the forth leaf of the host plant. Two clip cages were used for one plant. Two aphids were allowed to acclimatize to the cage and to feed on the host plants, referred to as the acclimatized generation. While the acclimatized generation produced new nymphs, only one new-borne nymph (I-II instar) was left in the clip cages, referred to as the maternal generation. The development of the maternal generations were monitored, and when they became adults, the amount of new progeny produced within 96h was recorded for the first generation (F_1). The experiment was set up in an environment-controlled room (temperature: 21°C±1°C, 70±5% r.h. under photoperiod: 16 L:8 D). Fifteen plants were used for each treatment (Figure 3).

Field experiment

Plant and treatments

One week old cabbage seedlings *(Brassica oleracea var. viridis)* were transferred to plastic pots (diameter: 11 cm) with a substrate comprising soil and sand (Ökohum® - Rindentorfsubstrat, Type RTS; 2:1 ratio) and were grown in a greenhouse [24±3°C, 80% relative humidity (r.h.) under photoperoid 16L : 8D]. After two weeks the cabbage plants were transplanted to big plastic pots (volume: 2L). Water was applied daily and fertilizer weekly (Wuxal® Super (10-10-8 w/v))

The methodology of culturing *A. alternatum* was the same used in the laboratory experiments. In this experiment, two controls (CoI, CoII) were included. Control I was treated by a fungicide with the active ingredient Iprodione (Rovral®: Rhone-Poulenc) (concentration: 2g/1L tap water). Control II was treated with normal water. Inoculated plants were watered with 70 ml, 10^6 conidia ml^{-1} *Acremonium alternatum* (E+) or 70ml fungicide solution (2g/L, CoI) or watered using the same volume of tap water

(CoII). 45 pots were set up for each treatment. After one week of inoculation, 135 pots plants were transferred to the field.

Field condition

The fallow field used for the experiment was isolated from other crop fields and has not been used for growing crops the previous years. 15 fenced exclosures (each edge: 2.5m) were established randomly in the field. In each plot, nine pots of plants treated with the endophyte, fungicide, and tap water were placed, following a Latin square design. Plants were exposed in the field on 17. May 2005 and the number of cabbage aphids on each plant was recorded every Tuesday and Thursday from 23. May to 23. June, 2005. Thereafter all plants were harvested and analyzed in the laboratory. A second set of plants, grown as described previously, were placed in the field plots on 21 July, 2005. Data collection was carried out every Tuesday and Thursday from 01 August to 01 September, 2005. After each field experiment, all plants were harvested and analyzed in the laboratory.

Laboratory analysis

Carbon/nitrogen ratio

Four young leaves (from 2^{nd} to 5^{th}) of each plant were washed using tap water to remove insects and dirt and dried at 60°C in paper bags for one week. Thirty plants from each treatment were analyzed.
Dry samples were ground to find powder and sent to the Institut für Pflanzenbau und Pflanzenzüchtung in Göttingen for analyses of total nitrogen and total carbon content. The ratio of C/N was calculated using these data.

Phytosterol profiles

Three weeks old plants (*Brassica napus/Brassica napus var.*) were watered with 70ml of a conidial suspension (10^6 conidia ml^{-1}) of *Acremonium alternatum.* The

same amount of sterilized culture filtrate was used for control plants. The plants were used for phytosterol extraction after three weeks of inoculation.

Leaves were cut from plants and pulverized into fine powder in liquid nitrogen using a mortar and a pestle. 0.5-0.6 g plant material was transferred to a 40 ml glass bottle with a screw-cup. Phytosterols were extracted following the protocol described by Newton (1989) with modification as follows: 20ml mixed solvent (5 ml 10 M KOH, 15 ml 96% ethanol and 0.06 g pyrogallol) was added to each bottle containing plant material. An ultrasonic homogenizer (Sonoplus HG 2200/UW 2200, 200 W, 20 kHz, Bandelin GmbH, Germany) was used for 30 sec for completely cracking the cells and degassing. Therefafter the bottles were placed in a shaker water bath at 80°C for 2.5 h. After cooling the samples to room temperature, 40 µl cholesterol was added as an internal standard (Cholesterol 5 µg/µl chloroform, Merck GmbH, Germany). Phytosterols were extracted with 10 ml hexane for 3 times. After thorough shaking for 20 sec, deposited for 5-10 min to separate phases, the supernatant was transferred to a rotary glass. One ml distilled water was added to wash the fractions; the separated lower phase was sucked out. Samples were distilled with a rotary-evaporator at 42°C. The concentrated sterols were resolved in 1.5ml hexane and transferred to 2 ml Eppendorf cups. After centrifuging with 10,000 rpm for 10 min, the supernatant was transferred to a GC-vial and the hexane was evaporated overnight at 50°C in a thermal block. The concentrated sterols were resolved in 240 µl hexane and 80 µl N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)(Fluka/Riedel-deHaen GmbH, Germany) and incubated in a thermal block at 70°C for 20 min. After cooling down to room temperature, 100-150 µl of the extract was transferred to a GC-vial equipped with 300 ul inserts.

Phytosterol identification and quantification was based on the internal standard method (peak area and retention time). 1 µl of each sample with a split of 1:50 of extracted sterols were analyzed using a gas chromatograph (Shimadzu GmbH, Model: GC14/15A) with a flame ionization detector. The samples were run on a fused silica column (SPB-1; 1.3m x 0.32mm, 0.25µm film thickness, Supelco Inc.; Sigma-Aldrich, Germany). Helium was used as a carrier gas; the make-up gas was synthetic air with a linear velocity of 35cm/s. The temperature program was initially 3 min at 180°C then increase to 290°C at a heating rate of 10°C/min to keep 36 min. the total analyzing time is 50min. The detector temperature was 300°C. Peak areas were calculated using an integrator and the internal standard. A set of typical peaks

indicating phytosterols, such as brassicasterol, camphsterol, stigmasterol and sitosterol individually had been identified beforehand using GC-MS with synthetic sterols as referent. The phytosterol content in one gram plant material was calculated following this equator:

$$G = \frac{200 \times p_2}{p_1 \times g}$$

G: weight of unkown phytosterol in one gram plant material p_1: peak area of cholesterol, p_2: peak area of unkown phytosterol, g: weight of plant material

Total phytosterol in one sample was calculated by summing up all the four phytosterols.

Statistics

Computations were carried out using SPSS software (SPSS, 2000).

Dual-choice experiment

Repeated measures ANOVA was used to analyze the result with regard to the dual-choice of cabbage aphids to leaf-discs and intact leaves of infected/uninfected host plants.

Cabbage aphid performance experiment

On *B. napus* (7dpi): The numbers of nymphs of F_1 and the developmental duration were analyzed using a Mann-Whitney test. The nymphs of F_2 and F_3 were analyzed using one-way ANOVA. On *B. napus* var. (7dpi): The numbers of nymphs of F_1 and F_2, and the development time were analyzed using a Mann-Whitney test. The numbers of nymphs of F3 was analyzed using a one-way ANOVA. On *B. napus* var. (28dpi): The number of nymphs of F_1 and the development duration were analyzed using a Mann-Whitney test. The number of nymphs of F_2 and F_3 were analyzed using a one-way ANOVA.

Field experiment

Repeated measures ANOVA was used to analyze the results of the cabbage aphid populations. One-way ANOVA was used to analyze the ratio of carbon/nitrogen.

4.3 Results

4.3.1 Preference experiment

Cabbage aphids significantly preferred the leaf-discs from E- of *B. napus* ($F_{1,1}$= 7.402, P=0.014<0.05) and V- *Brassica napus* var.($F_{1,1}$= 8.631, P=0.009< 0.01), but did not discriminate between E+/E- or V+/V- on intact host plants (Table 1-3).

Table 1 Dual-choice of cabbage aphids (*Brevicoryne brassicae* L.) on leaf-discs and intact leaves of *Brassica napus* infected by *Acremonium alternatum* and controls (leaf disc: $F_{(1,1)}$= 7.402, P=0.014<0.05; intact leaf: $F_{(1,1)}$= 0.095, P=0.761, n.s: not significant).

Time series	leaf-discs of *B. napus*		intact leaf of *B.napus*	
	E+	E-	E+	E-
Min15	3.6 ± 2.4	3.7 ± 2.2	2.6 ± 1.6	3.9 ± 1.7
Min30	3.1 ± 2.2	3.9 ± 2.2	3.0 ± 1.2	4.3 ± 1.8
Min45	3.4 ± 2.6	4.0 ± 2.5	3.3 ±1.4	4.2 ± 1.6
Min60	3.6 ± 1.7	4.6 ± 1.6	3.9 ±1.2	3.7 ± 1.5
Min75	3.7 ± 1.8	5.2 ± 1.8	3.5 ± 1.7	3.5 ± 1.8
Min90	3.0 ± 1.5	5.6 ± 1.8	3.6 ± 1.7	3.8 ± 1.8
Min105	3.3 ± 1.3	5.8 ± 1.6	3.8 ± 1.8	3.5 ± 1.8
Min120	2.7 ± 1.6	6.2 ± 1.8	3.8 ± 2.0	3.3 ± 1.9
Min135	3.0 ± 1.3	5.5 ± 1.9	3.5 ± 1.9	3.4 ± 1.7
Min150	3.2 ± 1.1	5.9 ± 1.7	3.4 ± 2.1	3.3 ± 1.9
Min165	3.4 ± 1.7	5.7 ± 1.6	3.7 ± 1.9	3.7 ± 1.6
Min180	3.1 ± 1.5	5.1 ± 2.1	3.8 ± 2.1	3.8 ± 1.8
p-vaule	0.014*		n.s	

Table 2 Dual-choice of cabbage aphid (*Brevicoryne brassicae* L.) on leaf-discs and intact leaf of *Brassica napus* var. infected by *Acremonium alternatum* and controls (leaf disc: $F_{(1,1)}$= 2.064, P=0.168; intact leaf: $F_{(1,1)}$= 0.790, P=0.386, n.s: not significant).

Time series	leaf-discs of *B. napus* var.		intact leaf of *B. napus* var.	
	E+	E-	E+	E-
Min15	4.0 ± 1.4	4.6 ± 1.7	2.1 ± 0.4	1.6 ± 0.6
Min30	4.1 ± 1.6	4.7 ± 1.9	1.8 ± 0.9	1.9 ± 0.4
Min45	4.1 ± 1.7	4.8 ± 2.0	1.8 ± 0.9	1.7 ± 0.5
Min60	4.2 ± 1.9	4.6 ± 1.8	1.9 ± 0.9	1.6 ± 0.5
Min75	3.4 ± 1.6	5.3 ± 1.9	1.9 ± 0.9	1.8 ± 0.4
Min90	3.7 ± 1.6	4.9 ± 2.3	1.7 ± 1.1	1.7 ± 0.4
Min105	3.8 ± 2.0	4.8 ± 2.3	1.7 ± 1.1	1.6 ± 0.5
Min120	4.1 ± 2.0	4.5 ± 2.4	1.8 ± 0.9	1.5 ± 0.5
Min135	3.1 ± 2.1	5.1 ± 1.8	1.9 ± 0.9	1.5 ± 0.5
Min150	3.5 ± 1.4	5.0 ± 1.3	1.9 ± 0.9	1.5 ± 0.4
Min165	3.6 ± 1.3	3.8 ± 2.0	1.9 ± 0.9	1.5 ± 0.5
Min180	3.9 ± 1.9	4.6 ± 2.2	2.0 ± 0.9	1.5 ± 0.5
p-vaule	n.s		n.s	

Table 3 Dual-choice of cabbage aphids (*Brevicoryne brassicae* L.) on leaf-discs and intact leaf of *Brassica napus* var. infected by *Verticillium longisporum* and controls (leaf disc: $F_{(1,1)}$= 8.631, P=0.009< 0.01; intact leaf: $F_{(1,1)}$= 0.496, P=0.49, n.s: not significant)

Time series	leaf-discs of *B. napus* var.		intact leaf of *B. napus* var.	
	V+	V-	V+	V-
Min15	3.7±1.6	4.1±2.3	3.4±2.1	4.1±1.5
Min30	4.1±1.6	4.7±2.2	3.8±1.7	3.5±1.2
Min45	3.9±1.5	4.2±1.9	3.7±1.5	3.9±0.9
Min60	3.7±2.0	5.4±2.0	3.6±1.7	3.9±0.7
Min75	3.6±1.5	5.1±1.5	3.5±1.8	4.5±1.7
Min90	3.0±0.9	5.7±1.5	3.9±1.9	4.2±1.6
Min105	2.6±1.7	5.9±1.37	4.1±1.7	4.3±1.3
Min120	3.3±2.3	5.6±1.7	4.3±1.6	4.2±1.2
Min135	2.9±1.8	5.8±1.9	4.2±1.8	4.3±1.5
Min150	2.9±2.5	5.9±2.6	3.5±1.9	4.6±2.0
Min165	3.1±1.9	5.4±2.5	3.5±1.7	4.8±2.1
Min180	2.6±1.8	5.5±2.8	3.7±1.7	4.5±1.9
p-vaule	0.009*		n.s	

4.3.2 Aphid performance experiment

Neither the number of nymphs produced by adult feeding on E+/E- host plants (*B. napus*, *B. napus* var.) at 7dpi and 28 dpi (days past inoculation), nor the development time was significantly different (Figure 1-6). Cabbage aphids needed a shorter developmental time from nymph to adult when feeding on *V. longisporum* infected *B. napus* var ($F_{1,37}$= 4.536, P=0.04) in comparision with on control, however the nymphs produced by adults within 96 h was no significantly different on V+/V- host plants ($F_{1,35}$=0.136, P=0.715) (Figure 7).

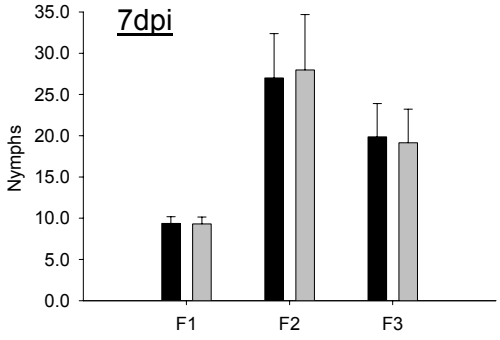

Figure 1

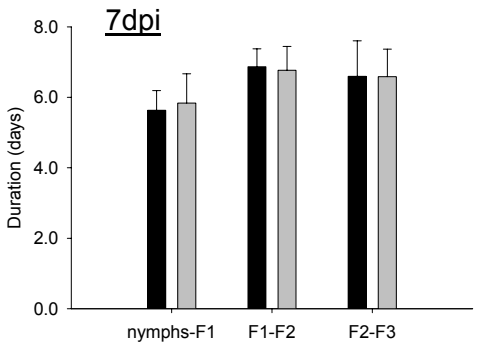

Figure 2

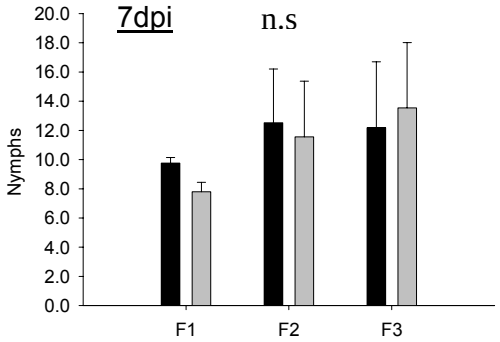

Figure 3

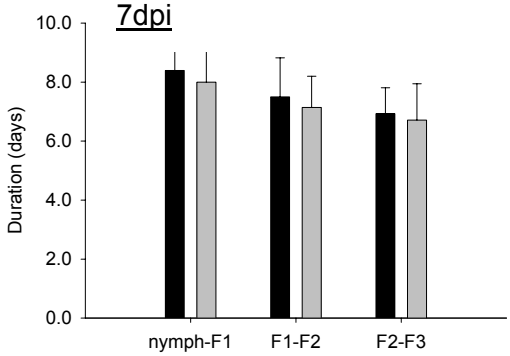

Figure 4

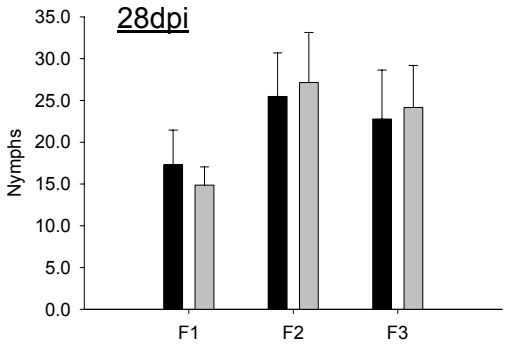

Figure 5

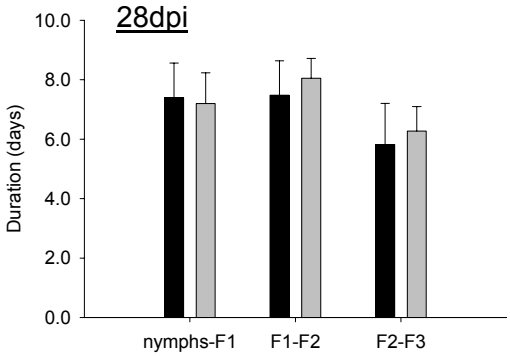

Figure 6

Figure 1 Nymphs produced by cabbage aphids within three successive generations (nymphs of F_1 in 72h, nymphs of F_2 in 96h and nymphs of F_3 in 96h) fed on *Brassica napus* var. inoculated with *A. alternatum* for one week (black column: E+, gray column: E-).

Figure 2 Duration of development (nymphs to F_1 adults, nymph of F_1 to adults of F_2, nymphs of F_2 to adults of F_3) of cabbage aphids fed on *Brassica napus* var. inoculated with *A. alternatum* for one week (black column: E+, gray column: E-).

Figure 3 Nymphs produced by cabbage aphids within three successive generations (nymphs of F_1 in 72h, nymphs of F_2 in 96h and nymphs of F_3 in 96h) fed on *Brassica napus* inoculated with *A. alternatum* for one week (black column: E+, gray column: E-).

Figure 4 Duration of development (nymphs to F_1 adults, nymphs of F_1 to adults of F_2, nymphs of F_2 to adults of F_3) of cabbage aphids fed on *Brassica napus* inoculated with *A. alternatum* for one week (black column: E+, gray column: E-).

Figure 5 Nymphs produced by cabbage aphids within three successive generations (nymphs of F_1 in 72h, nymphs of F_2 in 96h and nymphs of F_3 in 96h) fed on *Brassica napus* var. inoculated with *A. alternatum* for four weeks (black column: E +, gray column: E-).

Figure 6 Duration of development (nymphs to F_1 adults, nymphs of F_1 to adults of F_2, nymphs of F_2 to adults of F_3) of cabbage aphids fed on *Brassica napus* var. inoculated with *A. alternatum* for four weeks (black column: E+, gray column: E-).

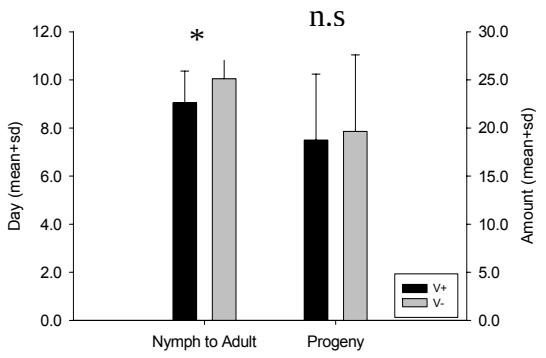

Figure 7

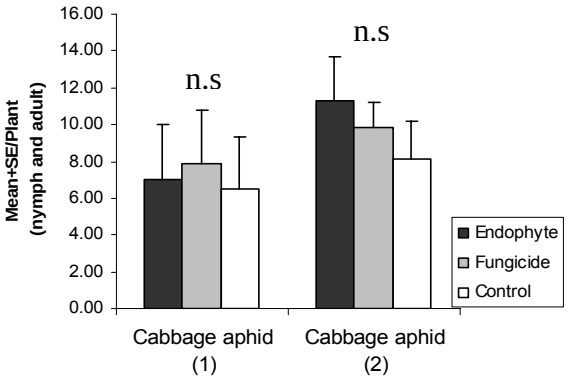

Figure 8

Figure 7 Nymphs produced by cabbage aphids within 96h feeding on *Brassica napus* var. inoculated with *V. longisporum* or control host plant (3 weeks past inoculation) and the developmental time of the new-borne nymphs to adults

Figure 8 The accumulative cabbage aphid population (*Brevicoryne brassica* L.) on each *Acremonium alternatum* infected / fungicide / control treated cabbage plants (*Brassica oleracea var. viridis*) in the two set of the field experiments

Given the two host plant cultivars, more nymphs were produced on *B. napus* var than on *B. napus,* no matter with regard to the treatment. Within the three generations (F_1-F_3), the numbers of nymphs produced by the F_2 adults were higher than the other two generations on *B. napus* spp.

4.3.3 Field experiment results

In the field cabbage aphids had no obvious preference for cabbage plants of the three different treatments, which was consistent with the findings in the laboratory (Figure 8, 9, 10). The accumulated populations of cabbage aphids in the second field experiment were higher than that in the first experiment, related to higher temperatures. No significant difference was found for the content of total carbon, total nitrogen and the ratio of carbon/nitrogen (Table 4).

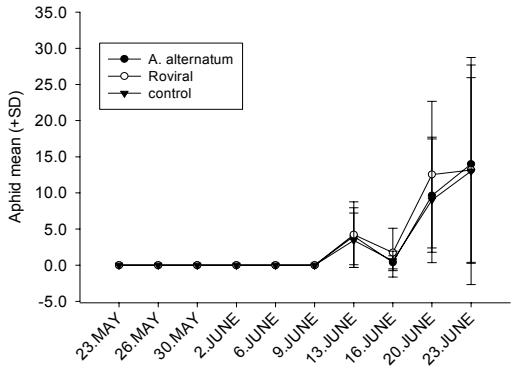

Figure 9

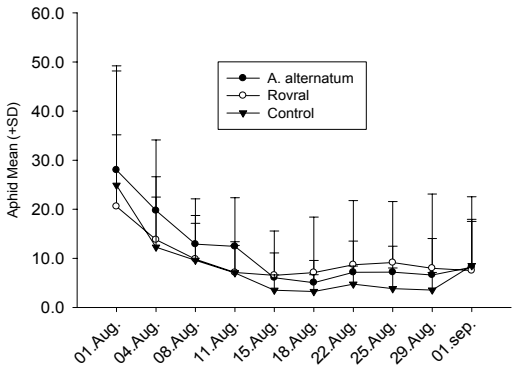

Figure 10

Figure 9 Populations of cabbage aphids (*Brevicoryne brassica* L.) on *Acremonium alternatum* infected / fungicide / control treated cabbage plants (*Brassica oleracea var. viridis*) in the first field experiment

Figure 10 Population of cabbage aphids (*Brevicoryne brassica* L.) on *Acremonium alternatum* infected / fungicide / control treated cabbage plants (*Brassica oleracea var. viridis*) in the second field experiment

Table 4 Content of total carbon, total nitrogen and the ratio of C/N contained in young cabbage leaves treated with *Acremonium alternatum* (E+), Iprodione (CoI) or controls (CoII). (Values: mean±STD; ANOVA; n.s: not significant)

	E+	CoI	CoII	$F_{(2, 87)}$	p
carbon	41.8±1.23	41.2±1.53	41.2±1.07	1.85	n.s
nitrogen	5.94±1.03	5.02±0.78	5.90±0.99	0.12	n.s
C/N	7.24±1.29	6.97±1.10	7.19±1.28	0.41	n.s

4.3.4 Phytosterols Profiles

Four major phytosterols were analyzed and summed up to calculate the total and relative phytosterol content: Brassicasterol, Campesterol, Stigmasterol and Sitosterol. The infection with the endophytic fungus *Acremonium alternatum* resulted in higher an amount of phytosterols in the leaves. In both treatments, sitosterol was the most prominent phytosterol followed by Brassicasterol, Campesterol and Stigmasterol. Regarding the relative proportion of phytosterols, in the leaves of infected host plants, the proportion was 63.7% Sitosterol, 16.1% Brassicasterol, 11.7% Campesterol and 8.5% Stigmasterol; in mock plants, the composition was 62.2% Sitosterol, 18.2% Brassicasterol, 11.3% Campesterol and 8.3% Stigmasterol; The relative composition of Brassicasterol was significantly less in endophyte infected leaves as compared to control leaves (one way-ANOVA test, $F_{(1, 14)}=5.191$, $p=0.039<0.05$) (Table 5).

Table 5 Phytosterols content (ug/g leaf) and relative composition in *Acremonium alternatum* in infected or uninfected *Brassica napus* leaves. Relative proportion of Brassicasterol, one way-ANOVA test, $F_{(1,14)}=5.191$, $p=0.039<0.05$ (* $p\leq0.05$; n.s: no significant difference)

Phytosterol	E+	E-	P value
mean of Brassicasterol	47.73±5.70	52.55±6.75	n.s
mean of Campesterol	35.02±4.19	32.87±4.16	n.s
mean of Stigmasterol	25.27±1.97	23.83±1.90	n.s
mean of Sitosterol	189.7±15.38	179.42±12.50	n.s
Sum of phytosterols	297.7±18.93	288.66±20.84	n.s
relative proportion of Brassicasterol	0.161±0.02	0.182±0.018	*
relative proportion of Campesterol	0.117±0.01	0.113±0.008	n.s
relative proportion of stigmasterol	0.085±0.01	0.083±0.004	n.s
relative proportion of sitosterol	0.637±0.02	0.622±0.02	n.s

4.4 Discussion

Dual-choice of cabbage aphids on infected/uninfected *Brassica spp*.

In this study, cabbage aphids (*Brevicoryne brassicae* L.) significantly preferred the leaf-discs cut from E- *B. napus* or V- *Brassica napus* var., however, they did not

discriminate between E+/E- or V+/V- intact host plants. The orientation flight and landing of aphids is mediated by volatiles and perceived via olfactory receptors on the antennae (Pickett *et al*., 1992; Visser *et al*., 1996; Guerrieri *et al*., 1999). By probing with the stylet, chemical cues present on the leaf surface are determined resulting in host plant acceptance (Bones *et al*, 1991; Cole, 1997). Pathogen-induced quantitative and qualitative variation in volatiles and non-volatiles have been documented a few times (Cardoza *et al*., 2003; Huang, *et al*., 2003). The content of glucosinolates and the release of isothiocyanates in *Brassica napus* and *B. rapa* was found to be altered when host plants were infected by the dark leaf spot pathogen *(Alternaria brassicae*) (Doughty *et al*., 1991, 1996). Gluocosinolates are secondary metabolites typical for all Brassicaceae (Gabrys *et al*., 1997; Fahey *et al*., 2001; Renwick, 2002). They are regarded as having protective functions in plants against pests and diseases (Mithen, 1992; Cole, 1996; Zukalova & Vasak, 2002). Simultaneously they act as stimulants for specialist herbivores including cabbage aphids, and trigger host plant acceptance and feeding, particularly sinigrin and a range of breakdown products of glucosinolates (i.e. isothiocyanates) (Fenwick *et al*., 1983; van Emden, 1990; Cole, 1997). However, sinigrin contained in oilseed rape in minute amounts was not the major cue for cabbage aphids' orientation to the host plants and a higher amount of isothiocyanates is released in crushed or damaged plant tissues as compared to intact host plants (Ettlinger 1968; Finch 1978; Kirk 1992), explaining the orientation behavior of cabbage aphids to leaf-discs cut from E- and V- host plant in the current study.

Performance of cabbage aphids feeding on E+/E- or V+/V- *Brassica spp*.

In the performance bioassays, the number of progeny produced by adult aphids was not significantly influenced by either *A. alternatum* or *V. longisporum* infection. The development time from nymph to adult was accelerated feeding on *B. napus* var. inoculated with *V. longisporum*. A similar study using arbuscular mycorrhiza (VAM) showed VAM fungi *Glomus intraradices* prompted the development of aphids *Myzus persicae* feeding on *Plantago lanceolata*, however the fungus did not influence the number of nymphs produced (Wurst *et al*., 2004). Negligible effects on the weight and reproduction of the aphid *S. graminum* was reported, when aphids were fed on

sorghum inoculated with *Glomus fasciculatum* (Pacovsky *et al*., 1985). However, more papers reported a positive impact of a microorganism infection, such as an accelerated development time, improved aphid weight and enhanced reproduction in the aphid *Euceraphis betulae* feeding on leaves of silver birch (*Betula pendula*) inoculated with a fungal pathogen (*Marssonina betulae*) (Johnson *et al*., 2003); in the melon aphid *Aphis gossyppi* (Glover) feeding on cucumber leaves locally infected by the cucurbit scab fungus (*Cladosporium cucumerinum*)(Moran, 1998) and in two aphid species (*Myzus ascalonicus* and *M. persicae*) reared on *Plantago lanceolata* plants with root colonization by the arbuscular mycorrhizal fungus (*Glomus intraradices*). (Gange & West, 1994; Gange *et al*., 1999) and two species of aphid (*Drepanosiphum platanoidis* and *Periphyllus acericola*) feeding on host plants infected by endophytic fungus *Rhytisma acerinum* (Gange, 1996). In these cases the improved performance of aphids was attributed to the enhanced plant suitability to aphids by adjusting the soluble nitrogen and phosphorus and amino acid content in the host plants. Amino acids act not only as dispensable nutrient compounds for aphid development (Dixon, 1970; van Emden & Bashford, 1971; Mattson 1980) but also they are attractants for aphid colonization and the basis for biosynthesis of glucosinolates (Halkier & Du, 1997; Klingauf, 1987).

Host preference and performance of cabbage aphids in the field

The application of fungicide Iprodione (Rovral) had no influence on plant growth, however effectively depressing mycorrhizal infection of plants in the field (Gange &West, 1994; West *et al*., 1993). In this study, no obvious effect on plant size was observed as well (Li, unpublished data). Therefore the impact of plant size on host choice of cabbage aphids could be neglected. Under field conditions, cabbage aphids had no obvious preference for host plants treated differently (E+: endophyte; CoI: fungicide; CoII: control) as observed in the laboratory bioassay. However, slightly lower numbers were found colonizing plants treated by the endophyte as compared to the other treatments (CoI, CoII). Our finding is consistent with the results reported by Gange and West (1994) that damage levels caused by mining insects in the field were lower on *Plantago lanceolata* L. infected by arbuscular mycorrhizal fungi as compared to those treated by Iprodione. The ‘mothers knows

best' principle refers to the crucial role of plant nutrients on adult oviposition and offspring performance (Johnson *et al*., 2006). Host plant quality, such as the level of nitrogen, carbon and defensive metabolites influences insect performance (Awmack & Leather, 2002). Reduced leaf carbohydrate caused impaired insect performance, but elevated leaf nitrogen levels increased insect performance (Gange & West, 1994). As carbon-heterotrophic organisms, fungi are thought to influence the nutritional quality of the plant (i.e. carbon-nitrogen balance) and the content of C- and N-based defense metabolites in the plants (Jones & Last 1991; Bazzaz *et al*., 1987). For the foliar fungus *Uromyces rumicis* enhanced nitrogen and non-structural carbohydrate levels were found in *Rumex obtusifolius* plants (Hatcher *et al*., 1997). In this study the content of carbon, nitrogen and the ratio of C/N were not significantly different in E+/E- leaves of the host plants; however the ratio of C/N did slightly increase in host plants inoculated with the endophyte. This point to the possibility of higher carbon-based defense metabolites produced in E+ than that in control leaves. Because the plant samples for C/N analysis were collected only in the second field experiment, the result reported here could explain the cabbage aphid populations found in the second field experiment only.

Aphids harbor symbiotic bacteria which upgrade nonessential to essential amino acids in the ingested phloem sap needed for a normal development and fecundity (Baumann *et al*., 1993; Douglas, 1998). Only slight differences in the nutrient sap (such as the fluctuation in nitrogen content: E+: 5.94±1.03, CoI: 5.02±0.78, CoII: 5.90±0.99) were caused by the treatments, their final impact on aphid performance was assumed to be minimal by symbiotic bacteria.

The development of aphids from nymphs to adults and their survival is affected by temperature regimes (Dean, 1974). The cumulated cabbage aphids populations in the second field experiment were higher as compared to that found in the first experiment, which was attributed to the more suitable mean temperatures in August than in June. In the field, many insect species including chewing (e.g. *Pieris rapae*, *Pieris brassicae*, *Plutella xylostella*, *Phyllotreta atra*), sucking insects (e.g. *Aleyrodes brassicae*, *Brecvicoryne brassicae*) and soil-burrowing insects (e.g. *Delia radicum*) shared the same host plant together with some natural enemies of the respective insects.

Phytosterols

Aphids exclusively suck plant phloem sap, however, the nutrients (e.g. amino acid) are only found at low concentrations (Sandstrom & Moran, 1999; Lehrer *et al*., 2000). By harboring intracellular symbiotic bacteria essential amino acids are produced for normal development and reproduction in aphid species, such as (*Myzus persicae*), Pea aphids (*Acyrthosiphon pisum*), *Aphis fabae*, *Uroleucon sonchi* L. (Buchner, 1966; Mittler, 1971; Douglass, 1996, 1998; Douglass *et al*., 2001; Bernays & Klein, 2002). However, these endosymbionts are not involved in sterols synthesis (Douglass, 1988). Exogenous sterols (i.e. phytosterols) are still required. The greenbug aphid, *Schizaphis graminum* (Rondani) was capable of sequestering campesterol, stigmasterol and sitosterol from their host plant *Sorghum bicolor* L. and converted them to cholesterol by dealkylation (Campbell & Nes, 1983). Phytosterols are crucial for the cabbage aphid development and reproduction as in other herbivorous insects. In this study the relative composition of brassicasterol significantly decreased and sitosterol slightly increased in E+ leaves of *B. napus* resulting in a consistent retarded development of aphids feeding on these plants within the successive three generations. The proportion of essential nutrients may affect the rate of growth and development of insects (House 1969). As primary metabolites, changes in the phytosterol composition (e.g. the ratio of utilizable sterols to nonutilizable sterols) contained in artificial diet had a negative effect on the survival, growth and reproduction of different insects, including the corn earworm *Heliothis zea* (Ritter, 1984; Nes, *et al*., 1997), the grasshopper *Schistocerca americana* (Behmer & Elias, 1999, 2000), and the Diamondback moth P*lutella xylostella* (Behmer & Grebenok, 1998). Fungicide treatment led to changes in sterol profiles in host plants and caused the impaired development of the armyworm (*Mamestra configurata*), flea beetles (*Phyllotreta cruciferae*), and two aphids species (*Lipaphis erysimi* (Kaltenbach) and *Myzus persicae* (Sulzer)) (Bodnaryk *et al*., 1997). By establishing a correlation between the phytosterols contents in the leaves from Brussels sprouts infected by *A. alternatum* and the pupal weight of the Diamondback moth P*lutella xylostella* feeding on it, Brassicasterol was categorized as a utililzable whilst Sitosterol as a non-utilizable phytosterol (Dugassa-Gobena *et al*. 1998). The reduction of Brassicasterol and the increase of Sitosterol in E+ *B. napus* might

explain the delayed development from neonates to adults. However, the variation in the phytosterol composition did not result in a significant impact on nymph production feeding on E+/E- *B. napus*. Phytosterols are not essential for herbivorous insects, but also for fungal growth and reproduction (Hendrix, 1970), potentially resulting in a competition between the two organisms for the same source supplied via their shared host plant. Moreover the metabolites produced by microorganisms might reduce the availability of dietary sterols and inhibit the cholesterol uptake by insect (Bernays, 1993). These factors complicate the allocation of nutrients from the host plant to the insects and the fungi. Our result are only feasible when using a simplified model, in which the phytosterols from the host plants are supposed to be supplied for the herbivorous insect performance only and the metabolites from the fungi will not influence the phytosterol uptake of the insects.

Acknowledgment

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4.5 Literature

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