Chapter 3

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

Introduction

While tripartite interactions between plants, phytopathogens, and herbivores have been considered in several studies (Agrawal *et al.* 1999; Paul *et al.*, 2000), the possible impact of such interactions on a fourth party, the entomopathogens, has almost been neglected. Phytopathogens are well-known to alter plant chemistry (Ayres, 1992; Hammerschmidt & Nicholson, 1999), and thus, the plant's susceptibility towards herbivores (Hatcher, 1995). However, even though several studies consider the effect of plant chemistry on insect pathogens (e.g. Duffey *et al.* 1995), knowledge on how phytopathogens may influence the herbivore's vulnerability towards entomopathogens is almost lacking.

Herbivorous insects often suffer reduced growth rates when feeding on suboptimal host plants. Although slow growth is not always associated with higher mortality it is assumed that sublethal effects are nevertheless detrimental to herbivores. The insect has to spend longer in the vulnerable stages which may finally lead to higher levels of natural enemy attack. These ideas on the detrimental effects of slow growth of herbivorous insects caused by suboptimal food plants have provided the base for the slow-growth, high-mortality hypothesis (Feeny, 1976).

In spite of widespread acceptance, the empirical evidence for this hypothesis is equivocal (Clancy & Price, 1987; Benrey & Denno, 1997). It seems that a more differentiated view is needed: e.g. while predators are more likely to consume slow-growing herbivores, parasitoids are not (Williams, 1999). Equally, contradictory results in studies with fungal entomopathogens have been found. It was suggested that this might reflect differences in their

dependence on host insects (Hajek & St.Leger, 1994). Further testing of the slow-growth, high-mortality hypothesis is necessary and in particular entomopathogens should be taken into consideration as important natural enemies of herbivores (Elliot *et al*., 2000) since so far studies have been biased in favour of predators and parasitoids.

Only few studies have addressed the question of how a decrease in host plant suitability might affect the efficacy of entomopathogens. For example, reduced host plant suitability over time, due to the feeding activity of larvae of *Spodoptera exigua* and *Trichoplusia ni,* correlated with an increased efficacy of *Bacillus thuringiensis* (Meade & Hare, 1994). However, induction of the defence-related plant enzyme peroxidase, caused by larval feeding, protected *Helicoverpa zea* and *Heliothis virescens* from baculovirus disease (Hoover *et al.* 1998).

The mustard leaf beetle *Phaedon cochleariae* F. (Col., Chrysomelidae) attacks leaves of several species of Brassicaceae, as well as the plant pathogenic fungus *Alternaria brassicae* (Berk.) Sacc. Beetle larvae reared on Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) leaves, partially infected by the phytophathogenic fungus, develop slower but do not suffer higher rates of mortality. Duration of larval development from hatching to pupation was increased by about 9% when larvae fed upon fungus-infected leaves (Rostás & Hilker, in press). With respect to the slow-growth, high-mortality hypothesis this study focused on the question whether the prolonged development of *P. cochleariae* larvae feeding on diseased plants would result in higher mortality if larvae are challenged by the generalist entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorok.

Materials and methods

The plant. Chinese cabbage (*Brassica rapa* ssp. *pekinensis* cv. 'Kantonner') was grown in a greenhouse under natural daylight conditions from July to August. After germination each seedling with a first true leaf unfolded was transferred into a plastic pot (7 x 8 x 7 cm) containing standard potting soil (Einheitserde Typ P, Kausek GmbH, Mittenwalde, Germany). Plants were used in the experiments when the 5th leaf (1st leaf = oldest leaf) had reached maximum size (after 4-5 weeks).

The phytopathogenic fungus. Strain BBA 64878 of *Alternaria brassicae* (Biologische Bundesanstalt Berlin-Dahlem, Germany) was cultured on potato-dextrose agar at 20°C and L18:D16 without induction of sporulation. Cultures used for inoculating the experimental plants were five days old.

Fungal inoculation of the plant. Chinese cabbage plants were inoculated with *A. brassicae* following a method adopted from Gröntoft & O'Connor (1990). Small mycelium-containing agar discs (Ø 5 mm) were punched out from behind the growth front of the Petri dish cultures using a cork borer. Four discs were laid with their mycelium-containing side onto the adaxial surface of the 5th leaf. On top of each agar disc, and underneath it on the abaxial side of the leaf, a piece of firm foam material (10 x 10 x 7 mm) was placed. The 'sandwich' construction was fixed by piercing an insect needle (No. 00) through foam material, inoculum, and leaf. Controls were treated in the same manner but agar discs were without mycelium. All test and control plants were placed under an incubation tent (100 % r.h.) for 5 d in a completely randomised design. After this time period lesion size was 10-15% of the total leaf area. No visible symptoms of fungal infection were recognized on the control plants.

The herbivore. The mustard leaf beetle *Phaedon cochleariae* (Chrysomelidae) was reared in transparent plastic boxes (20 x 20 x 6 cm) and provided with Chinese cabbage leaves every second day. Conditions in the climate chamber were 20°C, 70% r.h., and a photocycle of L18:D16.

The entomopathogenic fungus. Metarhizium anisopliae strain U23 was a gift from Dr. A. Vey (INRA, St. Christol-les-Ales, France). The fungus was maintained on Sabouraud agar with 2% glucose at room temperature. Conidia from stationary cultures were used as inoculum for subsequent propagation in submerged culture with nutrient broth (25 g Standard I, Merck and 20 g glucose dissolved in 1 l demineralised water). For the experiments a suspension was prepared by harvesting conidia from 7-14 day old cultures. The conidia were counted (Bürker chamber) and then diluted in 0.1% aq. Tween 20.

Fungal inoculation of the herbivore. Larvae were inoculated externally by dipping them into a suspension of 8.5 x 10^6 conidia per ml 0.1% aq. Tween 20 from *M. anisopliae* (≅ LD_{50}, personal communication Jürgen Gross, Freie Universität Berlin, Institute of Biology). The suspension was constantly stirred by a magnetic stirring bar to ensure homogenous distribution of the conidia. For control, larvae were dipped in 0.1% aq. Tween 20 without conidia.

Experiment 1

This experiment was conducted to test, whether larvae feeding upon a plant infected by a phytopathogen, differ in their susceptibility towards an entomopathogenic fungus, when compared with larvae feeding on leaves of a healthy plant.

Herbivore feeding. Neonate larvae of *P. cochleariae* were set singly into one Petri dish (Ø 55 mm) containing a moist filter paper. Individuals of the treatment group (n = 50) were supplied with a leaf disc (Ø 10 mm) that had been punched out from *A. brassicae*-infected leaves. Leaf discs were taken only from green, symptom-free parts of the infected leaves. Larvae belonging to the control group (n = 50) received discs from healthy leaves of the uninfected control plants. All discs were replaced once a day. The experiment was carried out in a constant environment room with 25°C, 70% r.h., and L16:D8. At least four experimental plants were used per treatment per day to avoid pseudoreplications. After a feeding period of 3 days larval weight was measured. As predicted weight of larvae feeding on diseased leaves (1.2 ± 0.30 mg) was significantly lower after 3 days compared to larvae fed with leaf discs from control plants (1.6 ± 0.56 mg) (P = 0.016, t-test for independent samples). Previous experiments showed that larvae on diseased leaves not only had lower body weight but also needed significantly more time to complete development (Rostás & Hilker, in press).

Inoculation of the herbivore. Young, 3-day-old larvae either feeding upon diseased leaf discs or control discs were inoculated with the entomopathogenic fungus. After inoculation, larvae continued to feed either on *A. brassicae*-infected or healthy leaf discs until they died or pupated. Mortality rates were recorded daily by counting the numbers of dead larvae. Dead individuals were left in their Petri dishes until the entomopathogen sporulated, ensuring that mortality had been caused by the pathogen. Eleven days after inoculation the experiment was stopped as all test larvae had died.

Experiment 2

Since larvae feeding upon a fungus-infected plant gain less weight within 3 days compared with larvae feeding on healthy plants (see above), we wanted to know whether larval body weight *per se* is an indicator for the susceptibility towards the entomopathogen *M. anisopliae*. Thus, the herbivore was reared on healthy plants and individuals were classified according to their weight prior to infection by the entomopathogen.

Herbivore feeding. Approximately 300 neonate larvae were reared on healthy Chinese cabbage leaves in a rearing box. After 3 days 120 larvae were selected and assigned to one of four treatment groups according to their weight. Treatment groups consisted of (a) larvae with high body weight (1.6 ± 0.20 mg); here referred to as large larvae and (b) larvae with low body weight (1.2 ± 0.20 mg); here referred to as small larvae. Mean body weight of large larvae was equivalent to the body weight of larvae feeding on healthy control plants, while small larvae had the same mean body weight as larvae feeding on fungus-infected plants (compare experiment 1).

Inoculation of the herbivore. 50% of the invidivuals belonging to the groups “large” and “small” larvae were inoculated with the entomopathogenic fungus. The remaining half of either group was mock-inoculated for control with 0.1% aq. Tween 20. As in experiment 1 larval mortality rates were recorded daily for a period of 11 days.

Statistics. Mortality rates in both experiments were analysed using chi-square tests adjusted by Bonferroni correction.

Results and Discussion

Experiment 1 revealed that larvae of *P. cochleariae* feeding upon fungus-infected Chinese cabbage suffered significantly higher mortality rates when exposed to the entomopathogenic fungus *M. anisopliae* than larvae feeding on healthy plants. While none of the entomopathogen-inoculated larvae feeding on the diseased plants survived, 46% of the inoculated larvae feeding on healthy plants survived the attack by the entomopathogen and pupated 11 days after inoculation with *M. anisopliae* (Fig. 1) ($P < 0.001$). This mortality of 54% was consistent with results from the preliminary LD_{50} experiments (see materials and methods). Following an entomopathogenic incubation period of two days most individuals died on day 3 and day 4. Thereafter, survival rate decreased at considerably slower pace.

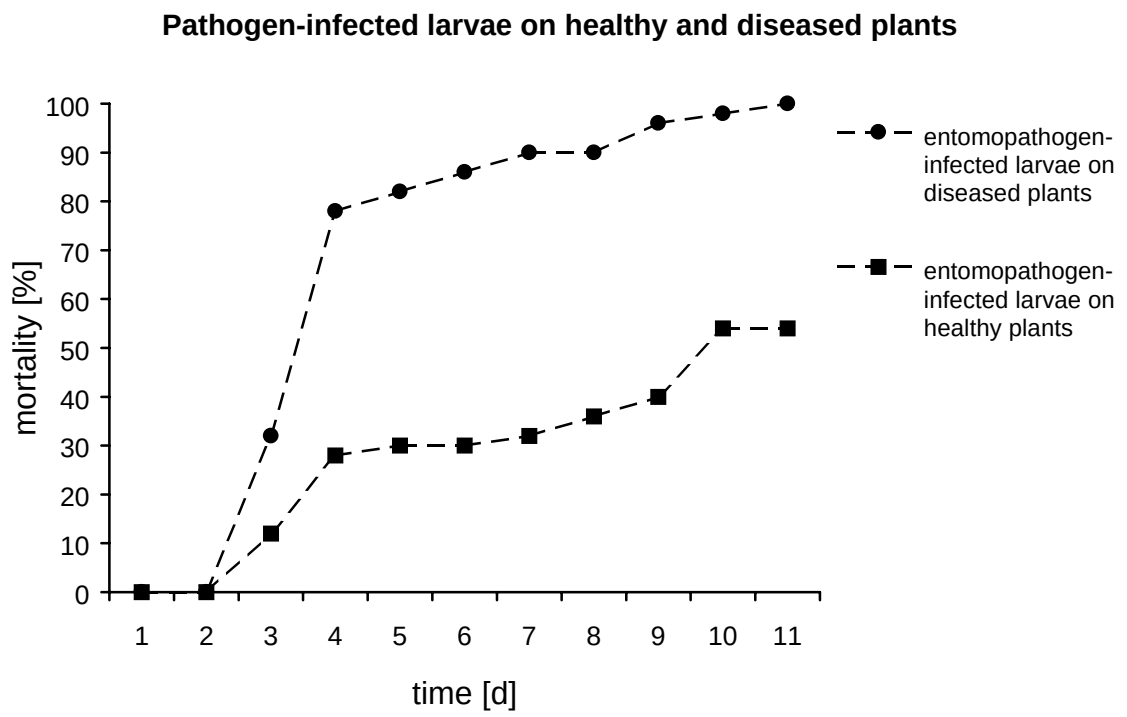

Figure 1 *Mortality [%] of larvae of* Phaedon cochleariae *infected by the entomopathogenic fungus* Metarhizium anisopliae *when feeding on either healthy cabbage leaves or cabbage leaves infected with the phytopathogen* Alternaria brassicae. *Mortality was recorded from L1 (day 1 after inoculation of 3-day-old larvae) until pupation (day 10 or 11). N = 50. Chi-square test.*

Conduction of experiment 2 was stimulated by the following results: (a) a former study showed that feeding upon fungus-diseased plants results in lower weight of *P. cochleariae* larvae (Rostás & Hilker, in press) and (b) feeding upon fungus-diseased plants results in higher susceptibility towards infection by an entomopathogen (Fig. 1, this study). To examine whether low larval weight *per se*, regardless of host plant disease, is an indicator for entomopathogenic susceptibility, larvae feeding upon healthy plants were selected for small and large individuals and inoculated by the entomopathogen. About 83% of the small larvae died when infected with *M. anisopliae* while approximately half of the infected large individuals survived fungal treatment (infected small larvae vs. infected large larvae: $P < 0.001$) (Fig. 2). The differences in mortality rates between small and large infected larvae feeding on healthy plants (experiment 2) parallel those between infected larvae feeding on diseased and healthy plants (experiments 1). Thus, small larval body weight might indicate high susceptibility towards infection by the entomopathogen, regardless whether larvae had fed on healthy or diseased plants. This lower susceptibility of *P. cochleariae* larvae with larger weight (and size) towards entomopathogens may be due to their more effective immune response. A relationship between larval stage (and thus body weight) and susceptibility was also observed by Inyang and co-workers (1998). Only the 1st and 2nd instars of *P. cochleariae* were affected by *M. anisopliae* at doses below 10^7 conidia/ml when sprayed onto Chinese cabbage leaves.

In experiment 2 larval mortality of mock-inoculated small and large control larvae on healthy plants was equally low (small larvae: 3%, large larvae: 6%) (Fig. 2). Likewise, no difference was found when mortality rates of infected larvae feeding on healthy plants in experiment 1 were compared to mortality rates of large large infected also feeding on healthy plants in experiment 2 (same mean larval weights; see material and methods). However, a comparison of mortality

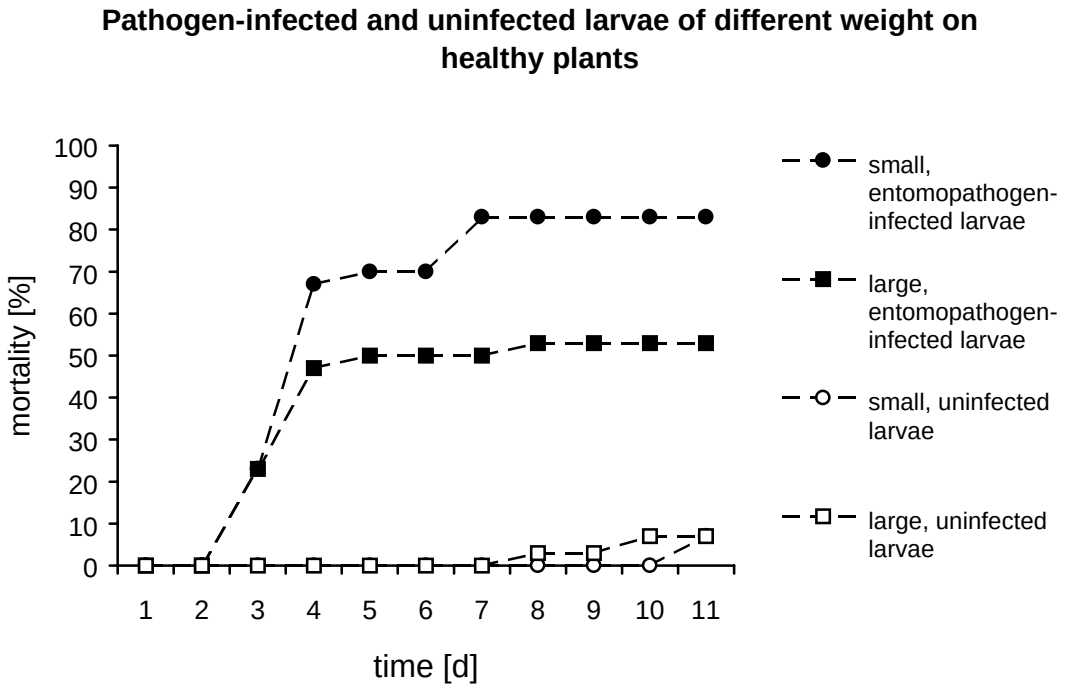

Figure 2 *Mortality [%] of healthy larvae of* Phaedon cochleariae *or larvae infected by the entomopathogen* Metarhizium anisopliae *in dependence of their body weight. All individuals were fed healthy Chinese cabbage leaves. Small (1.2 ± 0.20 mg) and large (1.6 ± 0.20 mg) larvae were either treated with the entomopathogen (infected) or mock-inoculated (control). Mortality was recorded from L1 (day 1 after inoculation of 3-day-old larvae) until pupation (day 10 or 11). N = 30. Chi-square test and Bonferroni correction.*

rates of infected larvae feeding upon diseased plants (experiment 1) and infected small larvae from the second experiment feeding upon healthy plants revealed a significant difference (P = 0.006) (same mean larval weights; see material and methods): infected small larvae feeding on diseased plants (experiment 1) had a higher mortality rate than infected small larvae on healthy plants. This shows that reduced larval size, indicated as low body weight, may only partly explain the effect why feeding on a diseased plant results in higher susceptibility towards entomopathogenic infection.

Other factors must be considered as e.g. the induction of defensive plant compounds by the phytopathogen. Chinese cabbage is known to increase glucosinolate concentrations and to synthesize phytoalexins when attacked by pathogens (Ludwig-Müller *et al.*, 1997; Pedras *et al.*, 2000). Effects of plant allelochemicals influencing insect susceptibility towards entomopathogens are well-known (Elliot *et al.*, 2000). Artificial diet containing plant secondary metabolites may enhance insect mortality caused by entomopathogens (Berenbaum, 1983). However, the opposite may also occur (Krischik *et al.*, 1991). This may depend on

whether it is the microbe or the herbivore that is more sensitive to the allelochemicals (Berenbaum, 1988).

The slow-growth, high-mortality hypothesis is supported by the results of this and a former study (Rostás & Hilker, in press) with respect to a generalist entomopathogen as the mortality factor: *P. cochleariae* larvae fed on fungus-infected Chinese cabbage displayed prolonged larval development (Rostás & Hilker, press) and a significantly higher susceptibility towards infection by the entomopathogen *M. anisopliae* (this study, experiment 1). Several other studies addressed herbivore susceptibility towards pathogens in conjunction with varying growth rates. Feeding on different host plant species influenced the duration of larval development and pathogenicity but the relationship was not unequivocal. The noctuid *Anticarsia gemmatalis* was most susceptible to a nuclear polyhydrosis virus when reared on the host plant species that provided fastest growth rates (Peng *et al*., 1997). Similar results were obtained by Hajek and co-workers (1995): caterpillars of *Lymantria dispar* feeding on the suboptimal host *Acer rubrum* demonstrated reduced growth and slower development of the entomopathogenic fungus *Entomophaga maimaiga*. In contrast, slow growing larvae of *Leptinotarsa decemlineata* suffered higher mortality from the entomopathogen *Beauveria bassiana* (Hare & Andreadis, 1983). It has been suggested that entomopathogens closely associated with their host, like e.g. viruses or obligate fungi, benefit from fast growing herbivores, while the opposite may be true for generalist or facultative saprophagous entomopathogens like *B. bassiana* (Hajek & St.Leger, 1994). Our results support this suggestion as the unspecific fungus *M. anisopliae* is able to successfully infect a higher rate of *P. cochleariae*-larvae when their body weight was low and their developmental time was prolonged.

To our knowledge we provide the first report on indirect interactions between a plant pathogenic and an entomopathogenic fungus. Simultaneously, our results support the hypothesis that feeding upon a suboptimal host plant imposes a general stress on the herbivore that leads to higher mortality caused by natural enemies (Schoonhoven *et al*., 1998).

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