UNIVERSITY OF HANNOVER

FACULTY OF HORTICULTURE

INSTITUTE OF PLANT DISEASES AND PLANT PROTECTION

The potential of entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sorokin, *Paecilomyces fumosoroseus* (Wize) Brown & Smith and *Verticillium lecanii* (Zimm.) Viégas (Deuteromycotina: Hyphomycetes) for control of the green leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae) and potential side effects on the egg parasitoid *Anagrus atomus* L. (Hymenoptera: Mymaridae)

By

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Thesis submitted in partial fulfilment for the requirements of the degree of

Master of Science in Horticulture

September 2002

DECLARATION

I, Agbeko Kodjo Tounou, hereby declare that the work presented in this thesis is my own work, which has not been and will not be submitted for a degree in any other university.

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ACKNOWLEDGEMENTS

This study was supported by the University of Hannover and partly by the German Academic exchange Service (DAAD). I would like here to express them all my gratitude.

I am grateful to PD Dr. Christian Borgemeister for initiating this project and for his continuous discussion, corrections, comments and suggestions during this research work.

I express my deepest thanks to Prof. Dr. Hans-Michael Poehling and Prof. Dr. Thomas Rath for supervising my thesis.

I am grateful to Dr. Gisbert Zimmermann, for providing us the entomopathogenic fungal strains and offering me the opportunity to learn the methodology of this research work and to undertake partly the experiments in his laboratory but also for his helpful discussion, comment and answers to my questions. I also thank all the technicians in his laboratory for their valuable technical assistance.

Last but not least, l address my sincere gratitude to my parents for supporting me and for all the confidence that they always placed on me.

I am solely responsible for the views expressed in this thesis and hope that the assembled information will provide a useful tool for students and scientists working on the microbiological control of leafhoppers.

ABREVIATIONS USED IN THE TEXT

ANOVA	Analysis Of Variance
df	degree of freedom
e.g.	for example
F	F-value
Fig.	Figure
CL	Confidence Limit
i.e.	that is
LD	Lethal Dose
log	Logarithm
Ø	diameter
NS	non-significant
p	P-value
R^2	regression/correlation coefficient
RH	Relative Humidity
SAS	Statistical Analysis System
SE	Standard Error of the mean

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ABSTRACT

The present study is part of a bigger project seeking to develop new strategies for biological control of the greenhouse leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae). This includes the use of entomopathogenic fungi and the egg parasitoid *Anagrus atomus* L. (Hymenoptera: Mymaridae). Hence in the present study, the efficacy of five entomopathogenic fungal strains against *E. decipiens* and the potential side effects of *A. atomus* were investigated both under laboratory and greenhouse conditions. The fungal strains screened included two *Metarhizium anisopliae* (Metsch.) Sorokin strains (Ma43 and Ma57), and one strain of *Beauveria bassiana* (Bals.) Vuill. (Bba113), *Paecilomyces fumosoroseus* (Wize) Brown & Smith (Pfr12) and *Verticillium lecanii* (Zimm.) Viégas (Vl23). In screening experiments, all strains proved to be highly virulent to late fifth instar nymphs of *E. decipiens*. Mortality of fifth instar nymphs after seven days exposure to a dose rate of 1×10^7 conidia/ml varied between 65% (*M. anisopliae* strain Ma57) and 98% (*P. fumosoroseus* strain Pfr12). Reisolated conidia after single host insect passage resulted in significantly faster and/or higher mortality rates of *M. anisopliae* strain Ma57 and *P. fumosoroseus* strain Pfr12 than recorded in the initial screening, with final mortality ranging from 72% (*V. lecanii* strain Vl23) to 100% (*M. anisopliae* strain Ma43 and *P. fumosoroseus* strain Pfr12). In dose-response experiments, mortalities significantly increased with rising dose rates (1×10^3, 1×10^4, 1×10^5, 1×10^6). Older nymphs (3rd and 5th instar) were more susceptible than younger ones (1st instar). LD_{50} values ranged from 1.3×10^4 to 9.4×10^5 conidia/ml, from 6×10^3 to 2.2×10^4 conidia/ml and from 6.4×10^3 to 1.5×10^4 conidia/ml for Bba113, Ma43 and Pfr12 respectively.

Application of leafhopper-infested plants in greenhouse resulted in more than 93% mortality and 100% infection. Leafhopper mortality declined significantly with increasing time after

application in residual assays. Release of adult leafhopper three and five days after fungal application resulted in similar mortality rates, being significantly lower when compared to the mortality recorded when insects were released immediately after treatment. Experiments on potential side effects of two entomopathogenic fungi, i.e. Ma43 and Pfr12, on *A. atomus* showed that the tested isolates had no influence on adult emergence and longevity; however a distinct reduction of the parasitation rate at spore concentrations of 1×10^5 and 1×10^7 conidia/ml compared to the control was noted. The latter either might be due to density effects and/or could indicate repellent features of the fungi on *A. atomus*. The results indicate that entomopathogenic fungi are potential candidates to be used as bio-insecticide to control the green leafhopper, and that its common egg parasitoid *A. atomus* may be less susceptible to the pathogens than the host itself.

1 GENERAL INTRODUCTION

1.1 Origin and distribution of leafhopper species

Leafhoppers are classified in the phylum Arthropoda, class Insecta, and order Homoptera, family Cicadellidae. The family comprises some 5,500 species of polyphagous insects, cosmopolitan in distribution, which feed on several economically important crops (Afscharpour, 1960; Loginova, 1992).

The exact worldwide distribution of many leafhopper species is not precisely known because of the difficulty in differentiating between them. However, according to Ossiannilsson (1981), *Empoasca decipiens* Paoli (Homoptera: Cicadellidae) also called the green leafhopper, is common in Central and Southern Europe, Northern Africa and Central Asia. In Egypt, the green leafhopper has been reported as a main pest on cotton, beans and potatoes (Habib *et al.*, 1972). More recently, *E. decipiens* was recorded as an important pest of cucumbers in southern Germany (Schmidt and Rupp, 1997). The potato leafhopper, *E. fabae* Harris (formerly *E. flavescens* [Fabricius]) is reported as serious pest in the eastern United States, where it causes damage symptoms commonly known as hopperburn on potatoes. It damages many other plants, including apples, beans, and clover.

1.2 Biology and ecology

Some adult leafhoppers are brightly colored, others are green to brown; they generally measure less than 6 mm in length (Schmidt and Rupp, 1997; Ossiannilsson, 1981). The female leafhoppers, usually larger than the males (Hamilton, 1979), lay eggs as a result of mating, although parthenogenic reproduction without fertilization may occur in some cases (DeLong, 1971). Leafhoppers may overwinter in any life stage, but few species pass the winter in more than one life stage. Adult stage is a common for of overwintering (*E. fabae*),

however in some other species (e.g. *E. inimica* Say), overwintering in eggs form ha been reported (DeLong, 1971). Eggs are inserted singly or a few at a time beneath the epidermis of leaves or stems of plants in slits made by the ovipositor. Females begin egg laying 6–7 days after the final nymphal moult. They can live for several months and lay 200-300 eggs during their lifetime (DeLong, 1971).

The eggs are cylindrical, slightly curved, broader and more bluntly rounded at the posterior end (Habib *et al.*, 1972). The duration of the incubation periods is negatively correlated with temperature (e.g. Habib *et al.*, 1972). Raupach *et al.* (2002) recorded in *E. decipiens* (8.19 days) egg duration of 8.19 days at 35°C and 14.88 days at 20°C.

The green leafhopper *E. decipiens* passes through five nymphal instars similar in form except for the size and the wings, which appear from the third instar onwards. In *E. decipiens*, Habib *et al.* (1972) reported a total nymphal development time of 11.8 days at 27°C in Egypt, while in Germany, Raupach *et al.* (2002) recorded 14.8 days at 24°C and observed no larval development at 37.5°C.

1.3 Host plants and economic importance of leafhoppers

Leafhoppers are plant-sucking insects, feeding on vascular tissue mainly on the phloem (Schmidt and Rupp, 1997), whereas Koblet-Günthardt (1975) described it as parenchyma and phloem feeder. Leafhopper damage typically begins with slight leaf curling, progressing to severe downward cupping of the leaves. In highly susceptible plants, the leaf curl is accompanied by leaf yellowing which, in most severe cases, leads to necrosis and subsequent browning of the leaf margins and interveinal areas. These symptoms are referred to as hopperburn as a result of insects injecting toxins into the plant tissues. Other symptoms of damage on bean include stunting, as well as fewer pods with fewer and smaller seeds per pod

(Van Schoonhoven *et al.*, 1978). The economic importance of the leafhoppers is reflected by the wide variety and comparatively large number of host plants (cultivated and non-cultivated) on which they have been recorded (Müller, 1956; Günthart, 1971; Le Quesne and Payne, 1981).

In Egypt, leafhoppers such as *E. decipiens* cause economical important damage on cotton, beans, potatoes and vegetables, including tomatoes, cucumbers, cabbage, sugar beets, sweet pepper and carrots (Habib *et al.*, 1972; El-Dessouki and Hosny, 1969). In Turkey, *E. decipiens* was recorded on sesame (Kersting *et al.*, 1997), while in Syria severe damages by green leafhoppers are common on cucurbits, cotton, grapevine and sugar beet (Silti and Ibrahim, 1991). The potato leafhopper *E. fabae* is the most common insect pest found on field beans in Ontario, Canada (DeLong, 1971) and can be responsible for heavy yield losses if not controlled (Gonzales and Wyman, 1991). It is also a key pest of alfalfa in the north-eastern and north-central United States and southern provinces of Canada (DeLong, 1971). Yield losses of up to 79% have been reported for severe infestation of *E. kraemeri* Ross & Moore in bean fields in Columbia (Kornegay and Cardona, 1990).

1.4 Management options

1.4.1 Chemical and cultural control

At present, control strategies for *E. decipiens* and other leafhoppers mainly rely on either conventional chemical control or breeding for host plant resistance .

The insect growth regulator Buprofezin (Applaud®) has shown high efficacy against the nymphal stages of several leafhopper species including *E. decipiens*, but little activity if adults and leafhopper eggs are considered (Helyer and Talbalaghi, 1994). In Germany however no pesticide is registered for specific control of the green leafhopper.

A study on varietal resistance of castor beans to *E. fabae* showed a positive correlation between organic acid contents in the plants and the preference of the pest for feeding and oviposition (Jayaraj, 1966, cited in Jayaraj, 1967). According to Poos *et al.* (1943), the development and conditions of plants at the time of oviposition and subsequent nymphal feeding may influence the frequency and intensity of *Empoasca* spp. infestations. The same authors reported increased oviposition and enhanced development of nymphs of *E. fabae* on young compared to older peanut plants. However, to date no report on selection of resistant varieties in practical control of *E. decipiens* is available in the literature.

1.4.2 Biological control as alternative to conventional pesticides

Bugs of the genus *Orius* (Het.: Anthocoridae), mainly *O. minutus* (L.) and *O. insidious* Say are frequently cited as predators of *Empoasca* spp. (Vietmeier *et al.*, 1996). According to Kühne (1998), *Coenesia* spp. (Dipt.: Muscidae) are the only natural enemies of adult leafhoppers. However, first releases of *Coenesia* spp. under practical conditions on the island Reichenau in southern Germany failed to prevent high infestations of *E. decipiens* (Schmidt and Rupp, 1997). Because of the high mobility of leafhopper nymphs and adults biological control of *E. decipiens* by means of releasing predators has so far failed (Helyer and Talbalaghi, 1994).

Several mymarids in the genus *Anagrus* such as *A. atomus* L. have been reported to parasitize the eggs of leafhoppers (Cronin and Strong, 1990; Williams and Martinson, 2000). While *A. atomus* is well known as an egg parasitoid of *Hauptidia marocana* Melichar (Hom.: Cicadellidae), an important leafhopper pest attacking tomatoes in greenhouses in the UK (Wardlow and Tobin, 1990), little is known about the efficacy of *A. atomus* for control of *Empoasca* species. First releases of the parasitoid in southern Germany did not lead to sufficient control in *E. decipiens* (Rupp, 1999). Due to these constraints, the development of

alternative methods for control of *E. decipiens* within the context of IPM has become indispensable, particularly in the greenhouse environment, since chemical control of leafhoppers threatens to disrupt the otherwise successful biological control of key pests in greenhouses like white flies, spider mites, leaf miners and aphids.

Microbial pest control, using entomopathogenic fungi could become a valid and environmentally more friendly alternative to chemical control of leafhoppers, particularly since various leafhopper species have shown to be susceptible to fungal infections (Bachchhav and Hapase, 1980; Nayak and Srivastava, 1982; Soper, 1985). In addition, the great mobility of leafhoppers could facilitate a fast spread of the pathogens, finally resulting in epizootics. Entomopathogenic deuteromycete fungi of the genera *Beauveria*, *Metarhizium*, *Paecilomyces* and *Verticillium* have been greatly exploited as microbial control agents against various pests and have shown considerable impact in both field and laboratory studies (e.g. Vestergaard *et al.*, 1995; Genthner *et al.*, 1997; Langewald *et al.*, 1999).

1.5 Entomopathogenic fungi

1.5.1 Diversity and geographic distribution

Entomopathogenic fungi are important pathogens of many insects and other arthropods and frequently cause epizootics that can significantly reduce pest populations all over the world. Approximately 750 species of entomopathogenic fungi are known to infect numerous economically important arthropod pests and have thus received considerable attention as potential microbial control agents (Gillespie and Moorhouse, 1989; Mulock and Chandler, 2000; Haraprasad *et al.,* 2001). The majority of these entomopathogenic fungi belong to the classes of Zygomycetes, Ascomycetes and Deuteromycetes. Entomopathogenic fungi are commonly found in aquatic, terrestrial and subterranean habitats (Ferron, 1978a). Among

them, *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sorokin, *Paecilomyces fumosoroseus* (Wize) Brown & Smith and *Verticillium lecanii* (Zimm.) Viégas (Deuteromycotina: Hyphomycetes) represent the most frequently used species and are known to infect many insect species, with sometimes rather species specific impact against their hosts. Hence, it is necessary to evaluate the host specificity of the strains intended to be used within the framework of IPM.

***Beauveria bassiana* (Bals.) Vuill.**

The genus *Beauveria* includes different species, among which *B. bassiana* is the most commonly identified entomopathogenic fungus. It infects both immature and adults of many insect species in the orders of Lepidoptera, Coleoptera, Homoptera, Hemiptera, and Hymenoptera. The fungus was the first microorganism to be identified as a disease agent of insects by Bassi in 1835. *Beauveria bassiana* has been reported on a wide range of insects, including the rice green leafhopper, *Nephotettix nigropicus* Stål, and the green paddy leafhopper *N. virescens* (Distant) (Nayak and Srivastava, 1982). This fungus has a great potential as microbial control agent due to its easy mass production and formulation, as well as its strain-specific pathogenicity to many target organisms (Crawford *et al.,* 1998, cited in Haraprasad *et al.,* 2001; Zurek *et al.*; 2000).

***Metarhizium anisopliae* (Metsch.) Sorokin**

The genus *Metarhizium* includes three species, i.e. *M. anisopliae* with four varieties (var. *acridium, anisopliae, lepidiotum,* and *majus*), *M. flavoviride* (Gams and Roszypal) with five varieties (var. *flaviride, minus, novazealandicum, pemphigumand* var. type E) and *M. album* (Petch) (Tulloch, 1976; Driver *et al.*, 2001).

Metarhizium anisopliae occurs on a wide range of insects in the orders of Orthoptera, Coleoptera, Lepidoptera, Hemiptera and Hymenoptera, as well in Arachnida. Various studies have shown the promising potential of *M. anisopliae* as a microbiological control agent of important pests. For instance *M. anisopliae* has been reported from the sugarcane leafhopper, *Pyrilla purpusilla* (Walker) in India (Bachchhav and Hapase, 1980). Treatment of Western Flower Thrips *Frankliniella occidentalis* (Pergande) adults by *M. anisopliae* resulted in at least 94% mortality 7 days post-inoculation (Vestergaard *et al.,* 1995). In addition, *M. anisopliae* has also a great potential to control several domestic pests such as cockroaches (Pachamuthu *et al.*, 1999). A product of *M. anisopliae* called BIO 1020 is registered in Germany for control of the black vine weevil, *Otiorhynchus sulcatus* and *Hepialus* spp. This product has also been successfully tested by Malsam *et al.* (2002) against whiteflies in the greenhouse.

***Paecilomyces fumosoroseus* (Wize) Brown & Smith**

Paecilomyces fumosoroseus is a filamentous soil-inhabiting fungus, and a common pathogen of many insects. It has been isolated from a wide variety of insects from different orders located throughout the world (Humber, 1992). *Paecilomyces fumosoroseus* is a promising candidate for control of the silverleaf whitefly *Bemisia argentifolii* (Bellows & Perring) and the tobacco whitefly *B. tabaci* (Gennadius) (Lacey *et al.*, 1993, cited in Vidal *et al.*, 1997a; Wraight *et al.*, 1998) both major pests in the field and greenhouses. The fungus is characterized by its rapid growth both on artificial medium and on infected insects (Wraight *et al.*, 1998). Different isolates are able to tolerate low and high temperatures, with intraspecific variability related to the microclimatic requirements of the fungus biotypes (Vidal *et al.*, 1997b).

***Verticillium lecanii* (Zimm.) Viégas**

Verticillium lecanii is an ubiquitous fungus with a wide host spectrum attacking insects, mites, spiders, nematodes, and phytopathogenic fungi and it is currently commercialised in Europe for the control of aphids and whiteflies in the greenhouse (Askary *et al.*, 1999). Recently, a revision of the genus *Verticillium* section Prostrata has been published (Zare and Gams, 2001), and the species *V. lecanii* has been divided into four new species *Lecanicillium lecanii*, *L. muscarium*, *L. longisporum* and *L. nodulosum.* The fungal mycelium of *V. lecanii* produces different toxins, which have been shown to possess insecticidal activities (Gindin *et al.*, 1994, cited in Askary *et al.,* 1999). Certain strains have also been reported to be candidates for biological control of powdery mildew on cucumbers (Verhaar *et al.*, 1996; Verhaar *et al.*, 1997) and rust (Allen, 1982, cited in Askary *et al.*, 1999).

1.5.2 Life cycle of entomopathogenic fungi and mode of action

In general, the life cycle begins with the attachment of the spore to the cuticle, then spore germination and active penetration of the host's cuticle, followed by a rapid proliferation of the fungal cells in the host's body. The death of the infested host is followed by the outgrowth of the fungus and the production of infective spores, which can immediately repeat the cycle. The processes of spore germination and growth on the cuticle are highly dependent on both biotic and abiotic factors. The importance of abiotic factors, such as temperature and relative humidity and light, on the infection by entomopathogenic fungi has been intensively investigated (e.g. Milner *et al.,* 1997; Estrada *et al.*, 2000). Abiotic factors affect both fungal sporulation and the survival of the conidia (Milner *et al.*, 1997). On the other hand, biotic factors influencing fungal infections include microbial antagonists on the host insect integument, host susceptibility and, most important, the varying degree of virulence of the fungal strains.

In contrast to entomopathogens like bacteria, nematodes and viruses that infect the host insect through its gut, fungal infection occurs through the insect's cuticle. The fungal spores contact the host and release enzymes, which attack and dissolve the cuticle. The adhesion of the insect cuticle appears to be a prerequisite for successful invasion of all entomopathogenic fungi (Quintela and McCoy, 1998). Once the spore has attached to the insect cuticle, it germinates and produces a germ tube, which then actively penetrates the host integument. Some strains of *V. lecanii* with low pathogenicity are reported to take longer to germinate and grow extensively over the cuticle surface with limited penetration, whilst strains with high pathogenicity germinate faster and penetrate directly the epicuticle (Schreiter *et al.*, 1994). In the insect body, the fungus rapidly multiplies throughout the body and uses it as a nutrient source. Mortality is due to tissue destruction and occasionally evoked by toxins produced by the fungi (Kershaw *et al.*, 1999).

1.6 Statement of the research work

The green leafhopper *E. decipiens* is an important greenhouse pest, which causes serious damages to economically important plants by feeding directly on leaves and/or fruits. Conventional control strategies mainly focus on the application of synthetic pesticides. Moreover, so far no biological and/or microbiological control strategies have been developed against the pest.

Although entomopathogenic fungi are potentially promising microbial control agents of *Empoasca* spp. (e.g. Zang *et al.*, 1976; Ben-Ze'ev and Kenneth; 1981), no in-depth study has yet been carried out elucidating their role as microbiological agents against *E. decipiens*.

To identify specifically effective strains of entomopathogenic fungi species against *E. decipiens*, initially various fungal strains need to be screened under laboratory conditions.

Thereafter, the effect of the most promising strains on the development of the pest and on possible side effects on its common natural enemy the egg parasitoid *A. atomus* have to be investigated. Such information can contribute to the development of an environmental friendly IPM strategy against *E. decipiens*.

1.7 Objectives of the research work

The main goal of this study is to gather basis data on the development of IPM strategies for control of the green leafhopper *E. decipiens* using entomopathogenic fungi. The study was divided into two parts: (i) the first part of the study aimed to select the most effective fungal strains against *E. decipiens* under laboratory conditions and to evaluate the effect of selected strains on various life table parameters of the leafhopper; (ii) in the second part, greenhouse tests with selected strains were done to evaluate their efficacy against *E. decipiens* under more practical conditions and to determine the residual activity of their applications on the bean foliage over time. In additional laboratory tests, potential side effects of selected strains on the egg parasitoid *A. atomus* were investigated under laboratory conditions because *A. atomus* is the most promising natural enemy for biocontrol of *E. decipiens* and a combination of fungi and parasitoid may be an interesting IPM approach. .

1.8 Hypothesis

To achieve our objectives, we started with the following hypotheses:

(i) Entomopathogenic fungi are important natural control factors for insects. However, host range and host specificity vary between fungal strains (Castillo *et al.*, 2000; Gindin *et al.*, 2001). Moreover, because the insect cuticle is the first barrier in the fungal infection process, insect developmental stages may greatly affect the efficacy of entomopathogenic fungi (Bittencourt *et al.*, 1994; Gindin *et al.*, 2001). Thus screening of different fungal isolates and

study on different developmental stages of the green leafhopper could be important parameters to evaluate the potential of entomopathogenic fungi to control the pest.

(ii) Entomopathogenic fungi with a broad host range such as *M. anisopliae*, *B. bassiana* and *P. fumosoroseus*, may not kill only the target insects but also negatively affect non-target ones including beneficial organisms. For example parasitoid wasps have proven susceptible to direct *B. bassiana* application (e.g. Danfa and van der Valk, 1999) and to *M. anisopliae* (e.g. Husberg and Hokkanen, 2001). Hence, investigation on interactions between entomopathogenic fungi and the natural enemy *A. atomus*, the most promising egg parasitoid of *E. decipiens* could be an important prerequisite for any further combination of the two control agents in IPM program for the control of the pest.

2 SUSCEPTIBILITY OF NYMPHAL NYMPHAL STAGES OF EMPOASCA DECIPIENS TO SELECTED STRAINS OF ENTOMOPATHOGENIC FUNGI

2.1 Abstract

The efficacy of five entomopathogenic fungal strains against the green leafhopper *Empoasca decipiens* was investigated under laboratory conditions. The fungal strains screened included two *Metarhizium anisopliae* strains (Ma43 and Ma57), and one strain of *Beauveria bassiana* (Bba113), *Paecilomyces fumosoroseus* (Pfr12) and *Verticillium lecanii* (Vl23). In screening experiments, all strains proved to be highly virulent to late fifth instar nymphs of *E. decipiens*. Single host insect passage of the isolates resulted in faster and/or higher virulence of the pathogens. The most virulent strains, Bba113, Pfr12 and Ma43, yielded nymphal mortality ranging from 88-97% within seven days of exposure at a spore concentration of 1×10^7 conidia/ml. In dose-response experiments significantly increasing mortalities with rising dose rates (i.e. 1×10^3, 1×10^4, 1×10^5, 1×10^6) were recorded. Older nymphs (3^{rd} and 5^{th} instars) were more susceptible than younger ones (1^{st} instar). LD_{50} values ranged from 1.3×10^4 to 9.4×10^5 conidia/ml for Bba113, 6×10^3 to 2.2×10^4 conidia/ml for Ma43 and 6.4×10^3 to 1.5×10^4 conidia/ml for Pfr12. All tested strains could complete their life cycle by forming conidiospores on the cadavers, usually one to two days after the host had died. The fastest and most profuse growth on cadavers was observed after treatments with *P. fumosoroseus* strain Pfr12.

2.2 Introduction

The green leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae) is an important pest of a wide range of economically important crops (Loginova, 1992). It is common in Central and Southern Europe, Northern Africa and Central Asia where it is found both in greenhouses and in the field (Ossiannilsson, 1981). In Germany, *E. decipiens* is a particularly

serious pest of vegetables under protected cultivation, where it was first recorded attacking cucumbers in 1995 on the Reichenau Island in southern Germany (Schmidt and Rupp, 1997). Subsequent outbreaks in cucumbers in 1997 led to a complete loss of marketable fruits (Schmidt and Rupp, 1997).

The economic importance of *E. decipiens* is reflected by a large number of host plants (cultivated and non-cultivated) on which it has been recorded and can be reared on (Paoli, 1930). Females lay eggs in the plant tissue and both nymphs and adults of *E. decipiens* damage plants directly by sucking on the leaves and fruits (Helyer and Talbalaghi, 1994). Characteristic damage symptoms vary from yellowish discoloration of leaves (hopperburn) to lines of punctures that resemble stitch marks when *E. decipiens* feeds on fruits. Such damage often leads to downgrading of the produce.

Currently, control strategies for leafhoppers mainly rely on the use of synthetic insecticides. The insect growth regulator Buprofezin is recommended for chemical control against *E. decipiens* and has proved to effectively control the pest and cause little to no harmful effects on natural enemies in greenhouses. However, it does not control the egg stage and adults of leafhoppers (Helyer and Talbalaghi, 1994). Chemical control of leafhoppers is difficult due to the lack of appropriate insecticides and the still unclear relationships between infestations and economic losses caused by many *Empoasca* spp. (Maixner *et al.*, 1998). Moreover, insecticide applications against leafhoppers most often cause harmful side effects on beneficial organisms, particularly natural enemies like predators and parasitoids (e.g. El-Nawawy *et al.*, 1983).

Attempts for biological control of leafhoppers have mainly concentrated on the use of predators and parasitoids. Insect parasitoids of three taxonomic groups, i.e. Mymaridae,

Dryinidae and Pipunculidae, have been reported to parasitize leafhoppers in German vineyards (Maixner *et al.*, 1998). However, egg parasitism by Dryinidae and Pipunculidae is often too low for significant effects on their host population density. Moreover, biological control of leafhoppers through releases of predatory arthropods is difficult and often not very successful since the adults and nymphs move too fast to be captured by commonly used predators in greenhouses such as *Orius* spp. (Heteroptera: Anthocoridae) (Helyer and Talbalaghi, 1994).

The lack of appropriate biological control strategies for *E. decipiens*, and the resulting reliance on chemical control might threaten the otherwise very successful biological control of other important pests in greenhouses like aphids, spider mites, leafminers and white flies. Thus, the development of alternative control methods for *E. decipiens* has become of paramount importance for the successful use of biological control within the context of integrated pest management (IPM) in European greenhouses. The feeding behaviour of leafhoppers (i.e. feeding on the plant sap by piercing the plant cuticle with their sucking mouth-parts), does not allow the use of viral, bacterial or protozoan pathogens that invade their host perorally. However, germinating spores of entomopathogenic fungi infect their hosts percutaneously, and might be better suited for microbial control of leafhoppers. Although many species of entomopathogenic fungi are known to infect various leafhoppers species (Soper, 1985), at present no specific data on the efficacy of these pathogens for the control of *E. decipiens* are available. The main objective of the present study was to gather basic information on the host-pathogen interactions between strains of *Metarhizium anisopliae* (Meschnikoff), *Beauveria bassiana*, (Bals.), *Paecilomyces fumosoroseus* (Wize) Brown & Smith and *Verticillium lecanii* (Zimm.) Viégas (Deuteromycotina, Hyphomycetes) and *E. decipiens.*

2.3 Materials and Methods

2.3.1 Insect and fungal cultures

Specimens of *E. decipiens* were originally obtained from the Federal Biological Research Centre for Agriculture and Forestry (BBA) in Braunschweig, Germany. The insects were reared continuously on broad beans (*Vicia faba* L.) at 24°C at 16:8 h photoperiods and RH of 60-70%, following the protocol developed by Raupach *et al.* (2002).

All entomopathogenic fungal strains (Table 1.1) tested in this study were provided by Dr. Gisbert Zimmermann (BBA, Institute for Biological Control, Darmstadt, Germany).

Table 2.1 Entomopathogenic fungal strains tested

Strains	Codes	Hosts
Beauveria bassiana	Bba113[a]	*Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae)
Metarhizium anisopliae	Ma43[b]	*Carpocapsa pomonella* L. (Lepidoptera: Tortricidae)
M. anisopliae	Ma57[c]	*Deois flavopicta* Stål (Homoptera: Cercopidae)
Paecilomyces fumosoroseus	Pfr12[d]	*Bemisia argentifolii* Bellows & Perring (Homoptera: Aleyrodidae)
Verticillium lecanii	Vl23[e]	*B. tabaci* Gennadius (Homoptera: Aleyrodidae)

[a] Isolate provided by Dr. J.B. Speakman, BASF, Germany.

[b] Infected insects provided by Dr. Russ, Austria. This strain formed the basis of the commercial myco-insecticide Bio 1020™, Bayer Ltd., Germany.

[c] Isolate provided by Dr. Roberts, USA; strain originally isolated from *Deois flavopicta* Stål (Hemiptera: Cercopidae) in Brazil.

[d] Isolated from the commercial myco-insecticide Preferal™, Biobest Ltd., Belgium, originally isolated from *B. argentifolii* in Florida, USA (the so-called Apopka strain 97).

[e] Isolate from the commercial myco-insecticide Mycotal™, Koppert Ltd., The Netherlands.

The fungi were grown at 25°C on malt extract-peptone agar medium between ten days to two weeks and prepared for bioassays. Conidia were harvested by washing the plates with an sterile aqueous solution of 0.1% Tween 80. This suspension was agitated for four minutes on a shaker to break up conidial clumps. The final concentration of the suspension was estimated using standard Thomas chamber counting techniques (Goettel and Inglis, 1997).

The average germination rate of conidia used in various tests was over 95%, as determined on yeast extract-peptone-glucose agar medium in dark after 24 hours at 25°C. The percentage of germinated conidia was estimated counting under the microscope 100 germinated and non-germinated conidia from three separate (1 x 1 cm^2) squares of agar. Any spore with a germinating tube was considered as germinated.

2.3.2 Bioassay procedure: screening and insect host passage re-infection tests

Discs (3 cm in diameter) from fleshly picked bean leafs were treated by dipping into 2 ml of conidia-0.1% Tween 80 suspension (1 x 10^7 conidia/ml) or 0.1%Tween 80 (control) for about one minute. The leaves were then dried at ambient temperature for 10 to 15 minutes under a laminar flow hood, and transferred to experimental units (9 cm diameter Petri dishes containing three 4 cm diameter Petri dishes with the leaf discs). Using a paintbrush, fifth late instar *E. decipiens* nymphs were carefully transferred to the experimental units containing the treated leaf discs. The leaf discs were changed every two to three days. Following this methodology leafhoppers could be kept on fresh leaves throughout the whole experimental period of seven days. Test insects were kept in the discs under nearly saturated relative humidity (95 ± 5 %), by placing moistened sterile 85 mm filter paper inside the 9 cm Petri dish, at a temperature of 25 ± 1°C under a 16:8 L:D light regime. Mortality was assessed daily for seven days.

To determine whether the virulence of the fungi was altered after the host passage, and to test the strains according to Koch's postulates, the conidia were re-isolated after a single passage through *E. decipiens* nymphs (see the results). Thereafter conidia of the tested strains were further grown on malt extract-peptone agar for two weeks, and tested for virulence against late fifth instar nymphs, again at a dose of 1×10^7 conidia/ml using the same experimental procedure as previously described. In both screening and re-infection tests four replications with 12 nymphs each were used per treatment.

2.3.3 Dose-response tests on three insect developmental stages

Based on the results of the initial screening experiments, three fungal strains, i.e. *M. anisopliae* strain Ma43, *B. bassiana* strain Bba113 and *P. fumosoroseus* strain Pf12, identified as most virulent against fifth instar nymphs of *E. decipiens*, were selected for further investigations. Fungal preparations were prepared as described above and tenfold serial dilutions were prepared from a standard concentration of 1×10^7 conidia/ml to 1×10^3 conidia/ml. The four concentrations (1×10^6, 1×10^5, 1×10^4 and 1×10^3 conidia/ml) were tested against three nymphal instars (first, third and fifth) of *E. decipiens*. For the dose-response test, the bioassay procedure described above was used. Each dose rate for a given strain was applied on each developmental stage separately, tested with four replications (12 nymphs each) and repeated three times.

2.3.4 Mycosis on dead insects

In case dead insects were encountered, cadavers were immediately removed from the experimental units and transferred onto sterile microscope slides placed inside sterile Petri dishes, containing 85 mm diameter moistened sterile filter paper. Cadavers were monitored

daily under a stereomicroscope for fungal outgrowth and sporulation on the insect cuticle. Spores from dead insects were re-isolated and identified.

2.3.5 Statistical analysis

In initial screening and re-infection tests, cumulative mortalities were analysed using multivariate analysis (profile analysis, repeated measures two-ways ANOVA), with one between-factor (isolates) and one within-factor (time) with seven levels (day1 – day 7). Dose response cumulative mortalities were analysed for each developmental stage using ANOVA, profile analysis repeated measures, with one between-factor (Isolates) and two within-factors, (Dose) with four levels (1×10^6, 1×10^5, 1×10^4 and 1×10^3 conidia/ml) and (time) with seven levels (day 1 – day 7). To identify particular time intervals in which treatment effects are different, individual ANOVAs (F-tests) were computed on each of the six different contrasts of the adjacent days (time_N: the nth successive differences in time) and the overall α of 0.05 was maintained using a Bonferroni adjustment of $\alpha/6 = 0.0083$ for each contrast (Scheiner and Gurevitch, 1993).

The efficacy of the different isolates on different developmental stages of the host was compared using the final mortalities (seven days cumulative mortalities) after arcsine transformation to normalize variances for all analyses. The normalized percent mortalities were subjected to ANOVA. In case of significant F-values, differences in mean cumulative mortality at different levels of each factor were compared using Tukey's multiple mean comparison procedure (SAS Institute, 1996). SAS probit analysis was used to evaluate LD_{50}, LD_{90} and Confidence Limits (CL). Significant differences in susceptibility were defined by non-overlap of 95% CL of LD_{50} and LD_{50} (Savin *et al.*, 1977). SAS regression procedures (PROC REG, SAS Institute, 1996) were used to determine the relationships between mortalities and tested dose rates. A significance level of $\alpha = 0.05$ was used in all analyses.

2.4 Results

2.4.1 Initial screening and re-infection host passage tests against late fifth instar nymphs of *E. decipiens*

With 12.5 % (SE = 2.4) and 8.3% (SE = 3.4) control mortalities in the initial screening and re-infection tests, respectively, were comparatively low. However, all fungal strains tested were pathogenic to *E. decipiens*, causing significantly higher mortality levels than in the control ($P < 0.0001$). In both tests, significant differences in mortality of fifth instar nymphs were observed between the tested fungal strains ($F = 5.73$; $df = 4$; $P = 0.0070$ for the initial screening) and ($F = 20.48$; $df = 4$; $P < 0.0001$ for the re-infection tests), with highest mortalities recorded in Ma43, Pfr12 and Bba113 and lowest in Ma57 and Vl23. However, in all fungal strains, total nymphal mortality exceeded 65% at the end of the experiments (i.e. seven days post treatment) (Figs. 2.1 and 2.2)

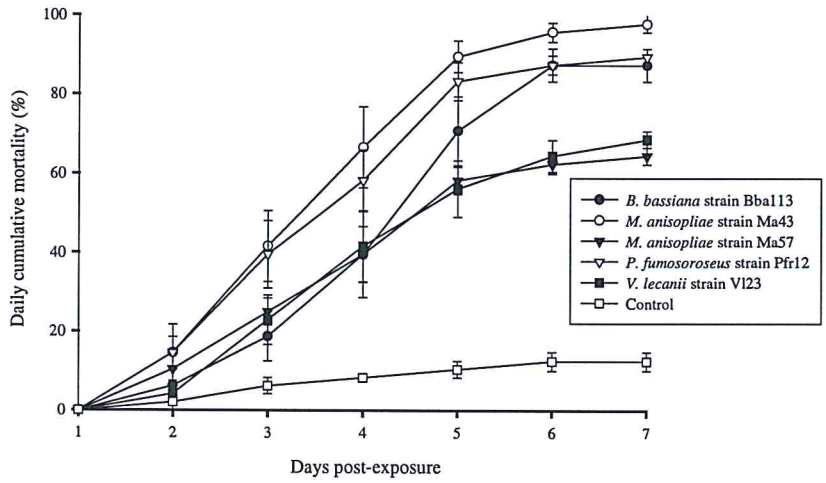

Figure 2.1 Daily cumulative mortality (mean ± SE) of late fifth instar nymphs of *Empoasca decipiens* caused by five different entomopathogenic fungal strains (*Beauveria bassiana* (Bba113), *Metarhizium anisopliae* (Ma43 and Ma57), *Paecilomyces fumosoroseus* (Pfr12) and *Verticillium lecanii* (Vl23)) at a concentration of 1 x 10^7 conidia/ml in initial screening.

In both tests, all isolates tested resulted in significant increasing mortality over time (Table 2.2), with significant increase in specifical time period from day one to days six. No further

increase in mortality was found after day six in the initial screening (F = 4.62; df = 1; P = 0.051) and re-infection tests (F = 2.00; df = 1; P =0.1778), respectively.

Single host passage resulted in significant increase in mortality caused by Pfr12 and Ma57 (100 % (SE = 0.0), and 81.25% (SE = 2.21) respectively) when compared to the initial screening (89.58% (SE = 2.08) and 64.60% (SE = 2.08) respectively). However, no significant differences were found in strains Vl23, Ma43 and Bba113 between the initial screening and re-isolation tests ($P > 0.05$).

Table 2.2 Repeated-measures ANOVA of cumulative daily mortality of late fifth instar nymphs caused by *Beauveria bassiana* strain Bba113, *Metarhizium anisopliae* strains Ma43 and Ma57, *Paecilomyces fumosoroseus* strain Pfr12 and *Verticillium lecanii* strain Vl23 in initial screening (A) and after host passage re-infection (B) tests

A: Initial screening

Between-factor source	df	MS	*F*	*P>F*
Isolate	4	2511.64	5.73	0.007
Error	13	438.22		
Within-factor source				
Time	6	19318.23	340.56	< 0.0001
Isolate x Time	24	217.57	3.84	< 0.0001
Error (Time)	78	56.73		

B: Host passage re-infection test

Between-factor source	df	MS	*F*	*P>F*
Isolate	4	2675.35	20.48	< 0.0001
Error	15	130.62		
Within-factor source				
Time	6	26811.84	971.01	< 0.0001
Isolate x Time	24	165.22	5.98	< 0.0001
Error (Time)	90	27.62		

Although all fungal strains tested showed high levels of pathogenicity in both initial screening and after single host insect passage experiments, and differed in the speed of infection of *E. decipiens* nymphs. The first insects showing signs of infection (i.e. leafhoppers becoming

immobile) were observed one-day post exposure, and initial mortality was recorded two days after inoculation.

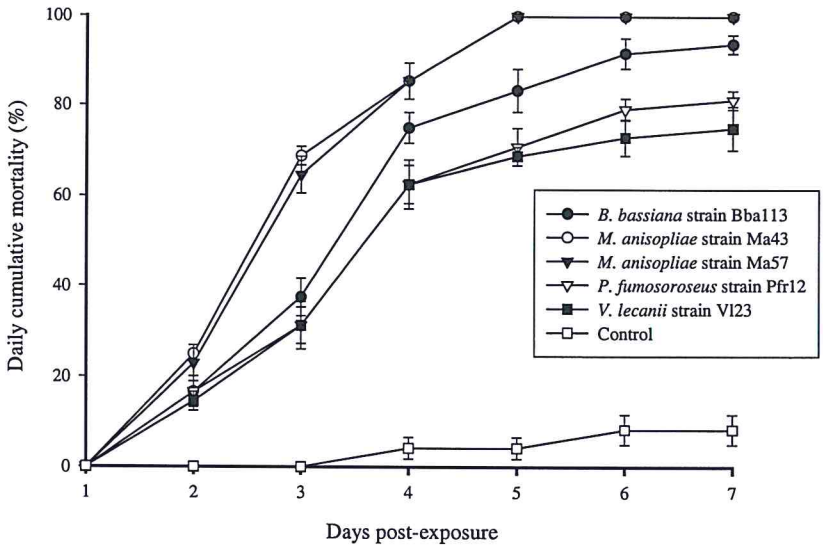

Figure 2.2 Daily cumulative mortality (mean ± SE) of late fifth instar nymphs of *Empoasca decipiens* caused by five different entomopathogenic fungal strains (*Beauveria bassiana* (Bba113), *Metarhizium anisopliae* (Ma43 and Ma57), *Paecilomyces fumosoroseus* (Pfr12) and *Verticillium lecanii* (Vl23)) at a concentration of 1 x 10^7 conidia/ml in re-infection tests after a single host passage.

For example four days after exposure, all tested isolates resulted in significantly higher mortality ($F = 35.14$; $df = 9$; $P < 0.0001$), i.e. 75, 85, 62.5, 85 and 62.5% respectively for Bba113, Ma43, Ma57, Pfr12 and Vl23 after single host insect passage compared to the initial screening, i.e. 39.6, 68.8, 39.6, 39.7, 56.3 and 41.7% respectively for Bba113, Ma43, Ma57, Pfr12 and Vl23.

2.4.2 Effect of conidia concentrations on mortality of different *E. decipiens* developmental stages

Based on the results of profile analysis repeated-measures ANOVA of the dose response tests, mortalities increased significantly over time in the first, ($F = 3131.09$; $df = 6$; $P < 0.0001$) third ($F = 199.74$; $df = 6$; $P < 0.0001$) and fifth instars nymphs ($F = 1815.83$; $df = 6$; $P <$

0.0001). Except for the time interval from day 4 to 5 in first instar nymphs (F = 3.69; df = 1; P = 0.1033) and in third instar nymphs for day 3 to 4 (F = 1.64; df = 1; P = 0.2474) and day 5 to 6 (F = 3.01; df = 1; P = 0.1334), mortality levels always significantly increased with time, particularly between day 1 to day 7. In contrast, in fifth instar nymphs, continuous increase in mortality was observed from day 3 to day 7, i.e. the end of the experiment. Hence, the efficacy of the different isolates on the three nymphal stages of *E. decipiens* was compared using the final mortality (i.e. seven days cumulative mortality).

In the control, mortality rates of 8.3% (SE = 3.2), 6.9% (SE = 1.4) and 10.4% (SE = 1.2) were recorded in first, third an fifth instar nymphs, respectively. All fungal strains, tested at all dose rates studied caused significantly higher mortality than in the control ($P < 0.0001$). Significant differences between fungal strains (F = 52.69; df = 2; $P < 0.0001$), spore concentrations (F = 343.41; df = 3; $P < 0.0001$) and insect developmental stages (F = 54.26; df = 2; $P < 0.0001$), with significant interactions between the three factors (F = 3.10; df = 18; P = 0.0003) were observed. No significant differences in mortality between the different developmental stages of *E. decipiens* were found in the control (F = 0.68; df = 2; P = 0.542). In contrast, all tested stages of *E. decipiens* were highly susceptible to the selected strains, but to varying degrees (Figs. 2.3; 2.4 and 2. 5). Except for Ma43 on 3rd instar nymphs (r^2 = 0.19; P = 0.74), the dose-response tests for all isolates resulted in significant and positive linear correlations between applied dose rates and nymphal mortality. The calculated LD_{50} and LD_{90} values and their 95% confidential limits show significantly higher LD_{50} and LD_{90} values of first instar nymphs than those of third and fifth instar nymphs (Table 2.2).

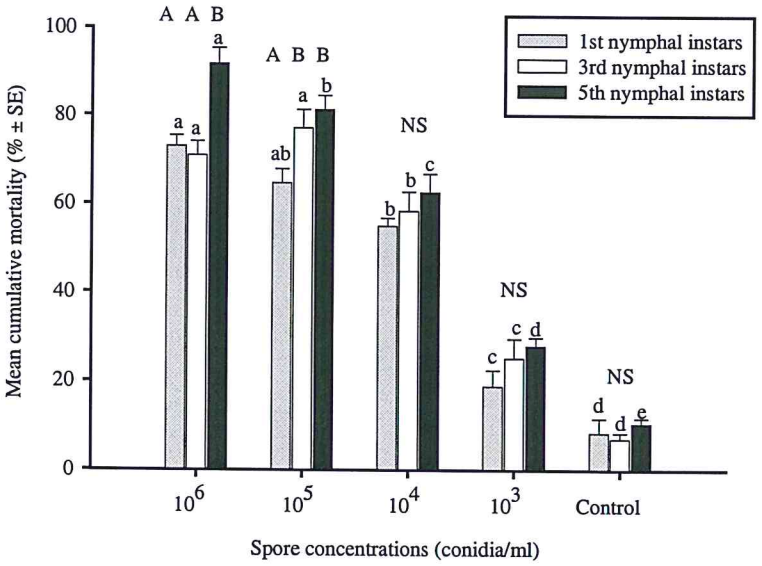

Figure 2.3 Seven days cumulative mortality (mean ± SE) of first, third and fifth instars of *Empoasca decipiens* caused by *Beauveria bassiana* strain Bba113 in dose rate studies. Lower case letters indicate significant differences per nymphal instar for all tested dose rates; upper case letters indicate significant differences per dose rate between the three tested nymphal instars.

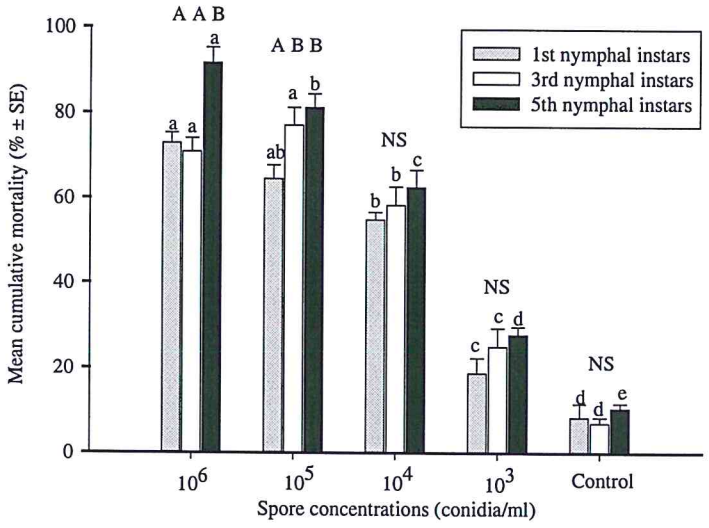

Figure 2.4 Seven days cumulative mortality (mean ± SE) of first, third and fifth instars of *Empoasca decipiens* caused by *Metarhizium anisopliae* strain Ma43 in dose rate studies. Lower case letters indicate significant differences per nymphal instar for all tested dose rates; upper case letters indicate significant differences per dose rate between the three tested nymphal instars.

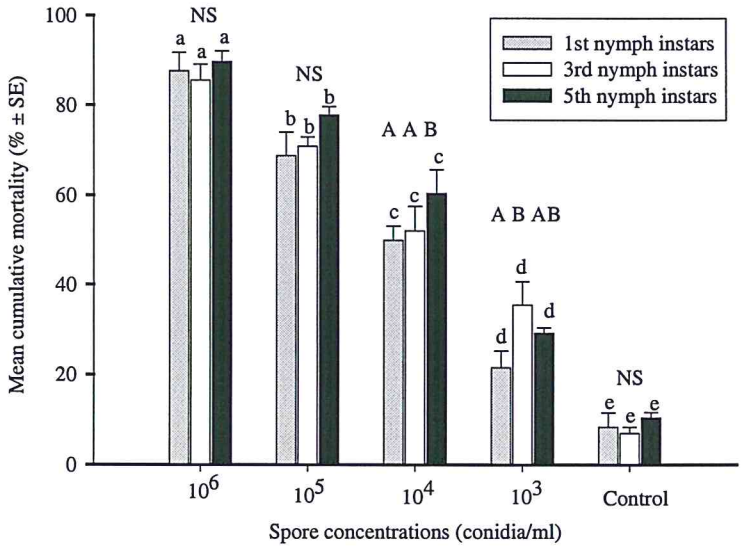

Figure 2.5 Seven days cumulative mortality (mean ± SE) of first, third and fifth instars of *Empoasca decipiens* caused by *Paecilomyces fumosoroseus* strain Pfr12 in dose rate studies. Lower case letters indicate significant differences per nymphal instar for all tested dose rates; upper case letters indicate significant differences per dose rate between the three tested nymphal instars.

2.4.3 Mycosis tests

Observations under the microscope of leafhoppers treated with the different fungal strains immediately after death, revealed that the insects had picked up the spores mainly with their legs and antennae. On leafhoppers that had died in the untreated controls, no sporulation was observed on the cadavers. However, nearly in all insects treated with entomopathogenic fungi, sporulation on the surface of the insect bodies commenced usually 24 to 48 hours after death. The initial external growth of the fungi began primarily on the antennae, legs, and pleural regions of the head and from intersegmental regions, especially between the head and the thorax, and between the thorax and the abdomen. Within four to six days the sporulating growth had gradually covered the cadavers and spread outwards.

Table 2.3 Dose effect of *Beauveria bassiana* (strain Bba113), *Metarhizium anisopliae* (strain Ma43) and *Paecilomyces fumosoroseus* (strain Pfr12) on three nymphal stages of *Empoasca decipiens* as determined by probit analysis.

Strains	Stages	Slope	LD_{50}[a]	95%CL[a]	LD_{90}[b]	95%CL[b]	r^2	p
Bba113	1st	0.31	8.9×10^5a	$3.2 \times 10^5 - 5 \times 10^6$	1.2×10^{10}a	$0.5 \times 10^9 - 0.4 \times 10^{12}$	0.46	0.794
	3rd	0.39	0.4×10^5b	$0.2 \times 10^5 - 0.7 \times 10^5$	7.2×10^7ab	$0.2 \times 10^7 - 0.1 \times 10^{10}$	1.30	0.523
	5th	0.56	0.1×10^5b	$0.5 \times 10^4 - 0.3 \times 10^5$	0.2×10^7b	$0.1 \times 10^7 - 0.2 \times 10^8$	7.02	0.03
Ma43	1st	0.47	2.2×10^4	-[c]	1.3×10^7	-[c]	14.43	0.0007
	3rd	0.46	0.1×10^5	-[c]	1.1×10^7	-[c]	23.91	0.005
	5th	0.67	0.5×10^4b	$0.3 \times 10^4 - 0.8 \times 10^4$	0.5×10^6b	$0.2 \times 10^6 - 1 \times 10^7$	3.43	0.1801
Pfr12	1st	0.63	0.2×10^5c	$0.9 \times 10^4 - 0.3 \times 10^5$	0.2×10^7b	$0.8 \times 10^6 - 0.4 \times 10^7$	1.31	0.52
	3rd	0.48	0.1×10^5bc	$0.4 \times 10^4 - 0.1 \times 10^5$	0.3×10^7b	$0.1 \times 10^7 - 0.2 \times 10^8$	0.15	0.929
	5th	0.60	0.1×10^5bc	$0.3 \times 10^4 - 0.1 \times 10^6$	0.1×10^7b	$0.4 \times 10^6 - 0.2 \times 10^7$	2.40	0.302

Regression equation following probit analysis: Arcsine (% Mortality) = log (Dose) + C

[a] LD50 and their 95% confidence limits as expressed in conidia/ml of solution (0.1 Tween 80%);

[b] LD90 and their 95% confidence limits as expressed in conidia/ml of solution (0.1 Tween 80%);

[c] Unable to estimate confidence limits from the data.

Values within the same row followed by the same letters are not significantly different ($P < 0.05$). Differences between LD_{50} and LD_{90} values are determined by probit analysis followed by pair wise comparisons using non-overlapping confidence limits (CL).

Although the sporulation level was not assessed in this study, particularly fast and profuse fungal growth was observed on leafhoppers killed by *P. fumosoroseus* strain Pfr12. Four days after death cadavers showed diffuse hyphal growth and sporulation with the entire cadavers coated in a dense mat of white conidiophores (Photo 2.1). Upon death, infected insect became rigid and showed external sign of melanization and mummification, with dark or reddish discoloration visible on the external surface of the nymph (Photo 2.2).

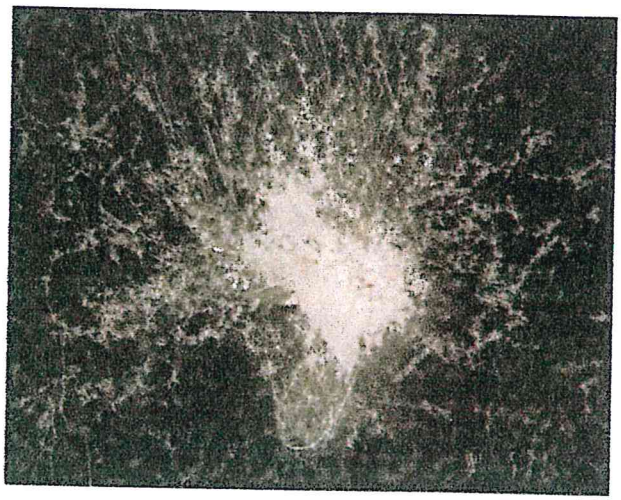

Photo 2. 1 Four days outgrowth of *Paecilomyces fumosoroseus* strain Pfr12 on dead *Empoasca decipiens*

Photo 2. 2 Melanization and mummification of *Empoasca decipiens* nymph infected by *Metarhizium anisopliae* strain Ma43

2.5 Discussion

2.5.1 Comparison of entomopathogenic fungal strains

All strains tested in this study revealed high virulence and thus possess considerable potential for microbial control of green leafhopper *E. decipiens*. The first signs of infection occurred only few hours after the treatment and became apparent by cessation of locomotion in treated insects. Although several entomopathogenic fungi, including *B. bassiana* (Nayak and Srivastava, 1978) and *M. anisopliae* (Bachchhav and Hapase, 1980), are know to infect leafhoppers, so far the potential of entomopathogenic fungi as control agents of leafhoppers

has received little attention. Kamala *et al.* (1981) reported high efficacy of *B. bassiana* against the groundnut leafhopper *E. kerri* Pruthi, while several fungi belonging to the entomophthorales have been isolated from different *Empoasca* spp. (e.g. Petch, 1935; Remaudière *et al.*, 1976). Differences in virulence among entomopathogenic fungal strains are common (e.g. Castillo *et al.*, 2000), corroborating results of this study. Gindin *et al.* (2001) reported high susceptibility of various developmental stages of *Boophilus annulatus* (Canestrini) (Acari: Ixodidae) to *M. anisopliae* and *B. bassiana* strains (among them the here tested strain Ma43) and lower mortality in *V. lecanii*, *P. fumosoroseus* and *M. flavoviride* strains. The high efficacy of the tested strains in our study can be attributed (i) to the general ability of entomopathogenic fungi to infect leafhoppers (Bachchhav and Hapase, 1980; Soper, 1985), and (ii) the high relative humidity and temperature in the experimental chambers, which correspond with the optimum growth conditions of all fungi (Milner *et al.*, 1997; Hallsworth and Magan, 1999; Ekesi *et al.*, 1999). Conidial viability is commonly associated with increasing virulence of entomopathogenic fungi, particularly for *V. lecanii*. Jackson *et al.* (1985) found high conidia germination, as well as sporulation rates correlated with virulence in several *V. lecanii* isolates. However, in other studies high virulence (in terms of mortalities) have been recorded for isolates with low viability of conidia in *V. lecanii* (Barson *et al.*, 1994) and *B. bassiana* (James and Jaronski, 2000).

The results show increasing virulence of the pathogens (e.g. Ma57 and Pfr12) following single host insect passage of the isolates. Increased virulence of entomopathogenic fungi following host insect passage is common. Hayden *et al.* (1992) reported 2.2- folds decrease in LT_{50} values after serial *in vivo* passages of *P. farinosus* (Holm ex. S.F. Gray) Brown & Smith through the English grain aphid *Sitobion avenae* (Fab.) (Homoptera: Aphididae). However, Hall (1980), cited in Hayden *et al.* (1992), showed that a host passage of *V. lecanii* did not

result in increased virulence of the pathogens and associated it to differences in mode of action of entomopathogenic fungi. According to Bidochka and Khachatourians, (1990), the host passage of entomopathogenic fungi such as *B. bassiana*, *M. anisopliae* and *P. fumosoroseus* that enter the insect body through the cuticle may enhance the pathogen virulence by means of cuticle degrading enzymes, while the virulence of other such as *V. lecanii* that penetrate host insects through natural orifices, such as stigmata, and between body segments, may no be altered after host insect passage, thus possibly leading to high inter-strain variability in terms of virulence.

2.5.2 Comparison of developmental stages and dose rates

In the dose rates study, both dose rates as well as insect developmental stages significantly affected mean mortality in *E. decipiens.* First instar nymphs were less susceptible than older nymphs, and fifth instar nymphs were the most susceptible development stage tested, yet comparatively high virulence levels against younger leafhopper developmental stages were recorded for Ma43 and Pfr12. These differences in virulence between different developmental stages of the host may be due to the fungal inoculum being shed with the exuvium following ecdysis in the younger instars. Higher proportions of first and third instar nymphs may be capable to avoid first infections by removing their cuticle as indicated by the non significant increase in mortality from day 4to 5 (first instar) and from day 4 to 6 (third instar). However, first and third instar nymphs might become infected after moulting as indicated by increasing mortalities from day 5 to 7 (first instar) and from day 6 to 7 (third instar). In general, efficacy of entomopathogenic fungi varies with fungal strains and host developmental stages and the insect cuticle can be an important resistance factor to fungal infections, particularly when the time interval between successive moults is short (Vey and Fargue, 1977; Gindin *et al.*, 2001) as it is in the case of *E. decipiens.* Garza and Arredondo (1993) recorded identical levels of

virulence of several Mexican strains of *B. bassiana* and *P. fumosoroseus* tested in the laboratory against tobacco whitefly *B. tabaci* (Gennadius). Lower LD_{50} values of *P. fumosoroseus* were observed against fourth instar of the closely related silverleaf whitefly *B. argentifolii* (Bellows & Perring) (Vidal *et al.*, 1997b) while Wraight *et al.* (1998) recorded higher LD_{50} values on second instar nymphs of the same species. Adane *et al.* (1996) and Carbollo (1998) found that *B. bassiana* caused high mortality in weevil larvae, while Vestergaard *et al.* (1995) showed that in western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), larvae were less susceptible to *V. lecanii* and *M. anisopliae* than the adult and pupal stages.

Decreasing LD_{50} with increasing nymphal developmental stage illustrate higher susceptibility of older compared to younger stages. More variability in susceptibility of younger than older *E. decipiens* instars to the tested strains was noted, as indicated by the range of variation in LD_{50} values (i.e. 2×10^4 - 8.9×10^5; 1×10^4 - 4×10^4 and 5×10^3 - 1×10^4 conidia/ml respectively for the first, third and the fifth instar nymphs). In Bba113 there was a considerable increase in LD_{50} values between the first instar and the two later instars (i.e. 22- and 89-folds increase from third to first, and from fifth to first instars, respectively). In Ma43 and Pfr12 these values were comparatively much lower (e.g. Ma43: 2.2- and 4.4-fold increase in LD_{50} values from third to first, and from fifth to first instars, respectively). Except for Bba113, only slight differences in LD_{50} values were observed between third and fifth instar nymphs, (e.g. Ma43: 2-fold increase in LD_{50} values from third to fifth instars). In general, virulence of entomopathogenic fungi expressed in terms of lethal concentrations may depend on the strain, host insect species and the mode of application (Ferron, 1978b; De La Rosa *et al.*, 2002).

Paecilomyces fumosoroseus strain Pfr12 and *M. anisopliae* strain Ma43 were the most virulent ones tested in this study, resulting in high mortality levels at high dose rates even against first instar nymphs of *E. decipiens*. The fast growth *P. fumosoroseus* (Wraight *et al.*, 1998), as observed in the experiments both on artificial medium and on *E. decipiens* cadavers, is a characteristic that might enhance the virulence of this fungus to first instar nymphs. Thus, it may allow Pfr12 to overcome the successive moults of the younger nymphal instars by entering the host before the removal of the previous cuticle. Similarly, germination speed of *M. anisopliae* (Jackson *et al.*, 1985), *V. lecanii* (Chandler *et al.*, 1993) and *P. fumosoroseus* (Altre *et al.*, 1999) was found to be positively correlated with infectivity of the fungi against mosquito larvae, adult aphids and diamondback moths, respectively.

2.5.3 Mycosis on dead insects

Although some of the results show low virulence of the tested strains to first instar nymphs of *E. decipiens* (Bba113), the recycling ability of the strains on infected cadavers can contribute greatly to the control of *E. decipiens* leafhopper populations. The potential of entomopathogenic fungi as microbial control agents against insect pests depends heavily on their degree of virulence, their species, strains and the ability to infect various developmental stages of the host, but also on the ability of the inoculum to be transferred from infected to non-infected hosts. According to Langewald *et al.* (1999) secondary pick-up is a key route for exposure in grasshoppers. Under the experimental conditions of the present study, nearly all cadavers showed clear external signs of fungal sporulation, indicating that all tested strains could complete their life cycle by forming conidiospores on the dead host. Ben-Ze'ev and Kenneth (1981) observed hyphal growth from ventral parts of the thorax and abdomen of dead *Empoasca* spp, attacked by *Zoophtora radicans* (Brefeld) Batko. (Zygomycetes: Entomophthorales). The emergence of a new generation of conidia on dead hosts ensures (i)

the persistence of infectious inoculum in the ecological niche of the pest (Gindin *et al.*, 2001), and (ii) opens up possibilities for transmission to non-infected hosts by wind, insect vectors and other means of transmission.

The dark or reddish colouration visible on the external surface in infected nymphs is commonly associated with pigments produced by entomopathogenic fungi (Kershaw *et al.*, 1999). Entomopathogenic fungi including *Beauveria*, *Metarhizium*, *Paecilomyces* and *Verticillium* spp. secrete a wide range of metabolites in culture (e.g. oosporein, beauvericin, destruxins), some of which are known to be important pathogenicity determinants or antagonistic factors (Samuels *et al.*, 1988).

Based on the results of this laboratory study, we believe that entomopathogenic fungi can be promising agents for control of *E. decipiens*. However, further studies, including testing of various isolates under greenhouse conditions, are needed to fully evaluate the impact of entomopathogenic fungi on *E. decipiens* populations. Moreover, information of potential side effects of entomopathogenic fungi on the egg parasitoid *Anagrus atomus* L. (Hymenoptera: Mymaridae), an important natural enemy of *E. decipiens*, are required before any possible combinations of the two control agents can be implemented in IPM strategies against the green leafhopper.

3 EVALUATION OF SELECTED STRAINS OF ENTOMOIPATHOGENIC FUNGI FOR CONTROL OF ADULT EMPOSCA DECIPIENS IN GREENHOUSE AND POTENTIAL SIDE EFFECTS ON THE EGG PARASITOID ANAGRUS ATOMUS

3.1 Abstract

The efficacy of *Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12 against adults of *Empoasca decipiens* and potential side effects on the egg parasitoid *Anagrus atomus* were investigated in greenhouse cage and laboratory experiments. Treating leafhopper infected bean plants at a dose rate of 1 x 10^7 conidia/ml resulted in up to 97% mortality seven days after the application and in a 100% infection rate. Experiments on the residual effects revealed a significant decrease in adult *E. decipiens* mortality with increasing time from application to insect release. The decrease in mortality over time corresponded well with data from conidia germination tests. The germination of conidia on sprayed plants' surface declined significantly from 95 and 96% immediately after application for *M. anisopliae* Ma43 and *P. fumosoroseus* Pfr12, respectively, to 29 and 27% five days later. Experiments on potential side effects of the entomopathogenic fungi on *A. atomus* showed that the tested isolates had no influence on adult emergence and longevity; however, the rates of parasitism were significantly reduced by the fungal treatments. The latter might be due to either density effects and/or could indicate that *A. atomus* does not avoid fungal treated plants.

3.2 Introduction

Empoasca decipiens Paoli (Homoptera: Cicadellidae) is one of the most damaging leafhopper species in European greenhouses. The pest is widely distributed in central and southern Europe, North Africa, the Middle East and central Asia (Ossiannilsson, 1981). Recent outbreaks of *E. decipiens* were reported in southern Germany (Schmid and Rupp, 1997), the UK and the

Netherlands (Helyer and Talbalaghi, 1994) and in Bulgaria (Loginova, 1992). To date *E. decipiens* is mainly controlled by extensive use of synthetic insecticides. However, these pesticides are in most instances harmful to beneficial organisms like predators and parasitoids, thus threatening the success of biological control programs against other important pests in greenhouses such as white flies, spider mites, leafminers and aphids. Entomopathogenic fungi are naturally occurring pathogens that attack a wide range of pest insects. These fungi are valid alternatives for synthetic insecticides and often used as biological control agents of insect pests in the field and greenhouses (e.g. Hirte *et al.*, 1989; Castineiras et *al.*, 1996; Selman *et al.*, 1997; Wraight *et al.*, 2000; Butt *et al.*, 2001).

Paecilomyces fumosoroseus (Wize) Brown & Smith (Deuteromycotina: Hyphomycetes) is an important entomopathogenic fungus used as biological control agent in both greenhouses and in the field (Wraight *et al.*, 2000). Because of its potential to cause epizootics, *P. fumosoroseus* has been developed and commercialised as a myco-insecticide (Jackson *et al.*, 1985; Bolckmans *et al.*, 1995). *Metarhizium anisopliae* (Metsch.) Sorokin (Deuteromycotina: Hyphomycetes) has been isolated from a wide range of insects, and due to its wide geographic distribution is next to *Beauveria bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes) the most commonly found entomopathogenic fungal species (Tulloch, 1976). Zimmermann (1993) summarized the safety data of *M. anisopliae* and concluded that no toxicological or pathological symptoms occur when the pathogen is applied by different methods to birds, fish, mice, rats, guinea pigs or rabbits.

One potential problem for using entomopathogenic fungi in pest control programs is their relatively short persistence when applied as foliar application in greenhouses and in the field (e.g. Inglis *et al.*, 1993). Moreover, entomopathogenic fungi with a wide host range like

M. anisopliae, may not only affect the target pest but also infect non-target organisms, e.g. natural enemies of pests like parasitoids and predators (Askary and Brodeur, 1999; Harris *et al.*, 2000).

Anagrus atomus L. (Hymenoptera: Mymaridae) is an important natural enemy of leafhoppers. It is a polyvoltine species that parasitizes leafhopper eggs as long as the host embryo has not developed (Vidano *et al.*, 1987). Under favourable conditions, *A. atomus* can reproduce continuously throughout the year (Cooper, 1993). Vidano *et al.* (1987) reported 50% egg parasitism in the closely related smaller green leafhopper *E. vitis* Goethe by *A. atomus* in Italian vineyards, and according to Schmidt and Rupp (1997), *A. atomus* can be also a potential control agent of *E. decipiens*.

Currently, entomopathogenic fungi and *A. atomus* are the most promising natural enemies of *E. decipiens*. However, before any combinations of the two control agents can be implemented in integrated pest management (IPM) programs, it is important to study whether the entomopathogens cause any side effects on the egg parasitoid. In numerous studies the effect of entomopathogenic fungi on insect pests have been investigated (e.g. Doust and Pereira, 1986; Gillespie and Moorhouse, 1989; Wraight *et al.*, 1998; Mulock and Chandler, 2000; Haraprasad *et al.,* 2001). However, few data are available on potential side effects of entomopathogenic fungi on non-target organisms in general, and on beneficials like predators and parasitoids in particular (Danfa *et al.*, 1999, Stolz *et al.*, 2002). In a previous study, we reported on the results of laboratory experiments that indicated the potential of entomopathogenic fungi for control of *E. decipiens* (Tounou *et al.*, 2002). The main objective of this study was to evaluate the efficacy of the two most promising isolates under more practical conditions against adult leafhoppers and to investigate potential side effects on the egg parasitoid *A. atomus*.

3.3 Materials and Methods

3.3.1 Insects rearing and fungal preparations

Leafhoppers were reared continuously on broad bean plants (*Vicia faba* L. [Fabaceae]) at 24 ± 1°C, 65-70% relative humidity (RH) and a photoperiod of 16:8 h (L:D), following the protocol developed by Raupach *et al.* (2002). Adult parasitoids were originally obtained from English Woodlands Biocontrol, the commercial supplier of *A. atomus* in the UK. Female parasitoids were released on *E. decipiens* eggs at 24 ± 1°C, 65-70% RH and a 16:8 h (L:D) light regime in Plexiglas cylinders (32 cm in height and 13.5 cm in diameter), equipped with two screened windows for ventilation, following the protocol developed by Triapitsyn and Moratorio (1998) for the closely related *A. nigriventris* Girault. Stems and leaves containing parasitized eggs, as indicated by their red colour (Cooper, 1993), were cut in small pieces (2.5–3 cm long) and placed into 1.5 ml Eppendorf tubes together with a piece of wet filter paper. Parasitized eggs were then reared at 24 ± 1°C until adult emergence. Emerged adult parasitoids were maintained in Eppendorf tubes and fed with honey through three small holes made at the top of the tubes.

Two entomopathogenic fungal strains, i.e. *M. anisopliae* strain Ma43 and *P. fumosoroseus* strain Pfr12, originally isolated from two commercial myco-insecticides (Ma43 from Bio 1020™, originally Bayer, Germany, and Pfr12 from Preferal™, Biobest, Belgium), were tested. The strains were grown at 25°C on malt extract-peptone agar medium. After about two weeks the spores were washed off the plates with 0.1% Tween 80. The average viability of conidia used in the experiments was 95% The germination rate was calculated by incubating four droplets of a suspension containing ca. 1 x 10^6 conidia/ml at 25°C in the dark for 24 h onto a 6 cm Petri dish containing malt extract-peptone-agar medium. Germinating conidia per 100 conidia in three separate (1 x 1 cm^2) squares of agar were counted under the microscope. All

conidia with visible germ tubes were scored as viable. The spore concentration was measured using a Thomas chamber (Goettel and Inglis, 1997).

3.3.2 Greenhouse cage experiments

Experiment 1: Direct treatment of leafhopper infested bean plants

Experiments were carried out in cages (46 cm x 46 cm x 122 cm). Four plastic pots (11 x 7.5 x 8.5 cm) each with three broad bean plants (6-leaf stage) were used per cage. Inside each cage, 90 to 100 2-3 days old adult leafhoppers were released at 28 ± 2°C and an approximate RH of 70-75%. Twenty-four hours after releasing the insects, 10 ml of an aqueous (Tween 80) spore suspension of a 10 to 15 days-old culture of Ma43 and Pfr12 at a dose rate of 1 x 10^7 conidia/ml were applied with a hand sprayer covering both sides of the leaves.

Experiment 2: Residual activity of the fungal strains

The residual activity of the two fungal strains, applied on the bean foliage, was evaluated over time. Foliar applications at a dose rate of 1 x 10^7 conidia/ml to non-caged bean plants (6-leaf stage) were carried out in the greenhouse as described in the previous section. Treated plants were moved into the cages immediately after application (0 h) and three and five days thereafter. Ninety to 100 adult leafhoppers were then introduced into each cage as previously described.

In addition, spore viability was quantified by sampling two bean leaves per treatment immediately after the application (0 h) and three and five days thereafter. The leaves were individually washed with 0.1% Tween 80 for 4 minutes on a shaker. The viability of conidia was determined using the Thomas chamber as describe before.

In both direct treatment and residual activity tests, treatments were repeated three times, and each treatment consisted of one cage with 12 bean plants. Plants sprayed with a wetting agent (i.e. 0.1% Tween 80) served as control. The insects were monitored daily for seven days and any mortality was recorded. Dead leafhoppers were collected daily directly from the leaves and/or inside the cage after gently shaking the plants.

Experiment 3: Effect of the isolates on emergence of leafhopper nymphs

The effect of the entomopathogens on *E. decipiens* eggs was investigated under laboratory conditions. Potted broad bean plants harbouring eggs were sprayed with fungal suspension at a concentration of 1 x 10^7 conidia/ml or with 0.1% Tween 80 (control) and allowed to dry for 24 h. Thereafter, the plants were individually transferred to Plexiglas cylinders (32 cm in height and 13.5 cm in diameter) and maintained in a climate chamber at 24 ± 1°C, 65–70% RH and a light regime of 16:8 h (L:D). Ten to 12 days later emergence of larvae were recorded daily for three days and the total number of emerged larvae was counted. The experiment was repeated three times, each repetition consisting of two plants.

3.3.3 Potential side effects on *A. atomus*

Experiments on potential side effects of the two fungal strains on the egg parasitoid *A. atomus* were conducted in a climate chamber at 24 ± 1°C, 65–70% RH and a light regime of 16:8 h (L:D).

Experiment 4: Effect on acceptance and suitability of host eggs

No-choice test for oviposition

Broad bean plants harbouring leafhopper eggs (five to six days-old) were treated as described before with two dose rates (i.e. 1 x 10^7 and 1 x 10^5 conidia/ml) of Ma43 and Pfr12 or with 0.1%

Tween 80 (control). Three days-old mated female parasitoids were individually released in a Plexiglas cylinder (32 cm in height and 13.5 cm in diameter) for oviposition.

Choice test for oviposition

In preliminary tests, parasitoids did not discriminate between plants treated with 0.1% Tween 80 and those treated with water (for details see results section). Hence parasitoids were given the choice between broad bean plants harbouring leafhopper eggs treated as previously described with Ma43 and Pfr12 at two dose rates (1×10^7 and 1×10^5 conidia/ml) and with 0.1% Tween 80 (control). Each group of plants (two plants per group) were transferred into oviposition cages (21 x 25 x 40 cm) and one three days-old mated female parasitoid was released in the centre of each cage.

In both choice and no-choice experiments, after 12 days the plants were gently shaken to remove leafhopper nymphs that had emerged from unparasitized eggs. Any parasitized eggs, as indicated by their red colour (Cooper, 1993), and emerged nymphs (dead or alive) were recorded and the rate of parasitism was calculated by dividing the number of parasitized eggs by the sum of parasitized eggs and number of emerged nymphs. Each treatment consisted of two replications, i.e. two Plexiglas cylinders (no-choice) and two oviposition cages (choice), per treatment, and the experiment was repeated three times.

Experiment 5: Effect on adult parasitoid emergence

Broad bean plants harbouring leafhopper eggs were first exposed to three days old mated female parasitoids. Three days before adult parasitoid emergence (i.e. nine to ten days after introduction of the *A. atomus* females), stems and leaves containing parasitized eggs, as indicated by their red colour (Cooper, 1993), were cut in small pieces (2.5–3 cm long) and

treated by immersion into spore suspensions of Ma43 and Pfr12 at a concentration of 1 x 10^7 conidia/ml for approximately one minute. The stem and leaf pieces were allowed to dry under ambient conditions for 30 minutes and were then placed into 1.5 ml Eppendorf tubes and kept in a climate chamber at 24 ± 1°C, 65–70% RH and a light regime of 16:8 h (L:D). Control treatments consisted of stem and leaf pieces harbouring parasitized eggs which were immersed into a wetting agent (i.e. 0.1% Tween 80) (control 1) and not immersed (control 2).

Experiment 6: Direct effect of Ma43 and Pfr12 on the longevity of adult *A. atomus*

Three days-old mated male or female parasitoids were individually exposed in 1.5 ml Eppendorf tubes to a leaf piece that had been previously immersed into two concentrations of Ma43 and Pfr12, i.e. 1 x 10^5 and 1 x 10^7 conidia/ml, or into 0.1% Tween 80 (for details refer to previous section). The parasitoids were provided with honey as food source as described before.

Experiments 5 and 6 were repeated three times, each repetition consisting of 15 to 20 stems or leaf pieces containing one to two parasitized eggs (experiment 5) or 15 to 20 adult parasitoids (experiment 6). Adult parasitoids emergence was recorded daily for five days and percentage of parasitoid emergence was calculated. Similarly, parasitoids were inspected daily until death and any mortality was recorded.

3.4 Statistical analysis

Cumulative mortalities were analysed using multivariate analysis (profile analysis, ANOVA repeated measure) with one between-factor (isolates) and one within-factor with seven levels (day1 – day 7). To identify particular time intervals in which treatment effects are different, individual ANOVAs (F-tests) were computed on each of the six different contrasts of the

adjacent days (Time_N: the nth successive differences in time) and the overall α of 0.05 was maintained using a Bonferroni adjustment of α/6 = 0.0083 for each contrast (Scheiner and Gurevitch, 1993).

The efficacy of the different isolates was compared using the final mortalities (seven days cumulative mortalities) after arcsine transformation to normalize variances for all analysis. ANOVA were also used to test for treatment effects on longevity, parasitism, performance and percentage emergence of adult *A. atomus* and on the number of emerged *E. decipiens* nymphs, after prior arcsine transformation of percentage data. Means were compared with Student's t-test (SAS Institute, 1996). All statistical analyses were performed using the general linear model (GLM) procedure of SAS (SAS Institute, 1996) and the probability level was set at α = 0.05.

3.5 Results

3.5.1 Greenhouse experiments and *E. decipiens* nymphs emergence tests

Experiment 1: Direct treatment of leafhopper infested bean plants

Dead insects immediately collected after spraying, i.e. 12.4% (SE = 1.0), 12.5% (SE = 1.4) and 13.1% (SE = 1.5) of the total number of released leafhoppers in Pfr12, Ma43 and the control, respectively, were not included for computing the final cumulative mortality values (Table 3.1).

Both isolates tested resulted in significant increasing mortality over time (F = 217.84; df = 6; $P < 0.0001$), with significant increase in time period from day one to days four (F = 66.51; df = 1; $P = 0.0012$), after which no further increase in mortality was observed. Moreover, no significance difference in time x isolate interaction was found (F = 0.61; df = 6; $P = 0.7179$. The majority of dead insects were recorded during the first three days after the applications

(i.e. 84.0% (SE = 1.9) and 78% (SE = 2.5) respectively for Ma43 and Pfr12.respectively. The two isolates resulted in significantly higher mortality than recorded in the control (F = 858; F = 2; $P < 0.0001$). However, no significant differences in mortality were found between the two strains tested (Table 3.1).

Table 3.1 Mortality of adult *Empoasca decipiens* after seven days following direct treatment of leafhopper infested bean plants with *Paecilomyces fumosoroseus* strain Pfr12 and *Metarhizium anisopliae* strain Ma43 in greenhouse cages.

Treatments	No. adults released per cage[a]	Mortality (% ± SE)[b]
Ma43	92	97.3 ± 1.0a
Pfr12	92	90.7 ± 2.0a
Control[c]	90	3.4 ± 1.5b

[a] means of four repetitions;

[b] means followed by the same letter in the same column are not significantly different ($P < 0.05$; Student t-test);

[c] treated with 0.1% Tween 80.

Experiment 2: Residual activity of Pfr12 and Ma43

Except when adult leafhoppers were released immediately after spraying (i.e., 4.49% (SE = 1.07)), no mortality was recorded in the control treatments (Table 3.2). In both fungal treatments, dead insects were found in most cases attached to the leaf under surface.

Significant effects of time from application to insect release were recorded for Ma43 (F = 39.53; df = 2; $P = 0.0004$) and Pfr12 (F = 41.72; df = 2; $P = 0.003$). However, no significant interactions between the two isolates and days after application were found (F = 0.27; df = 2; $P = 0.769$). Independent of the age of the spray residues, adult leafhopper mortality increased significantly from day 1 to day 7 in Ma43 (F = 88.49; df = 6; $P < 0.0001$) and Pfr12 (F = 185.70; df = 6; $P < 0.0001$). In both treatments, mortality was always significantly higher in

both Pfr12 and Ma43 than in the respective control treatments (Table 3.2). For both strains on three and five days-old spray residues similar mortality rates were recorded, which were significantly lower than those on fresh residues.

The decline in mortality over time (Table 3.2) corresponds well to the results of the conidia germination test (Fig. 3.1). No significant interactions between the two strains and the time after application were recorded ($F = 2.03$; $df = 2$; $P = 0.182$).

Table 3.2 Mortality of adult *Empoasca decipiens* on broad bean plants at different time intervals after treatment with *Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12 in greenhouse cages. Leafhoppers were introduced into the cages immediately and three and five days after the applications.

Time after application (days)	Mortality (± SE)[a]		
	Ma43	Pfr12	Control[b]
0	49.67 ± 4.86Aa	51.52 ± 5.88Aa	4.48 ± 1.67Ba
3	23.61 ± 5.65Ab	30.94 ± 0.48Ab	0 ± 0.0Bb
5	24.28 ± 0.79Ab	22.08 ± 0.76Ab	0 ± 0.0Bb

[a] means within columns followed by the same lower case and within rows followed by the same upper case letter are not significantly different ($P < 0.05$; Student t-test);

[b] treated with 0.1% Tween 80.

Hence, the two strains and the three time treatments were compared irrespectively of each other. The viability of conidia on broad bean leaf surfaces, as indicated by the results of the conidia germination assays, declined significantly with increasing time after application but no significant differences were found between the two strains (Fig.3.1).

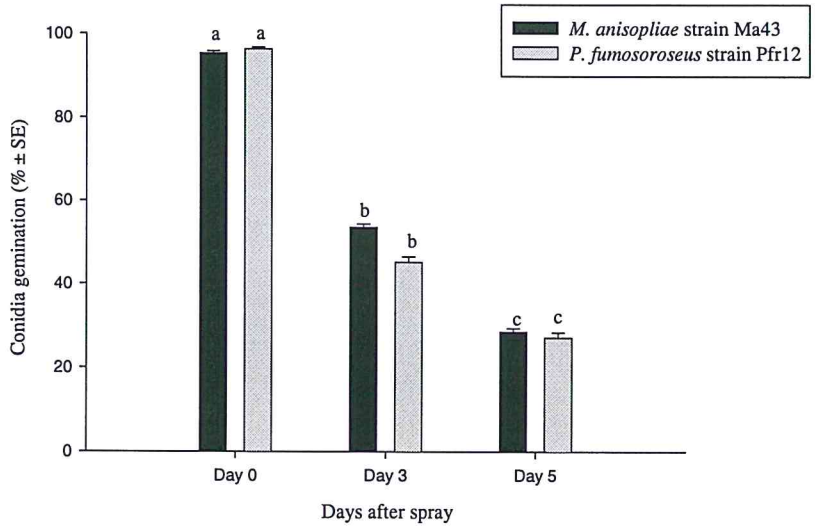

Figure 3.1 Conidia germination (% ± SE) of *Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12 as affected by time between application of broad bean plants and release of adult *Empoasca decipiens* in the greenhouse. For each strain bars with different letters are significantly different at $P = 0.05$.

Experiment 3: Effects of Pfr12 and Ma43 on emergence of leafhopper nymphs

An average, 49.3 (SE = 3.5) and 53.0 (SE = 2.6) nymphs per pot of three broad bean plants were recorded in the 0.1% Tween 80 and water treatments, respectively, which did not differ significantly (Fig. 3.2). However, with 25.7 (SE = 1.2) and 32.0 (SE = 2.2) nymphs for Ma43 and Pfr12, respectively, significantly lower number of nymphs were recorded in both fungal treatments compared to the controls (Fig. 3.2).

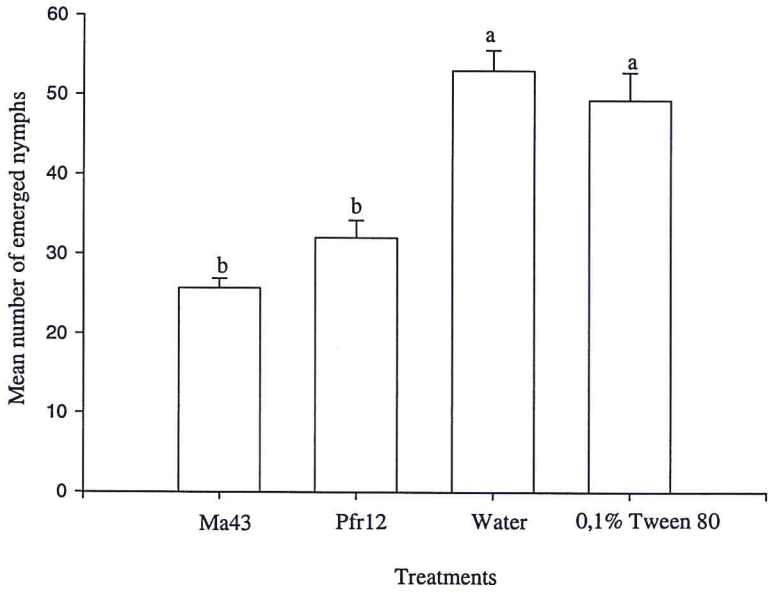

Figure 3.2 Mean numbers (± SE) of *Empoasca decipiens* nymphs emerging from plants exposed to two entomopathogenic fungi (*Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12). Bars with the same letters are not significantly different at $P = 0.05$.

3.5.2 Potential side effects of Pfr12 and Ma43 on *A. atomus*

Experiment 4: Effect on acceptance and suitability of host eggs

No-choice oviposition

The percentage of parasitized eggs was significantly lower in all fungal treatments, i.e. 6.9% (SE = 1.6) and 16.3% (SE = 1.9) for Ma43 and 9.8% (SE = 0.9) and 19.2% (SE = 1.6) for Pfr12 at concentrations of 1 x 10^5 and 1 x 10^7 conidia/ml, respectively, than in the control, i.e. 44.5% (SE = 0.9). No significant interactions between the two dose rates and the two isolates were found ($F = 0.22$; df = 1; $P = 0.67$). Therefore, the data were pooled for the two isolates and dose rates. No significant difference was found between the two tested strains (Fig. 3.3). However, *A. atomus* females parasitized significantly more eggs at the lower than at the higher dose rate tested (Fig. 3.4).

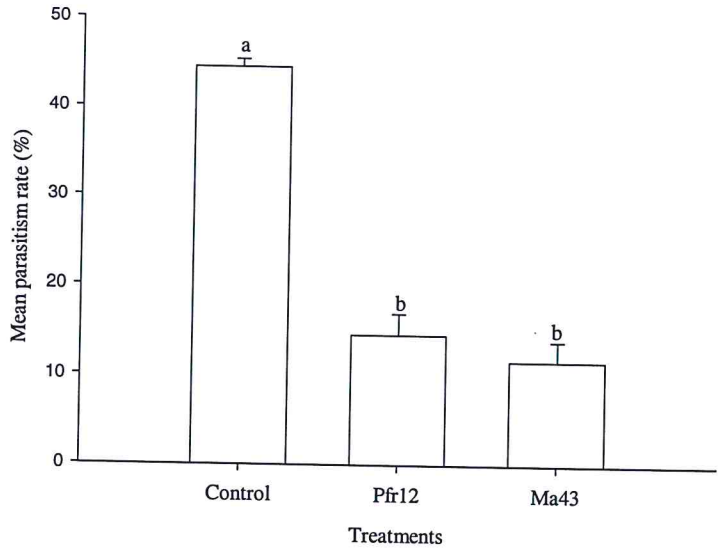

Figure 3.3 Effects of two entomopathogenic fungal strains (*Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12) applied at spore concentrations of 10^5 and 10^7 conidia/ml on percentage of parasitism by *Anagrus atomus*. No significant interactions between the two isolates and the two dose rates tested were recorded (for details see results). Hence isolates were compared regardless of the dose rates tested. Bars with different letter are significantly different at $P = 0.05$.

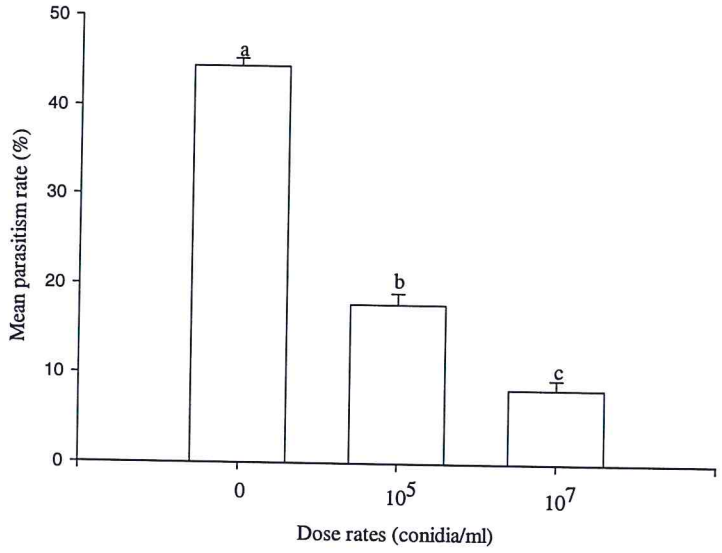

Figure 3.4 Effects of two entomopathogenic fungi (*Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12) applied at spore concentrations of 10^5 and 10^7 conidia/ml on percentage of parasitism by *Anagrus atomus*. No significant interactions between the two isolates and the two dose rates tested were recorded (for details see results). Hence dose rates were compared regardless of the isolates tested. Bars with different letter are significantly different at $P = 0.05$.

Choice oviposition

No significant differences were found when the parasitoids were given the choice between plants sprayed with water and those with 0.1% Tween 80 (mean numbers of emerged nymphs and parasitized eggs for water 85.7 (SE = 2.9) and Tween 85.5 (SE = 4.8), and mean percentage of parasitism for water 25.2 (SE = 0.9) and Tween 25.8 (SE = 1.7)). Thus in the choice experiments control plants were treated with 0.1% Tween 80. For both Ma43 and Pfr12 at a spore concentration of 1 x 10^5 conidia/ml, the parasitoids did not discriminate between fungal- and Tween-treated plants (Table 3.3).

Table 3.3 Effects of *Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12 on acceptance of *Empoasca decipiens* eggs by *Anagrus atomus* in choice oviposition experiments.

	Dose rates[a]			
	10^5		10^7	
Treatments	Total no. eggs[b]	Parasitism (% ± SE)[c]	Total no. eggs[b]	Parasitism (% ± SE)[c]
Ma43	53.2 ± 5.7	8.0 ± 1.0Aa	52.3 ± 6.8	6.2 ± 2.2Ba
Control[d]	50.0 ± 3.3	11.8 ± 0.4Ab	65.7 ± 7.0	18.4 ± 1.2Aa
Pfr12	61.5 ± 6.0	9.7 ± 1.6Aa	45.7 ± 2.2	7.3 ± 0.8Ba
Control[d]	63.8 ± 3.5	13.3 ± 1.9Ab	69.5 ± 7.4	20.2 ± 1.2Aa

[a] in conidia/ml;

[b] means of three repetitions, estimated by the sum of the number of emerged nymphs and the number of parasitized eggs;

[c] means within the same column followed by the same upper case and within the same row followed by the same lower case letter for each group of treatment are not significantly different ($P < 0.05$; Student t-test);

[d] treated with 0.1% Tween 80.

However, at a dose rate of 1 x 10^7 conidia/ml, mean rates of parasitism were significantly lower on plants sprayed with Ma43 and Pfr12 than in their respective controls. No significant differences were found between the two pathogens at the two tested doses. However, percentage parasitism differed significantly in the control treatments for both fungal strains between the two dose rates tested (Table 3.3).

Experiment 5: Effect on adult parasitoid emergence

No significant differences in terms of parasitoid emergence were found between the fungal- and Tween-treated stem and leaf pieces (Fig. 3.5). However, a significantly higher proportion of *A. atomus* emerged from the non-immersed stem and leaf pieces.

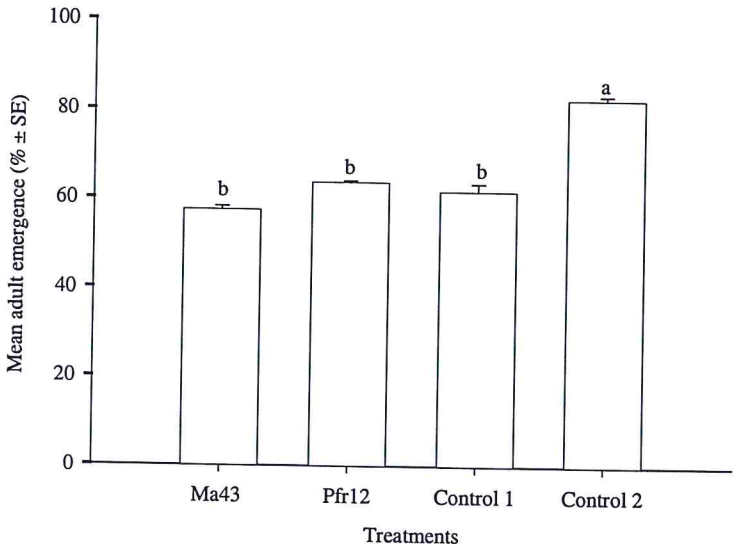

Figure 3.5 Mean emergence (± SE) of adult *Anagrus atomus* as affected by two entomopathogenic fungal strains (*Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12) at a spore concentration of 10^7 conidia/ml. In control 1 broad bean leaf and/or stem pieces were immersed in 0.1% Tween 80 and in control 2 they were not immersed. Means with the same letter are not significantly different at $P = 0.05$.

Experiment 6: Effect on adult parasitoids longevity

First dead adult *A. atomus* were recorded five and seven days post application in the control and the fungal treatments, respectively. The two tested strains at both dose rates did not

significantly affect the longevity of the parasitoids (Table 3.4). Under the experimental conditions (i.e. 25°C), 100% mortality was reached 17 to 19 days after the treatments.

Table 3.4 Effects of *Metarhizium anisopliae* strain Ma43 and *Paecilomyces fumosoroseus* strain Pfr12 on the mean survival time (days ± SE) of adult *Anagrus atomus*.

Dose (conidia/ml)	10^5	10^7
Ma43	11.2 ± 0.1a[a]	11.27 ± 0.1a
Pfr12	10.9 ± 0.1a	11.13 ± 0.1a
Control[b]	12.16 ± 0.2a	

[a] means followed by the same letter in the same column are not significantly different ($P < 0.05$; Student t-test);

[b] treated with 0.1% Tween 80.

3.6 Discussion

Effect on adult *E. decipiens* in the greenhouse

Direct applications of leafhopper infested bean plants with *M. anisopliae* strain Ma43 and *P. fumosoroseus* strain Pfr12 in the greenhouse lead to an efficient control of green leafhoppers, with up to 97% reduction of pest densities. These findings corroborate earlier results on the high virulence of Ma43 and Pfr12 in laboratory tests against *E. decipiens* (Tounou *et al.*, 2002) and confirm the great potential of entomopathogenic fungi for control of *E. decipiens*. However, only a direct contact between hosts and the spore suspensions lead to high leafhopper mortality. Increasing time after application resulted in significantly decreasing leafhopper mortalities, coupled with a likewise significant decrease in conidia germination. Conidia germination is often associated with the efficacy of entomopathogenic fungi (e.g. Altre *et al.*, 1999), although in some studies high mortalities were also obtained with low viability of conidia (e.g. James and Jaronski, 2000). Under field and greenhouse conditions, conidia are

exposed to degradation factors such as sunlight (in the greenhouse mostly by irradiation lamps), which possibly reduce the persistence of the conidia on the leaf surface and thus reduce their effectiveness (Inglis *et al.*, 1993). Direct spraying of spore suspensions on leafhopper-infested plants is more effective than later contact of *E. decipiens* with conidia on treated leaf surfaces. Hence, entomopathogenic fungi cannot be used as a preventive tool, except if better formulations with an increased persistence of the conidia are developed. Entomopathogenic fungi need to be applied immediately after leafhopper outbreaks have been recorded in the greenhouse. Exposure of second instar Colorado potato beetles to *B. bassiana* lead to significantly higher mortality when larvae were sprayed directly compared to those exposed to the treated foliage (Fernandez *et al.*, 2001). The pressure of the spray may contribute to enhance conidial lodging within cuticular folds, facilitating germination and penetration (Boucias *et al.*, 1998; Fernandez *et al.*, 2001). Foliar applications of entomopathogenic fungi result in conidia landing directly on the insect cuticle and/or the insect collecting the conidia by moving from one leaf to another. Moreover, insects that are not directly hit by the conidia during application have a greater chance to avoid infection than those sprayed directly on the foliage.

Potential side effects of the pathogens on the egg parasitoid *A. atomus*

In a no-choice situation *A. atomus* females parasitized significantly lower numbers of leafhopper eggs on fungi-treated plants than in the control. Moreover, in the choice experiments significantly lower rates of parasitism were recorded in both Ma43 and Pfr12 at the higher dose rate. However, no differences in rates of parasitism between the fungal treatments and the control were observed at the lower dose rate tested. These results indicate that the parasitoids only avoid fungi-treated plants if a certain concentration of conidia is exceeded. Many

parasitoids are repelled or have developed the ability to detect and then avoid hosts infected by entomopathogenic fungi (Jones and Wraight, 1996; Lord, 2001a). Landa (1984) and Fransen and van Lenteren (1993) recorded fewer eggs laid by the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) in greenhouse whiteflies, *Trialeurodes vaporarium* Westwood (Homoptera: Aleyrodidae), infected by the fungus *Aschersonia aleyrodis* Webber (Deuteromycotina: Coelomycetes) than in uninfected hosts. In rose-grain aphids, *Metopolophium dirhodum* (Walker) (Homoptera: Aphididae), infected by *Pandora* (*Erynia*) *neoaphidis* (Brefeld) Batko (Entomophthorales: Entomophthoraceae) three days before exposure to the parasitoid *Aphidius rhopalosiphi* de Stefani Perez (Hymenoptera: Braconidae) Brobyn *et al.* (1998) recorded reduced frequency in egg laying. According to Lord (2001a), while vision may be of little importance and the pigment associated with the fungal mycosis may not be an effective deterrent to oviposition by a parasitoid, such recognition is often based on the presence of cuticular chemical cues, perceived through antennae, and movements of the host once contacted. In *Aphelinus asychis* Walker (Hymenoptera: Aphelinidae) females can sense the presence of hyphal bodies or fungal metabolites in the hemolymph of aphid hosts infected by *P. fumosoroseus* when inserting their ovipositor, and consequently avoid oviposition in such diseased hosts (Mesquita and Lacey, 2001).

Direct exposure of *A. atomus* to Ma43 and Pfr12 did not affect the longevity of the parasitoids. To date few studies have addressed possible direct effects of entomopathogenic fungi to beneficial arthropods such as predators, parasitoids and honey bees. The pathogenicity of entomopathogenic fungi on the honeybee *Apis mellifera* L. (Hymenoptera: Apidae) has been reported to be significant at high dose rates under laboratory conditions, but no impact on the bee colony was recorded (Butt *et al.*, 1994). Several isolates of *M. anisopliae* are highly

virulent to *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae), an important parasitoid of the cassava mealy bug *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae) and the polyphagous parasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae) (Danfa, 1996). In *A. lopezi*, Stolz *et al.* (2002) reported 24% reduction in longevity after exposition to *M. anisopliae* var. *acridum* relatively to the untreated control. Up to 56% adult mortality in *Teretrius* (formerly *Teretriosoma*) *nigrescens* Lewis (Coleoptera: Histeridae), a predator of the larger grain borer *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae), was recorded after direct exposure to 1 x 10^9 conidia/ml of *M. anisopliae* and *B. bassiana* (Bourassa *et al.*, 2001). Direct inoculation of adult *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylidae), a parasitoid of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), to *B. bassiana* resulted in LC_{50} values in the range of 5.3 x 10^6 to 7.9 x 10^7 conidia/ml (De La Rosa *et al.*, 1997). Similar results were obtained for *Prorops nasuta* Waterston (Hymenoptera: Bethylidae), another parasitoid of the coffee berry borer, with LC_{50} values of 8.31 x 10^6 and 4.08 x 10^6 conidia/ml for *B. bassiana* and *M. anisopliae*, respectively (De La Rosa *et al.*, 2000). Hence these authors concluded that the pathogens did not affect the biocontrol potential of the two parasitoids. In contrast, Lord (2001b) reported that exposition of females of *C. tarsalis* Ashm., an ectoparasitoid of several post-harvest pests, to 100 mg of *B. bassiana*/kg of wheat for 3 h resulted in 52.7% mortality, that eggs which were deposited on hosts showing signs of mycosis did not survive and, hence, concluded that the parasitoid was as susceptible to the fungus as the hosts.

Ovicidal effects of entomopathogenic fungi have been mainly reported when eggs were directly exposed to the pathogens (e.g. Zimmermann, 1982; Long *et al.*, 1998; Gindin *et al.*, 2001). Our results suggest that once *E. decipiens* eggs have been successfully parasitized by *A. atomus*, the

two tested fungal isolates do not affect the emergence of the adult parasitoids. Treating broad bean plants with *M. anisopliae* strain Ma43 and *P. fumosoroseus* Pfr12 immediately after oviposition of *E. decipiens* females had no effect on the numbers of emerging leafhopper nymphs except for Ma43. The latter was possible due to still viable conidia at the time of nymphal emergence and not to ovicidal impact of the isolate. Since *E. decipiens* females lay their eggs deep in the plant tissue, they are thus unlikely to be affected by entomopathogenic fungi.

In conclusion to this second part, the two tested fungal isolates caused high mortalities in adult *E. decipiens* under greenhouse conditions. Moreover, side effects on the egg parasitoid *A. atomus* were rather limited, opening up possibilities for a combined use of the two antagonists for control of green leafhopper outbreaks in greenhouses.

4 GENERAL DISCUSSION AND RECOMMENDATIONS

Entomopathogenic fungi and the egg parasitoid *A. atomus* may be become key components in integrated control of the green leafhopper *E. decipiens*, an important new pest in European greenhouses. The main objective of this study was to investigate the potential of entomopathogenic fungi for control of *E. decipiens*.

Results from laboratory experiments demonstrate that entomopathogenic fungi can be promising tools for control of the green leafhopper. In both initial screening and single host insect passage tests, all tested isolates proved to be highly pathogenic to late fifth instar of *E. decipiens*. Different concentrations of spore suspensions of *B. bassiana* strain Bba113, *M. anisopliae* strain Ma43 and *P. fumosoroseus* strain Pfr12 were exposed to three nymphal stages of *E. decipiens*. All strains proved to highly efficient against *E. decipiens*, with younger nymphs generally being less susceptible to the tested fungi than older ones. Cumulative mortality (seven days exposure) varied between 17% (Bba113 at a concentration of 1×10^3 conidia/ml against first instar nymphs) and 92% (Ma43 at a concentration of 1×10^6 conidia/ml against fifth instar nymphs).

The general ability of entomopathogenic fungi to infect leafhoppers (Bachchhav and Hapase, 1980; Nayak and Srivastava, 1982; Soper, 1985), and the high relative humidity (leading to a > 95%) and temperature (25°C) in the experimental chambers, which correspond with the optimum growth conditions of all fungi (e.g. Hallsworth and Magan, 1999), might have contributed to the recorded high efficacy of all tested fungal strains. Short time interval between successive moults of *E. decipiens* nymphs are probably the reason for the lower mortality rates in younger compared to older nymphal stages of *E. decipiens*. Higher proportions of first and third instar nymphs may be capable to avoid fungal infections by

removing their cuticle before successful penetration. However, the ability of *P. fumosoroseus* to grow fast (Wraight *et al.,* 1998), observed in this study both *in vitro* and *in vivo*, might enhance the virulence of this fungus to first instar nymphs by allowing this pathogen to enter the host body before the removal of the cuticle.

The host passage of entomopathogenic fungi such as *B. bassiana*, *M. anisopliae* and *P. fumosoroseus* that enter the insect body by penetration of the cuticle may enhance the virulence of the pathogen by increased production of cuticle degrading enzymes. However, the virulence of other fungi like *V. lecanii* that penetrate host insects through natural orifices, such as stigmata, and between body segments, may no be altered after host insect passage, thus possibly leading to high inter-strain variability in terms of virulence.

Decreasing LD_{50} values with nymphal developmental stage, as recorded in the dose rate experiments, illustrates higher susceptibility of younger compared to older stages. Although some of the results show low virulence of the tested strains to first instar nymphs of *E. decipiens* (e.g. Bba113 against first instar nymphs), the recycling ability of the strains on infected cadavers can contribute greatly to the control of *E. decipiens* populations. All tested strains could complete their life cycle by forming conidiospores on dead hosts, consequently opening up possibilities (or producing sources of infective material) for transmission to non-infected hosts by means of wind, insect vectors and other means of transmission.

The greenhouse study expands the previous laboratory observation on the high virulence of *M. anisopliae* strain Ma43 and *P. fumosoroseus* strain Pfr12. The data show that direct treatment of leafhopper infested bean plants in the greenhouse can be very effective for control of green leafhopper populations. The majority of *E. decipiens* mortality is probably a result of direct contact between insects and the applied spore suspensions. However, the

significant decline in adult leafhopper mortality in residual study a indicate a strong decline in activity of infectivity of conidia applied to leaf surfaces. The infectivity of residual spore suspensions depends on the capability of the conidia to survive over time until contacting the host pest. Conidia viability, as also shown in the first part of this study, has been often associated with increasing virulence of entomopathogenic fungi (e.g. Altre *et al.*, 1999) although some other studies have shown that high mortalities can be obtained also with low viability of conidia (e.g. James and Jaronski, 2000). Under greenhouse conditions (compared to the laboratory studies), applied conidia are more susceptible to degradation factors such as sunlight, which may reduce the persistence of conidia on the leaf surface and hence subsequently their effectiveness (Inglis *et al.*, 1993).

Experiments on the potential side effect of the pathogens on the egg parasitoid *A. atomus*, showed that the parasitoid could partly avoid detrimental effects of the tested fungal strains. When the parasitoids were given the choice between fungal treated plants and untreated ones at both concentrations (i.e. 1×10^5 and 1×10^7 conidia/ml) a distinct reduction in the rate of parasitism by *A. atomus* could be observed on hosts treated with pathogens compared to the control. Moreover, the parasitoids showed the ability to detect and avoid fungal treated plants but only at the higher and not the lower dose rate tested. Thus parasitoids foraging on treated plants are substantially less susceptible to infections by the tested pathogens than its host *E. decipiens*.

A successful integration of the two control agents, i.e. *A. anagrus* and entomopathogenic fungi, depends on the possibility to minimize direct and indirect detrimental effects on the parasitoid. Despite of the lower rate of parasitism recorded on fungal treated plants, it is encouraging that the tested pathogens did not affect the rate of emergence and the longevity of the parasitoids. Results of this study indicate that once the host eggs have been successfully

parasitized, the fungal isolates have no negative effects on the rate of emergence and longevity of adult parasitoids. This is further supported by the fact that after treatment of plants infested with leafhopper eggs no differences in numbers of emerged nymphs between the control and the fungal treatments, except for Ma43, were observed. The latter might have been due to further infection of the emerged nymphs, collecting remaining viable conidia from the treated plants, rather than the direct effect of Ma43 on *E. decipiens* eggs. Eggs of *E. decipiens* located in the plant tissues are very likely to be protected against any contact effect of the fungi. The significant increase of successful parasitoid attacks on *E. decipiens* eggs with decreasing spore concentrations and high nymphal mortality rates at lower dose rates of the pathogens (e.g. Ma43 and Pfr12), indicate the possibility of combinated releases of the two biocontrol agents against *E. decipiens*. Although it is not possible in *E. decipiens* to determine the exact number of the host eggs laid and/or exposed to parasitoids, the relatively higher rate of parasitism in the choice oviposition tests where higher numbers of eggs were exposed to *A. anagrus* than in the no choice test (51 vs. 44% for the choice and no choice tests, respectively), indicates that the host egg density may have been responsible for the reduced rate of parasitism in *A. atomus* in the non choice oviposition test. Results from the residual and spore viability tests additionally suggest that applying the entomopathogens several days after releases of *A. anagrus* might further contribute to minimize detrimental effects of the fungi on the egg parasitoid.

In the present study some of the most promising fungal strains derived from commercial products (Ma43 from Bio 1020™, Bayer, Germany, and Pfr12 from Preferal™, Biobest, Belgium company), suggesting that microbial control of *E. decipiens* could be readily implemented. However, in further studies more fungal isolates should be screened for high virulence at low dose rates against *E. decipiens*. Moreover, different application techniques

and formulations should be tested to fully evaluate the impact of entomopathogenic fungi on green leafhopper populations. However, studies on potential side effects on other natural enemies of *E. decipiens* like the egg parasitoid *A. anagrus* always need to be an integral part of such an exercise to enable combined releases of several biocontrol against leafhoppers.

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