Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbst20

Laboratory investigations on the potential of entomopathogenic fungi for biocontrol of Helicoverpa armigera (Lepidoptera: Noctuidae) larvae and pupae

Nguyen T. H. Nguyen [a], Christian Borgemeister [b], Hans-Michael Poehling [a] & Gisbert Zimmermann [c]

[a] Institute of Plant Diseases and Plant Protection, Leibniz University Hannover, Hannover, Germany

[b] International Centre for Insect Physiology and Ecology, Nairobi, Kenya

[c] Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Darmstadt, Germany

Version of record first published: 26 Sep 2007.

To cite this article: Nguyen T. H. Nguyen , Christian Borgemeister , Hans-Michael Poehling & Gisbert Zimmermann (2007): Laboratory investigations on the potential of entomopathogenic fungi for biocontrol of Helicoverpa armigera (Lepidoptera: Noctuidae) larvae and pupae, Biocontrol Science and Technology, 17:8, 853-864

To link to this article: http://dx.doi.org/10.1080/09583150701546375

Biocontrol Science and Technology, 2007; 17(8): 853–864

Taylor & Francis
Taylor & Francis Group

Laboratory investigations on the potential of entomopathogenic fungi for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae and pupae

NGUYEN T. H. NGUYEN[1], CHRISTIAN BORGEMEISTER[2], HANS-MICHAEL POEHLING[1], & GISBERT ZIMMERMANN[3]

[1]*Institute of Plant Diseases and Plant Protection, Leibniz University Hannover, Hannover, Germany,* [2]*International Centre for Insect Physiology and Ecology, Nairobi, Kenya, and* [3]*Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Darmstadt, Germany*

(Received 6 February 2007; returned 24 April 2007; accepted 19 June 2007)

Abstract

The susceptibility of third instar *Helicoverpa armigera* to seven strains of three entomopathogenic fungal species, i.e. *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces fumosoroseus*, was tested under laboratory conditions using the larval immersion method. High efficacies ranging from 68 to 100% corrected mortality were recorded with more profound effects in treatments with *B. bassiana* and *P. fumosoroseus* strains. The median lethal concentration (LC_{50}) for L3 was 6.0×10^5 in *M. anisopliae* 79, 1.5×10^5 in *B. bassiana* 124 and 4.2×10^4 in *P. fumosoroseus* 14. These three strains were further used to characterize the age-dependent mortality of different larval stages (L2–L5) and the effect against pupae of *H. armigera.* Larval stages did not differ in their mortality but differed in median lethal time, with shorter values recorded in the second instar. Tested fungi also caused a high reduction between 74.4 and 100% in the emergence of pupae using the soil inoculation method and the pupal immersion technique. All three fungal species, especially *P. fumosoroseus*, have a high potential for biocontrol of *H. armigera* larvae and also as a soil treatment targeting the pupae.

Keywords: *Entomopathogenic fungi,* Helicoverpa armigera*, biocontrol, age-dependent mortality, dose–mortality relationship,* Beauveria bassiana, Metarhizium anisopliae, Paecilomyces fumosoroseus

Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a cosmopolitan polyphagous pest of many economically important crops and is able to complete its development on a wide variety of non-cultivated species (Zalucski 1986; Fitt 1989; King & Coleman 1989; King 1994). The larvae have a distinct feeding preference for high nitrogen plant structures, reproductive structures and growing points, enabling them to influence the crop yield directly. In addition, feeding on high value crops with

Correspondence: Christian Borgemeister, International Centre for Insect Physiology and Ecology, Nairobi, Kenya. Tel: +254 20 8561686. Fax: +254 20 8561690. E-mail: dg@icipe.org

ISSN 0958-3157 print/ISSN 1360-0478 online
DOI: 10.1080/09583150701546375

low damage thresholds like cotton, tobacco, sweet corn, and tomato often results in severe crop losses and damage (Fitt 1989).

Although synthetic pesticides continue to be the main pest control agents, recently increasing cases of resistance and reduced susceptibility of *H. armigera* to several insecticides have been recorded worldwide (e.g. Cameron et al. 1995; Ahmad et al. 1997; Gunning et al. 1998; Han et al. 1999; Martin et al. 2000). Hence, interest in bio-pesticides is growing as they are showing promise for the protection of agricultural crops (Inglis et al. 2001). One potential alternative in *H. armigera* management is the use of entomopathogenic fungi which are known for their environmental safety and pest selectivity (Carner & Yearian 1989; Jayaraj et al. 1989; King & Coleman 1989). *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *B. brongniartii* (Saccardo) Petch, *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Nomuraea rileyi* (Farlow) Samson have all been reported from Heliothinae species (Carner & Yearian 1989; Tang & Hou 1998; Hua et al. 1999). Laboratory and field studies using *B. bassiana* and *N. rileyi* have shown these species have high efficacy against different larval instars of *H. armigera* (Holdom & Van De Klashorst 1986; Hassani 2000; Sandhu et al. 2001; Sun et al. 2001). The studies showed that fungal virulence is determined by a variety of factors, including host and fungal physiology in interactions with various environmental factors. Germination rate and speed of hyphae penetration and proliferation in the insect hosts are species/strain-dependent (Hajek & St. Leger 1994; Altre & Vandenberg 2001).

The main objective of this study was to critically evaluate the potential of entomopathogenic fungi for control of *H. armigera* larvae and also for pupae. Therefore, the efficacy of several species/strains of the entomopathogenic fungi *M. anisopliae*, *B. bassiana* and of *Paecilomyces fumosoroseus* (Wize) Brown and Smith were evaluated. Subsequently, the best-performing strain of each tested species was used to further characterise the age-dependent mortality (LC_{50} and LT_{50}) against different larval stages and the pupae of *H. armigera.*

Materials and methods

Rearing of Helicoverpa armigera

Helicoverpa armigera larvae were originally obtained from the Institute for Biological Control of the Federal Biological Research Centre for Agriculture and Forestry (BBA) in Darmstadt, Germany, and reared individually in small plastic containers ($1.8 \times 1.8 \times 1.8$ cm) containing sufficient artificial diet for completion of larval development. The artificial diet contained 20 g agar, 2 g nipagin, 2 g benzoic acid, 2 g frisomycin, 8 g vitamin mixture for insects, 6 g ascorbic acid, 2 g Wesson salt, 40 g yeast, 40 g wheat germ, 4 g sunflower oil and 125 g white bean flour per 1 L diet. Pupae were kept in plastic boxes ($13 \times 15 \times 10$ cm) on a layer of tissue paper. Emerged adults were transferred to plastic cylinders (diameter 25 cm, height 40 cm) and fed on cotton buds soaked with 10% sucrose for oviposition. Insect rearing was carried out in a climate-controlled chamber at $27 \pm 2^{\circ}$C, 65 ± 5% relative humidity (RH) and a photoperiod of L16:D8. One-day-old larvae of all tested stages and pupae were used in experiments.

Plants

A Thai tomato *Solanum lycopersicum* L. (Solanaceae) cv. Phuang Chom Poo (East–West Seed Co. Ltd., Bang Bue Thong Nonthaburi, Thailand) was used as a base food crop. The tomato plants were planted in a commercial soil (Fruhstorfer Erde, Archut GmbH, Wallenrod, Germany) and grown in a greenhouse at $30 \pm 1^{\circ}$C and $65 \pm 5\%$ RH.

Fungal pathogens

Fungal isolates. Seven strains of three species were selected from the fungal culture collection of BBA, Darmstadt, Germany, including *M. anisopliae* strains 79 and 97, *B. bassiana* strains 108, 124 and 139 and *P. fumosoroseus* strains 14 and 15 (Table I). The selection was partly based on the original lepidopteran host from which they were isolated and their geographical origin. Fungi were cultured on malt extract peptone agar (MEPA), containing 3% malt extract, 0.5% soya peptone and 1.8% agar, following the protocol of Kassa et al. (2002). Fungal cultures were kept in an incubator at $25 \pm 1^{\circ}$C and $65 \pm 5\%$ RH in the dark for 14 days before use in experiments.

Preparation of conidial suspensions and viability test

Conidia were harvested from the MEPA culture by scraping them off the medium surface by a loop needle and transferring them to an Eppendorf tube containing 1 mL 0.1% Tween 80. Tubes were then shaken on a Vortex mixer for 3 min to homogenise the hydrophobic conidia. Spore concentration was determined using a hemocytometer (Thoma cell chamber). Conidial viability was tested according to Goettel and Inglis (1997). Three droplets of a diluted suspension containing ca. 1×10^6 conidia mL^{-1} were placed on MEPA and incubated at $25 \pm 1^{\circ}$C and $65 \pm 5\%$ in the dark for 24 h. After staining with lacto-phenol cotton blue, germination was checked under a microscope (Zeiss Axioplan, $\times 400$). Only spores with a germ tube as long as the conidium width were considered to have germinated. In all tested strains more than 90% of the conidia had germinated.

Efficacy screening of entomopathogenic fungi against H. armigera *third instar larvae*

The immersion method was used in this study as described by Goettel and Inglis (1997). *Helicoverpa armigera* third instars were immersed individually for 10 s into a

Table I. Entomopathogenic fungi used in the screening bioassays.

Fungal species	Strain	Host	Origin
M. anisopliae	79	*Agrotis segetum* (Lepidoptera: Noctuidae)	Germany
	97	Semi-looper (undefined) (Lepidoptera)	Philippines
B. bassiana	108	*Hepialus* sp. (Lepidoptera: Hepialidae)	Germany
	124	Re-isolation from commercial EPF product	China
	139	Re-isolation from commercial EPF product	China
P. fumosoroseus	14	*Bemisia tabaci* (Homoptera: Aleyrodidae)	Pakistan (Dr Lacey)
	15	*B. tabaci*	Taiwan (Dr Lacey)

fungal suspension containing 1×10^7 conidia mL^{-1}. In the control larvae were dipped into a 0.1% Tween 80 solution. Treated larvae were allowed to freely crawl in a Petri dish to remove excess moisture before placing them individually onto a filter paper, which was moistened on the first day and every next 2 days with 500 μL distilled water, in a Petri dish (diameter 7 cm). Excised parts of tomato leaves were added as food source for the larvae. Leaves were regularly replaced by fresh ones. The Petri dishes were held in a climate chamber at $27 \pm 1°C$, $65 \pm 5\%$ RH and 16L:8D photoperiod. Mortality of *H. armigera* was assessed daily for 2 weeks. Dead larvae were transferred to a Petri dish containing a piece of moistened cotton to promote outgrowth and sporulation of the respective fungi. Thirty individuals were tested in each treatment, and the experiments were repeated three times over time. The best-performing fungal strain of each species was then selected for subsequent experiments.

Dose–mortality effect of M. anisopliae *79,* B. bassiana *124 and* P. fumosoroseus *14 against* H. armigera *third instar larvae*

Third instar larvae were dipped into four different spore concentrations (1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia mL^{-1}) of *M. anisopliae* 79, *B. bassiana* 124 and *P. fumosoroseus* 14. For the control treatment, larvae were dipped into a 0.1% Tween 80 solution. Insect mortality and sporulation on cadavers were assessed daily over 14 days. The median lethal concentration (LC_{50}) was determined. Thirty *H. armigera* L3 larvae were used per treatment and the experiments were repeated three times over time.

Efficacy of M. anisopliae *79,* B. bassiana *124 and* P. fumosoroseus *14 against various larval and pupal stages of* H. armigera

The same immersion method as described above was used to evaluate the pathogenicity of the selected fungal species/strains at 1×10^7 conidia mL^{-1} to L2–L5 and pupae of *H. armigera*. Pupae after immersing into fungal suspension, were individually released onto soil surface in 28-well plastic trays ($4 \times 4 \times 5$ cm/well). Each well contained 5 g of a commercial soil substrate (Fruhstorfer Erde, Archut GmbH, Wallenrod, Germany), which composed of humus, clay and peat in a proportion of 15:35:50, respectively, and was moistened with 2 mL of tap water. For each larval and pupal stage, a control treatment was included by immersing in 0.1% Tween 80. Twenty to 30 individuals were tested per treatment and the experiments were repeated three times over time. Daily mortality and sporulation on larval cadavers were observed for 14 days as described above. The emergence of adults from pupae was monitored over 2 weeks.

Soil application of M. anisopliae *79,* B. bassiana *124* and P. fumosoroseus *14 against* H. armigera *pupae*

Two soil inoculation methods were used, i.e. soil inoculation and soil surface contamination. In the soil inoculation method, 5 g soil in each well of 28-well plastic trays ($4 \times 4 \times 5$ cm/well) were first moistened with 1 mL tap water, followed by 1 mL of the fungal suspension (1×10^7 conidia mL^{-1}) which was gently mixed into the soil to give a final concentration of 2×10^6 conidia/g soil. Pupae were released

individually into each well. In the control, the soil was treated with 1 mL tap water and 1 mL of 0.1% Tween 80. For the soil surface contamination method, plastic boxes ($12 \times 15 \times 10$ cm) were filled with a 1-cm layer of the soil substrate. Subsequently, 15 pupae were released into one box and then covered with another 2 cm layer of the same commercial soil substrate. A total amount of 100 g soil moistened with 20 mL of tap water was used per box. Finally, 15 mL containing 1×10^7 conidia mL^{-1} were applied to the soil surface. In the control, the same amount of a 0.1% Tween 80 solution was used. Thirty pupae were tested in each method and the experiments were repeated three times over time. All trays and boxes were covered by ventilated plastic lids and incubated at $27 \pm 1^{\circ}C$, $65 \pm 5\%$ RH, and 16L:8D photoperiod. The emergence of adults was monitored over 2–3 weeks. Pupae that failed to emerge into adults were observed for fungal outgrowth and sporulation as described above.

Statistical analysis

Corrected percent mortality was calculated using Abbott's formula (see Goettel & Inglis 1997) and prior to analysis these values were arcsine transformed. Data were then analysed by means of two-way ANOVA using the general linear model (PROC GLM) of SAS (1989). In case of significant F values, means were compared using Tukey's test. The significance level was set at $P < 0.05$. Median lethal time value was computed using the LIFETEST option of SAS. The LT_{50} values were derived using Kaplan–Meier estimates. The homogeneity of the survival curves among the treatments was tested using Log-rank tests. Probit analysis was used to analyse the median lethal concentration (LC_{50}) with 95% confidence interval (CI) using the PROBIT option of SAS. Percentages of sporulating cadavers were compared between isolates and across dosages for each experiment using two-way ANOVAs followed by Tukey's test where appropriate.

Results

Efficacy screening of entomopathogenic fungi against H. armigera *third instar larvae*

All tested fungal species/strains were highly virulent against third instar *H. armigera* (Table II). However, significantly higher virulence of *B. bassiana* and *P. fumosoroseus* strains than *M. anisopliae* strains was recorded ($F_6 = 21.1$, $P < 0.0001$). After 2 weeks, the highest corrected mortality was recorded for *B. bassiana* 124 (100%) and *P. fumosoroseus* 14 (100%); whereas the lowest mortality was recorded for *M. anisopliae* 97 (68.1%). In addition to high efficacy, *P. fumosoroseus* strains also caused significantly faster death of the third instar *H. armigera* compared to that treated with *M. anisopliae* strains ($F_6 = 5.82$, $P = 0.0048$). The shortest median lethal time was recorded in larvae treated with *P. fumosoroseus* 14 (3 days), followed by that treated with *B. bassiana* 124 (4.3 days).

A high rate of mycosis was also observed on larval cadavers following treatment with *P. fumosoroseus* and *B. bassiana* (Table II). In addition to causing lower mortality, sporulation on *M. anisopliae*-treated cadavers was also significantly lower than on *B. bassiana* or *P. fumosoroseus*-treated insects ($F_6 = 8.4$, $P = 0.001$) and did not exceed 50%.

Table II. Efficacy of entomopathogenic fungi tested (1×10^7 conidia mL^{-1}) on third instar *H. armigera* 2 weeks after treatment at 27°C.

Fungus	Corrected mortality (% ± SD)	Sporulation on cadavers (% ± SD)	LT_{50} (days ± SD)
M. anisopliae 79	82.0 ± 6.2 b	51.0 ± 23.2 bc	5.3 ± 0.8 a
M. anisopliae 97	68.1 ± 6.0 c	36.2 ± 23.8 c	6.5 ± 0.5 a
B. bassiana 108	98.6 ± 2.5 a	59.1 ± 15.1 abc	5.7 ± 1.5 a
B. bassiana 124	100.0 ± 0.0 a	82.1 ± 6.6 ab	4.3 ± 0.6 ab
B. bassiana 139	98.7 ± 2.3 a	88.7 ± 10.2 a	5.3 ± 1.2 a
P. fumosoroseus 14	100.0 ± 0.0 a	86.4 ± 20.8 a	3.0 ± 0.0 b
P. fumosoroseus 15	87.3 ± 7.5 ab	90.7 ± 1.6 a	5.2 ± 1.9 ab
0.1% Tween 80 (Mortality% ± SD)	18.9 ± 3.8		

Means within a column followed by the same letter are not significantly different (Tukey's test, $P < 0.05$). Data show the mean of three assays.

Based on these results, *B. bassiana* 124, *P. fumosoroseus* 14 and *M. anisopliae* 79 were selected among the fungal species/strain tested for further studies.

Dose–mortality effect of M. anisopliae *79,* B. bassiana *124 and* P. fumosoroseus *14 against third instar* H. armigera

The estimated LC_{50} values based on the mortality trends across dosages are presented in Table III. *Paecilomyces fumosoroseus* 14 showed the highest virulence with the lowest LC_{50} (4.2×10^4 conidia mL^{-1}), followed by *B. bassiana* 124 (1.5×10^5 conidia mL^{-1}) and *M. anisopliae* 79 (6.0×10^5 conidia mL^{-1}). Probit regression slopes were 1.5, 2.1 and 1.4 for *P. fumosoroseus* 14, *B. bassiana* 124 and *M. anisopliae* 79, respectively.

Table III. Median lethal concentration (LC_{50}) of *M. anisopliae* 79, *B. bassiana* 124 and *P. fumosoroseus* 14 (conidia mL^{-1}) to third instar *H. armigera* at 27°C.

Fungus/assays	LC_{50} (conidia mL^{-1})	Slope ± SE	χ^2	*P*	Confidence interval
M. anisopliae 79					
Assay 1	5.1×10^5	1.3 ± 0.3	16.84	< 0.0001	1.2×10^5–1.6×10^6
Assay 2	6.8×10^5	1.6 ± 0.5	10.3	0.0013	7.3×10^5–2.1×10^6
Assay 3	5.1×10^5	1.4 ± 0.4	12.02	0.0005	6.1×10^4–1.7×10^6
Mean	6.0×10^5	1.43			
0.1% Tween 80 (Mortality% ± SD)	24.4 ± 5.0				
B. bassiana 124					
Assay 1	2.1×10^5	2.2 ± 0.7	11.10	0.0009	2.6×10^4–5.4×10^5
Assay 2	1.3×10^5	2.4 ± 0.7	13.01	0.0003	2.8×10^4–3.0×10^5
Assay 3	9.4×10^4	1.9 ± 0.6	8.80	0.003	3.2×10^3–2.7×10^5
Mean	1.5×10^5	2.17			
0.1% Tween 80 (Mortality% ± SD)	26.7 ± 3.3				
P. fumosoroseus 14					
Assay 1	4.2×10^4	1.1 ± 0.3	15.66	< 0.0001	4.3×10^3–1.4×10^5
Assay 2	3.6×10^4	1.8 ± 0.5	15.41	< 0.0001	5.9×10^3–9.2×10^4
Assay 3	4.9×10^4	1.7 ± 0.4	22.55	< 0.0001	1.5×10^4–1.1×10^5
Mean	4.2×10^4	1.53			
0.1% Tween 80 (Mortality% ± SD)	12.2 ± 3.8				

Efficacy of M. anisopliae *79,* B. bassiana *124 and* P. fumosoroseus *14 against various larval and pupal stages of* H. armigera

Significant high mortalities were also recorded when the test of three selected fungi were extended to other larval and pupal stages of *H. armigera* (Table IV). There was no difference in the susceptibility of the different larval and pupal stages tested in terms of total mortality following treatments with all three fungal species at 1×10^7 conidia mL^{-1}. However, second instars succumbed faster to the pathogens than older ones (Table V). Median lethal times of different larval stages were significantly different ($F_3 = 4.28$, $P = 0.06$ for *M. anisopliae* 79; $F_3 = 8$, $P = 0.02$ for *B. bassiana* 124; $F_3 = 8.05$, $P = 0.02$ for *P. fumosoroseus* 14).

The percentage of cadavers of all larval stages showing fungal outgrowth and sporulation was 67.1–98.3% in *B. bassiana* 124 and 84.4–100% in *P. fumosoroseus* 14 compared to 18.0–74.4 in *M. anisopliae* 79 (Table VI). All pupae failed to emerge and showed outgrowth of the respective fungal species with which they had been treated. No significant differences were detected in sporulation on different larval stage cadavers previously exposed to *M. anisopliae* 79 and *P. fumosoroseus* 14. Sporulation occurred on a significantly lower proportion of fourth instar cadavers compared to the other larval stage cadavers previously treated with *B. bassiana* 124 ($F_3 = 9.56$, $P = 0.01$).

Soil application of M. anisopliae *79,* B. bassiana *124 and* P. fumosoroseus *14 against pupae of* H. armigera

High mortality (74.4–97.8%) was obtained when pupae were released into fungus-inoculated soil (Table VII). In this manner, *P. fumosoroseus* 14 performed significantly better than *B. bassiana* 124 ($F_2 = 8.16$, $P = 0.04$). In contrast, the soil surface contamination method had no effect on *H. armigera* pupae in all fungal strains tested. Significant differences in efficacy of the same fungus treated were detected between the two soil application methods ($t_4 = -14.7$, $P = 0.0001$ for *M. anisopliae* 79; $t_4 = -9.7$, $P = 0.0006$ for *B. bassiana* 124 and $t_4 = 20.1$, $P < 0.0001$ for *P. fumosoroseus* 14). Fungal outgrowth and sporulation was evident in all pupae which failed to emerge to adults.

Table IV. Corrected mortality of different larval and pupal stages 2 weeks after exposed to *M. anisopliae* 79, *B. bassiana* 124 and *P. fumosoroseus* 14 (1×10^7 conidia mL^{-1}) at 27°C.

	Corrected mortality (% ± SD)				
Fungus	L2	L3	L4	L5	Pupa
M. anisopliae 79	90.3 ± 12.5 a	82.0 ± 6.2 a	87.0 ± 15.1 a	98.6 ± 2.5 a	100.0 ± 0.0 a
B. bassiana 124	98.2 ± 3.0 a	100.0 ± 0.0 a	98.6 ± 2.5 a	100.0 ± 0.0 a	100.0 ± 0.0 a
P. fumosoroseus 14	100.0 ± 0.0 a	100.0 ± 0.0 a	97.3 ± 4.6 a	100.0 ± 0.0 a	100.0 ± 0.0 a
0.1% Tween 80 (Mortality% ± SD)	15.3 ± 4.8	18.9 ± 3.8	13.3 ± 12.0	15.6 ± 6.9	0.0 ± 0.0

Means for different larval stages treated with the same fungus followed by the same letter are not significantly different (Tukey's test, $P < 0.05$). Data show the mean of three assays.

Table V. Median lethal time (LT_{50}) of *M. anisopliae* 79, *B. bassiana* 124 and *P. fumosoroseus* 14 (1×10^7 conidia mL^{-1}) to different larval stages of *H. armigera* at 27°C.

	LT_{50} (days ± SD)			
Fungus	L2	L3	L4	L5
M. anisopliae 79	2.5 ± 0.9 b	5.3 ± 0.8 a	4.3 ± 0.6 ab	4.3 ± 0.6 ab
B. bassiana 124	3.3 ± 0.6 ab	4.3 ± 0.6 a	2.7 ± 0.6 b	4.3 ± 0.6 a
P. fumosoroseus 14	2.2 ± 0.3 b	3.0 ± 0.0 ab	2.7 ± 0.6 b	4.7 ± 1.2 a

Median lethal time for different larval stages treated with the same fungus followed by the same letter are not significantly different (Tukey's test, $P < 0.05$). Data show the mean of three assays.

Discussion

Entomopathogenic fungi have potential to control *H. armigera.* All seven tested strains from three species showed high efficacy against third instar *H. armigera* in the screening tests, corroborating previous findings of Hassani (2000). However, the *P. fumosoroseus* and *B. bassiana* strains were more virulent than the two tested *M. anisopliae* strains. These results were similar to previous laboratory findings demonstrating high virulence of *B. bassiana* against *H. armigera* larvae (Sandhu et al. 2001). In other laboratory experiments, *N. rileyi* (1×10^7 conidia mL^{-1}) caused 90.5 − 100% mortality in fourth instar *H. armigera* which were fed on fungus-treated corn silks, soybean leaves, tomato and chrysanthemum (Tang & Hou 1998). *Nomuraea rileyi* hyphal bodies (blastospores) were also virulent against first and third *H. armigera* instars (Holdom & Van De Klashorst 1986).

In addition to high virulence, outgrowth and sporulation of entomopathogenic fungi on cadavers is a key factor for proliferation and spread of the disease within a pest population in nature, and is an important consideration in the selection of entomopathogens for biological control (Hajek & St. Leger 1994; Inglis et al. 2001). In this study, the percentage of fungal outgrowth on cadavers varied among different *H. armigera* instars and the fungal species tested. Although conidiogenesis on cadavers usually require periods of high humidity, conidia are formed on cadavers independently of fluctuations in relative humidity as long as the cadavers do not rapidly desiccate (Inglis et al. 2001). Moreover, according to Hallsworth and Magan (1999) *B. bassiana* and *M. anisopliae*, as well as some other entomopathogenic fungi, can infect insects under conditions of low ambient humidity as long as there is sufficient moisture available on the host cuticle. However, Thomas et al. (2003) found

Table VI. Percentage sporulation on *H. armigera* larval cadavers instars following treatment with different entomopathogenic fungal species/strains at 25°C.

	Sporulation on cadaver (% ± SD)				
Fungus	L2	L3	L4	L5	Pupa
M. anisopliae 79	18 ± 22.7b	51 ± 23.2ab	31 ± 26.8b	74.4 ± 26.3ab	100.0 ± 0.0a
B. bassiana 124	95.8 ± 7.2ab	82.1 ± 6.6bc	67.1 ± 7.5c	98.3 ± 2.9a	100.0 ± 0.0a
P. fumosoroseus 14	100.0 ± 0.0a	86.4 ± 20.8a	96.7 ± 5.8a	87.8 ± 21.2a	100.0 ± 0.0a

Means for different larval stages treated with the same fungus followed by the same letter are not significantly different (Tukey's test, $P < 0.05$). Data show the mean of three assays.

Table VII. Efficacy of *M. anisopliae* 79, *B. bassiana* 124 and *P. fumosoroseus* 14 on pupae of *H. armigera* in two different soil application techniques after 14 days at 27°C.

Fungus	Percentage of pupae failed to emerge (% ± SD)	
	Soil surface contamination	Soil inoculation
M. anisopliae 79	6.7 ± 6.7 Ba	86.7 ± 6.7Aab
B. bassiana 124	11.1 ± 7.7 Ba	74.4 ± 8.4 Ab
P. fumosoroseus 14	5.5 ± 6.9 B[1]a[2]	97.8 ± 3.8 Aa
0.1% Tween 80 (Mortality% ± SD)	0a	0c

Means within a fungus ([1]) and method ([2]) followed by the same upper and lower case letter, respectively, are not significantly different (Tukey's test, $P < 0.05$). Data show the mean of three assays.

M. anisopliae to be a relatively weak saprophytic fungus. This may explain the relatively poor level of sporulation on *M. anisopliae*-treated cadavers in this study.

Virulence is an important factor for selection of candidate fungal strains for insect control (Inglis et al. 2001). In the dose–mortality study, *P. fumosoroseus* 14 was the most virulent strain tested against *H. armigera* as evidenced by its comparatively low LC_{50} value.

Different developmental stages of insects vary in their susceptibility to infection by entomopathogenic Hyphomycetes (Inglis et al. 2001). The resistance of caterpillar to pathogens and parasites is positively correlated to melanism on their cuticle and in the midgut (Wilson et al. 2001). Melanin strengthens the insect's cuticle, preventing the penetration of parasites and pathogens. On the other hand, melanin is toxic to micro-organisms (see Wilson et al. 2001). Melanin levels in the cuticle of larvae of the Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) were found to extensively vary across five developmental stages examined, i.e. late fourth to mid-sixth larval stages. This difference was more profound in larvae which were reared individually (Lee & Wilson 2006). Hafez et al. (1997) found that early larval instars of the potato tuber moth *Phthorimaea operculella* (Z.) (Lepidoptera: Gelechiidae) were more susceptible to *B. bassiana* than older larval stages. However, Vandenberg et al. (1998) reported that third and fourth instar diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) were more susceptible to *B. bassiana* than second instars. In our study, high mortality levels were obtained for *H. armigera* larval stages (L2–L5) treated with 1×10^7 conidia mL^{-1}. The shorter median lethal time value for second instars suggests that they are more susceptible to infection. The short life time of the second instar *H. armigera* of about 2 days may be the reason for their greater susceptibility to fungal infections. According to Lee and Wilson (2006) within a given larval stage, melanism of *S. littoralis* decreases markedly from the first to the last day, when caterpillars prepare to moult to the next stage. The 1-day-old second instars *H. armigera* in our study entered the second day or last day of their developmental stage when fungal conidia adhered to their cuticle were germinating. The reduction of melanin on their culticular at this time (Lee & Wilson 2006) may cause the larvae to succumb to fungal penetration. Thus, the fungus may act faster in killing the second instars than other larval stages tested. On the contrary, the early second and third larvae of diamondback moth and second instars of thrips were found to be more susceptible to *B. bassiana* than the older individuals of the same larval stage (Vandenberg et al. 1998; Ugine et al. 2005).

The thick and sclerotised pupal cuticle provides a great barrier to fungal infection (St. Leger 1993; Hajek & St. Leger 1994; De La Rosa et al. 2002). Pupae are also inactive and often difficult to target with a fungal treatment. Therefore, pupae have seldom been primary targets for biocontrol using fungal entomopathogens. However, in our study, we were able to induce high levels of infection in pupae by immersion in a fungal suspension or through soil inoculation prior to introducing pupae into the medium. This indicates that soil treatments targeting *H. armigera* pupae may provide a viable method for suppression. The high levels of mycosis we observed on the dead pupae also suggest that horizontal transmission of the pathogens can occur in the soil. Studies on effects of other entomopathogenic fungi against pupae showed that adult emergence rate is reduced (Hafez et al. 1997; Ekesi et al. 2002), or that the duration of the pupal stage is prolonged with a concurrent reduction in adult longevity (Kaaya & Okech 1990; Hafez et al. 1997). Malformation and reduced fecundity were also reported in adults emerging from fungal-treated pupae (Hafez et al. 1997). On the other hand, Hicks et al. (2001) reported that *B. bassiana* was ineffective against pupae of the pine beauty moth *Panolis flammea* Denis & Schiffermüller (Lepidoptera: Noctuidae). Similarly, *B. bassiana* had no effect on pupae of the Mexican fruit fly (De La Rosa et al. 2002). Sufficiently high fungal concentrations (Hafez et al. 1997; Hicks et al. 2001; Inglis et al. 2001) and contamination of pupae at the beginning of their development (Ekesi et al. 2002) are critical for infection to occur.

The success of soil applications of entomopathogenic fungi is strongly dependent upon pathogen virulence, good incorporation into the soil profile and persistence, which is heavily influenced by fungal strain, soil type, application method, environmental conditions and the presence or absence of a susceptible host (Inglis et al. 2001). Prophylactic applications of entomopathogenic fungi prior to the introduction of pupae into the soil have been recommended (Ekesi et al. 2002). Storey and Gardner (1989) showed that *B. bassiana* propagules significantly decreased (85–95%) in soil within 12 days of spraying the soil surface. Similarly, Vänninen et al. (2000) observed a rapid decline in *M. anisopliae* and *B. bassiana* soil populations in the first year after treatment, but populations stabilised thereafter. However, when *B. bassiana* was mixed into soil, persistence was enhanced (Gaugler et al. 1989). This may explain why, in our study, higher *H. armigera* mortality was obtained when soils were pre-inoculated with fungi than when only the soil surface was contaminated. In addition, pre-inoculation of fungus into the soil may enhance the contact effects of the fungus, both in time and with regard to coverage, as the late final larval stage of *H. armigera* drop on and pupate beneath the soil surface (King 1994), hence narrowing the 'window' for infection of the fungus applied on the soil surface.

Our results suggest that entomopathogenic fungi, particularly *P. fumosoroseus*, *B. bassiana* and *M. anisopliae*, are very effective against all *H. armigera* larval stages, and will infect pupae. Application of entomopathogenic fungi can be carried out in the presence of larvae at all developmental stages; however, for effects on larvae, sufficient concentrations of the pathogens are required. Building on the here reported promising laboratory results of entomopathogenic fungi against *H. armigera* pupae, appropriate soil application techniques need to be developed and tested under field conditions.

Acknowledgements

This study was partly conducted within the framework of the DFG Research Group 'Protected Cultivation' (FOR 431).

References

Ahmad M, Arif MI, Attique MR. 1997. Pyrethroid resistance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Pakistan. Bulletin of Entomological Research 87:343–347.

Altre JA, Vandenberg JD. 2001. Penetration of cuticle and proliferation on hemolymph by *Paecilomyces fumosoroseus* isolates that differ in virulence against Lepidopteran larvae. Journal of Invertebrate Pathology 78:81–86.

Cameron PJ, Walker GP, Herman TJB. 1995. Development of resistance to fenvalerate in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in New Zealand. New Zealand Journal of Crop and Horticultural Science 23:429–436.

Carner GR, Yearian WC. 1989. Development and use of microbial agents for control of *Heliothis* spp. in the USA. In: King EG, Jackson RD, editors. Proceedings of the workshop on biological control of *Heliothis*: Increasing the effectiveness of natural enemies, 11–15 November, 1985, New Delhi, India. Far Eastern Regional Research Office, US Department of Agriculture. pp 467–481.

De La Rosa W, Lopez FL, Liedo P. 2002. *Beauveria bassiana* as a pathogen of the Mexican fruit fly (Diptera: Tephritidae) under laboratory conditions. Journal of Economic Entomology 95:36–43.

Ekesi S, Maniania NK, Lux SA. 2002. Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. Biocontrol Science and Technology 12:7–17.

Fitt GP. 1989. The ecology of *Heliothis* species in relation to agro-ecosystem. Annual Review of Entomology 34:53–57.

Gaugler R, Costa SD, Lashomb J. 1989. Stability and efficacy of *Beauveria bassiana* soil inoculations. Environmental Entomology 18:412–417.

Goettel MS, Inglis GD. 1997. Fungi: Hyphomycetes. In: Lacey LA, editor. Manual of techniques in insect pathology. San Diego, CA: Academic Press. pp 213–247.

Gunning RV, Moores GD, Devonshire AL. 1998. Insensitive acetycholinesterase causes resistance to organophosphates in Australia *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Pesticide Science 54:319–320.

Hafez M, Zaki FN, Moursy A, Sabbour M. 1997. Biological effects of the entomopathogenic fungus, *Beauveria bassiana* on the potato tuber moth *Phthorimaea operculella* (Zeller). Journal of Pest Science 70:158–159.

Hajek AE, St. Leger RJ. 1994. Interactions between fungal pathogens and insect hosts. Annual Review of Entomology 39:293–322.

Hallsworth JE, Magan N. 1999. Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus*. Journal of Invertebrate Pathology 74:261–266.

Han Z, Wang Y, Zhang Q, Li X, Li G. 1999. Dynamics of pyrethroid resistance in the field population of *Helicoverpa armigera* (Hübner) in China. Pesticide Science 55:462–466.

Hassani M. 2000. Development and proving of biocontrol methods based on *Bacillus thuringiensis* and entomopathogenic fungi against the cotton pests *Spodoptera lituralis, Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Aphis gossypii* (Homoptera: Aphididae), PhD thesis. University of Giessen, Germany. p 130.

Hicks BJ, Watt AD, Cosens D. 2001. The potential of *Beauveria bassiana* (Hyphomycetes: Moniliales) as a biological control agent against the pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae). Forest Ecology and Management 149:275–281.

Holdom DG, Van De Klashorst G. 1986. Sporulation by hyphal bodies of *Nomuraea rileyi* and subsequent infection of *Heliothis* spp. Journal of Invertebrate Pathology 48:242–245.

Hua F, Liang F, Qin F, Yan W, Jun H, Fan X, Feng S, Fu Y, Wang R, Hu M. 1999. Field studies on the occurrence and epidemiology of entomopathogenic diseases infecting *Helicoverpa armigera* in Hebei. Chinese Journal of Biological Control (English abstract) 15:27–30.

Inglis GD, Goettel MS, Butt TM, Strasser H. 2001. Use of Hyphomycetous fungi for managing insect pests. In: Butt TM, Jackson C, Magan N, editors. Fungi as biocontrol agent progress, problems and potential. Wallingford, UK: CABI Publishing. pp 23–70.

Jayaraj S, Rabindra RJ, Narayanan K. 1989. Development and use of microbial agents for control of *Heliothis* spp. (Lepidoptera: Noctuidae) in India. In: King EG, Jackson RD, editors. Proceedings of the workshop on biological control of *Heliothis*: Increasing the effectiveness of natural enemies. 11–15 November 1985, New Delhi, India. Far Eastern Regional Research Office, US Department of Agriculture. pp 483–503.

Kaaya GP, Okech MA. 1990. Horizontal transmission of mycotic infection in adult tsetse, *Glossina morsitans morsitans.* BioControl 35:589–600.

Kassa A, Zimmermann G, Stephan D, Vidal S. 2002. Susceptibility of *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae) and *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) to entomopathogenic fungi from Ethiopia. Biocontrol Science and Technology 12:727–736.

King ABS. 1994. *Heliothis*/*Helicoverpa* (Lepidoptera: Noctuidae). In: Matthews GA, Tunstall JP, editors. Insect pest of cotton. Wallingford: CAB International. pp 21–75.

King EG, Coleman RJ. 1989. Potential for biological control of *Heliothis* species. Annual Review of Entomology 34:53–75.

Lee KP, Wilson K. 2006. Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait. Ecological Entomology 31:196–205.

Martin T, Ochou GO, Hala–N'Klo F, Wassal J, Waissayre M. 2000. Pyrethroid resistance in cotton bollworm *Helicoverpa armigera* in West Africa. Pest Management Science 56:549–554.

Sandhu SS, Unkles SE, Rajak RC, Kinghorn JR. 2001. Generation of Benomyl resistant *Beauveria bassiana* strains and their infectivity against *Helicoverpa armigera.* Biocontrol Science and Technology 11:245–250.

SAS Institute. 1989. SAS/STAT user's guide. Version 6, 4th edition, Vols 1&2. SAA Institute INC. Cary, NC, USA.

Storey GK, Gardner WA. 1989. Movement of aqueous spray of *Beauveria bassiana* into the profile on four Georgia soils. Environmental Entomology 18:835–839.

St. Leger RT. 1993. Biology and mechanism of insect-cuticle invasion by deuteromycete fungal pathogens. In: Beckage NE, Thompson SN, Fedrici BA, editors. Parasites and Pathogens of insects, Vol. 2: Pathogens. San Diego, CA: Academic Press. pp 211–229.

Sun LJ, Wu KM, Guo YY. 2001. The pathogenicity of *Beauveria bassiana* to *Helicoverpa armigera* under different temperatures and humidities. Acta Entomologica Sinica 44:501–506.

Tang L, Hou RF. 1998. Potential application of the entomopathogenic fungus, *Nomuraea rileyi*, for control of the corn earworm, *Helicoverpa armigera.* Entomologia Experimentalis et Applicata 88:25–30.

Thomas MB, Watson EL, Valverde-Garcia P. 2003. Mixed infections and insect-pathogen interactions. Ecology Letters 6:183–188.

Ugine TA, Wraight SP, Brownbridge M, Sanderson JP. 2005. Development of a novel bioassay for estimation of median lethal concentration (LC50) and doses (LD50) of the entomopathogenic fungus *Beauveria bassiana*, against western flower thrips, *Frankliniella occidentalis.* Journal of Invertebrate Pathology 89:210–218.

Vandenberg JD, Ramos M, Altre JA. 1998. Dose-response and age- and temperature-related susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae). Environmental Entomology 27:1017–1021.

Vänninen I, Tyni-Juslin J, Hokkanen H. 2000. Persistence of augmented *Metarhizium anisopliae* and *Beauveria bassiana* in Finnish agricultural soils. BioControl 45:201–222.

Wilson K, Cotter SC, Reeson AF, Pell JK. 2001. Melanism and disease resistance in insects. Ecology Letters 4:637–649.

Zalucski MP. 1986. The biology and ecology of *Heliothis armigera* (Hübner) and *H. punctigera* (Wallengren) (Lepidoptera: Noctuidae) in Australia: What do we know? Australian Journal of Zoology 42:329–334.