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A laboratory assessment of the potential of selected entomopathogenic fungi to control the green leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae).

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ABSTRACT

Objective: The present study was conducted to evaluate the effect of selected entomopathogenic isolates on various nymphal stages of the green leafhopper *Empoasca decipiens* Paoli.

Methodology and results: The efficacy of 5 fungal isolates from the genera of *Beauveria, Verticillium, Paecilomyces and Metarhizium* to the green leafhopper, *E. decipiens* nymphs, was tested under laboratory conditions. A single exposure concentration (1 x 10^7 conidia/ml) assays for each isolate were first conducted by contact with treated disc leaf for 5 min. These were followed by multiple-concentration assays on three of the most pathogenic isolates using four test concentrations ranging from 1 x 10^3 to 1 x 10^6 conidia/ml. All isolates proved to be highly virulent to late fifth-instar nymphs of *E. decipiens,* with mortality ranging from 60 to 98% and the mean survival time (MST), from 4.30 to 6.09 days. Among the tested isolates, three isolates, *M. anisopliae* (Ma43), *B. bassiana* (Bba113) and *P. fumosoroseus* (Pfr12) were the most pathogenic to the nymphs, resulting in significantly higher mortality and lower MST values. In the multiple-concentration assays, nymphal mortality was dose-dependent. The LC_{50} values ranged from 0.8 to 5.0 x 10^5 conidia/ml with the third- and fifth-instar nymphs being more susceptible than the first-instar. All tested strains were able to complete their life cycle by forming conidiospores on the dead insects; in general one to two days after the host had died.

Conclusion and application of findings: Our current study has identified virulent isolates of *M. anisopliae, P. fumosoroseus* and *B. bassiana,* the first two being commercially available, and that are good candidates for further development as biological control agents for *E. decipiens.*

Key Words: *Empoasca decipiens*, biological control, *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, *Verticillium lecanicillium*

INTRODUCTION

The green leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae) is a serious and extremely polyphagous pest of a wide range of economically important crops, and also attacks

various non-cultivated plants (Gencsoylu & Yalcin, 2004; Rassoulian *et al.,* 2005; Naseri *et al.,* 2007). The pest is common in Central and Southern Europe, Northern Africa and Central Asia where it is found both in greenhouses and in the field (Habib *et al.*, 1972; Schmidt & Rupp, 1997). 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; subsequent outbreaks in cucumbers in 1997 led to a complete loss of marketable fruits (Schmidt & Rupp, 1997). Females lay eggs in the plant tissue and both nymphs and adults damage plants directly by sucking on the leaves and fruits (Raupach *et al.,* 2002; Backus *et al.*, 2005). 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. Current control strategies mainly rely on the use of synthetic insecticides. The insect growth regulator buprofezin has been proven to effectively control *E. decipiens* with little to no harmful effects on natural enemies in greenhouses. However, it does not affect adults of leafhoppers (Helyer and Talbaghi, 1994). Chemical control of leafhoppers is generally difficult due to the lack of registered 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 (El-Nawawy *et al.*, 1983). So far 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 (Schmidt & Rupp, 1997; Maixner *et al.*, 1998; Rupp, 1999; Agboka *et al.*, 2003; Agboka *et al.*, 2004). 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 the releases of predatory arthropods is difficult and often not very successful since leafhopper adults and nymphs move too fast to be captured by commonly used predators in greenhouses such as *Orius* spp. (Heteroptera: Anthocoridae) (Helyer & Talbaghi, 1994). The lack of appropriate biological control strategies for *E. decipiens*, and the resulting reliance on chemical control threatens the otherwise very successful biological control of important pests in greenhouses like aphids, spider mites, leafminers and white flies. Hence, the development of alternative control methods for *E. decipiens* is of paramount importance to sustain the successful use of biological control 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 (Kamala & Padmavathamma, 1996), at present, only few specific data on the efficacy of these pathogens for control of *E. decipiens* are available (Tounou *et al*., 2003). Hence, the objective of this study was to gather more information on the host-pathogen interactions between strains of *Metarhizium anisopliae* (Metschnikoff) and *Beauveria bassiana* (Bals.), *Paecilomyces fumosoroseus* (Holm ex SF Gray) Brown & Smith and *Verticillium lecanicillium* (Zimmerman) Viegas (all Deuteromycotina: Hyphomycetes) and *E. decipiens.* These data, coupled with already existing information on the population dynamics of *E. decipiens* (Raupach *et al.*, 2002; Al-Suhaibani & Aldawood, 2005), could provide additional information for the development of a successful biological control strategy against this leafhopper species.

MATERIALS AND METHODS

Insect rearing: 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 and a photoperiod of 16:8 L:D and a relative humidity (RH) of 60-70%, following the protocol developed by Raupach *et al.* (2002). Leafhoppers of similar age structure were obtained by incubating adults on healthy plants in oviposition cages (46 x 46 x 122 cm). Adults were first kept for four days on healthy plants for oviposition. After gently shaking off the adult leafhoppers, he plants were then transferred to clean rearing cages (same dimensions as the oviposition cages) where the emerging nymphs were reared until maturity.

Fungal isolates: Single-concentration screening assays were first carried out on 5 isolates from four fungal species, including *B. Bassiana* (Bals.), *V. lecaniicillium* (Zimmerman) Viegas, *P. fumosoroseus* (Holm ex SF Gray) Brown & Smith and *M. anisopliae* (Metschnikoff) Sorokin (Table 1).

Table 1: Host and origin of entomopathogenic fungi isolates used in this study.

Isolates	Code	Hosts	Origin
B. bassiana	Bba113	*Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae)	Dr. J.B. Speakman, BASF, Germany
M. anisopliae	Ma43[a]	*Carpocapsa pomonella* L. (Lepidoptera: Tortricidae)	by Dr. Russ, Austria
M. anisopliae	Ma57	*Deois flavopicta* Stål (Homoptera: Cercopidae)	Dr. Roberts, USA
P. fumosoroseus	Pfr12[c]	*Bemisia argentifolii* Bellows & Perring (Homoptera: Aleyrodidae	Florida, USA
V. lecaniicillium	Vl23	*B. tabaci* Gennadius (Homoptera: Aleyrodidae)	Mycotal™, Koppert Ltd., The Netherlands

[a] This strain formed the basis of the commercial myco-insecticide Bio 1020™, Bayer Ltd., Germany; [b] strain originally isolated from *Deois flavopicta* Stål (Hemiptera: Cercopidae) in Brazil; [c] Isolated from the commercial myco-insecticide Preferal™, Biobest Ltd., Belgium

These isolates were originated from various insect hosts and substrates from Germany, Austria, Brazil, Netherland and the United States. The three most promising isolates were selected for subsequent multiple-concentration assays. All isolates were cultured on malt extract-peptone agar medium and held at 25°C in the dark for 14 days. Conidia were harvested by washing the plates with a sterile aqueous solution of 0.1% Tween 80 just before application. Conidia viability was assessed using methods described by Goettel and Inglis (1997). Only batches that were over 95% viable were used in the assays.

Bioassay Procedure

Single-concentration screening: A discriminatory singly concentration bioassay was conducted to select the most virulent isolates for further studies. For each isolate, an aqueous suspension (0.1% Tween 80 (v/v)) containing 1 x 10^7conidia/ml was prepared. Seventy (70) 12-day old fifth instar *E. decipiens* nymphs were inoculated per isolate in group of 10 individuals by direct contact with 9 cm diameter broad bean leaf discs treated by immersion in conidia suspension, for 5 minutes. A further 70 fifth instar nymphs were maintained in contact for 5 minutes with broad bean leaf discs treated with 0.1% Tween 80 (v/v) alone as control. Insects were thereafter transferred to clean plastic Petri dishes (150 x 20 mm^2) using a fine camel's-hair brush and incubated in seven groups of 10 for 7 days at 25 ±1°C, 65 ± 5 % R.H. and a photoperiod of 16:8 L:D light regime. Untreated leaf discs were used to feed the insects and changed every 24 hours. Following this methodology, leafhoppers could be kept on fresh leaves throughout the whole experimental period of seven days. Mortality was assessed at 24-h intervals, but to assure independence of values, each group of 10 insects per isolate was assessed only once, thus group 1 was assessed at 24 h only, group 2 at 48 h and so on to group 7 at 7 days. Three replicates of the strain selection test were performed at 2-week intervals.

Multiple-concentration assays: Based on the results of single-concentration screening, three fungal isolates, two of them being available as commercial myco-pesticide (*M. anisopliae* strain Ma43 (Bio 1020™, Bayer

Ltd., Germany), *P. fumosoroseus* strain Pf12 (Preferal™, Biobest Ltd., Belgium)) and *B. bassiana* strain Bba113, that readily induced higher mortality and lower Mean Survival Time (MST) values against the fifth instar nymphs, were selected for the second series of assays. The virulence of these isolates was assessed using multiple concentrations against first, third and fifth instar nymphs. In the multiple-concentration assays, nymphs were exposed to a range of conidial concentrations. Four concentrations (1 x 10^3, 1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia/ml) of the three selected isolates were prepared and used to treat the nymphs. For each concentration, seventy (70) individuals of each instar were inoculated as described in single-concentration screening assay and three replicate treatments per concentration were made in each experimental assay at 2-week intervals. Mortality was assessed at 24-h intervals, but to assure independence of values, each group of 10 insects per isolate was assessed only once, thus group 1 was assessed at 24 h only, group 2 at 48 h and so on to group 7 at 7 days.

Mycosis tests: In case dead insects were encountered, cadavers were immediately removed from the experimental units (Petri dishes and cages). Dead insects were surface sterilized in 70% ethanol, dried and incubated on moist filter paper inside sterile Petri dishes for 5 days to confirm infection by the strains.

All assays were conducted at the Institute of Plant disease and Plant Protection, University of Hannover, Germany.

Statistical Analysis: For the single-concentration assays, Median Survival Times (MST) was calculated using SPSS (SPSS, 1999). For multiple-concentration assays, the effect of isolate and concentration was determined by ANOVA and Fisher LSD ($\alpha = 0.05$). The Probit procedure was used to estimate LC_{50} values for the three selected isolates and for each tested instar (SAS Institute 1996). LC_{50} values were considered significantly different from one another when their 95% confidence intervals did not overlap (Tabashnick and Cushing 1987). The efficacy of the different isolates was compared using the final mortalities (i.e., 7 days cumulative mortalities). Mortality data were first corrected for natural mortality using Abbott's formula (Abbott, 1955) and then subjected to analysis of variance (ANOVA). All other analyses were performed using the general linear model (GLM) procedure of SAS (SAS Institute, 1996). All means were separated with the Student-Newman-Keuls test at 5%.

RESULTS

Single-concentration screening: The results of tests on the 5 isolates are given in Table 2. Late fifth-instar of *E. decipiens* was susceptible to the test fungal isolates under laboratory conditions, and mortality, 7 days after inoculation, ranged from 59.65 ± 1.85 to 95.37 ± 0.54 among the five isolates tested (Table 2). Among the tested isolates, *M. anisopliae* Ma43 demonstrated the shortest MST at 4.03 days and *V. lecaniicillium* Vl23 the longest at 6.03 days (Table 2). The evolution of cumulative mortality among late fifth instar of *E. decipiens* following contact with disc leaf treated with *M. anisopliae* indicates greater efficacy of *M. anisopliae* Ma43 compared to *M. anisopliae* Ma57 (Table 2). At the concentration used, 3 isolates were statistically as pathogenic.

Table 2: Seven-day cumulative percentage mortality (uncorrected) (± SE) and Median survival time (MST) of fifth instar *E. decipiens* after exposure to *B. bassiana* (Bba113), *M. anisopliae* (Ma43 and Ma57), *P. fumosoroseus* (Pfr12) and *V. lecaniicillium* (Vl23) in laboratory screening tests.

Isolates	Mortality (% ± SE)[ac]	MST[b] (days ± SE)
B. bassiana (Bba113)	86.52 ± 1.04b	5.33 ± 0.0b
M. anisopliae (Ma43)	95.37 ± 0.54a	4.30 ± 0.03a
M. anisopliae (Ma57)	59.65 ± 1.85c	5.57 ± 0.07b
P. fumosoroseus (Pfr12)	89.30 ± 0.23b	4.56 ± 0.04a
V. lecaniicillium (Vl23)	64.48 ± 0.19c	6.09 ± 0.08b
Control	3.06 ± 0.32d	>8[d]

[a] Seven days cumulative mortality; [b] median survival time; [c] means in the same columns followed by the same letter in the same column are not significantly different (Student-Newman-Keuls, $P \leq 0.05$); [d] MST exceeded the observational period.

Multiple-concentration assays: For the three test isolates nymphal mortality for *E. decipiens* was dose-dependent, is presented in Table 3. Irrespectively to insect developmental stage, control mortality never exceeded 5%. However, all tested isolates were highly virulent to *E. decipiens*, resulting in significantly higher mortality levels than in the control ($P < 0.0001$). For each developmental stage/isolate combinations, the Pearson chi-square for goodness-of-fit test were not significant ($P > 0.05$), indicating that all data fit the Probit model. Based on LC_{50} confidence intervals (Table 3), third- and fifth-instar nymphs were significantly more susceptible to the selected isolated than first-instar.

Mycosis tests: Microscopic observations of leafhoppers treated with the different fungal strains immediately after death revealed that all 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, on hundred percent of dead insects recorded in fungal treatments sporulation was observed usually 24 to 48 hours after death. On all cadavers, 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 (Figure 1A). Within four to six days after the application the sporulating growth had gradually covered the cadavers and spread outwards. 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 (Figure 1B). Although the sporulation level was not assessed in this study, particularly fast and profuse fungal growth was observed on leafhoppers killed by the *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 conidiospores (Figure 1C).

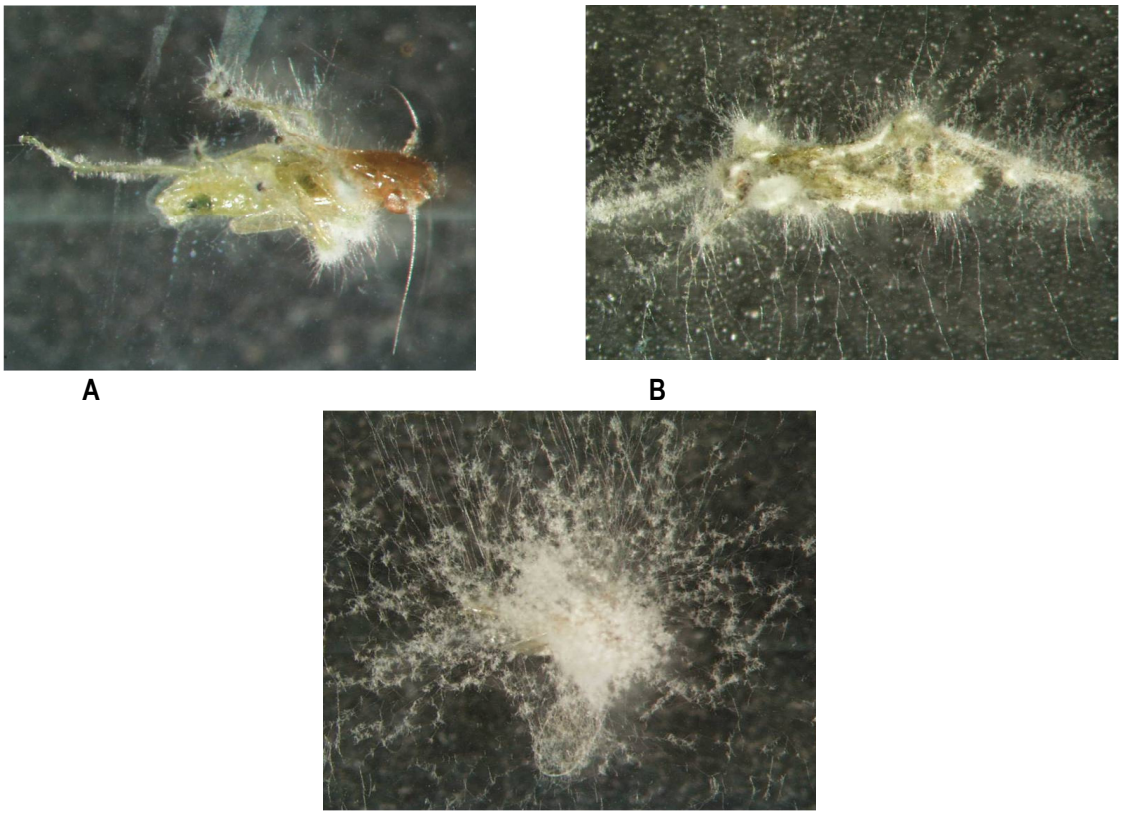

Figure 1. *Empoasca decipiens* nymphs showing sign of fungal infection. (A) infected nymph with initial external growth of the fungi on the antennae, legs, and pleural regions of the head and from intersegmental regions (60x); (B) nymph with darker pigment showing external signs of melanization and mummification, 3 days after infection (60x); (C) *E. decipiens* nymph dead 4 days after treatment with *P. fumosoroseus*, the fungus spreading outwards (60x).

DISCUSSION

All fungal strains tested revealed high virulence under laboratory conditions of high humidity and moderate temperature, and may thus possess good potential for microbial control of the green leafhopper *E. decipiens*. Although several entomopathogenic fungi, such as *M. anisopliae* (Kamala & Padmavathamma, 1996), are known to infect different *Empoasca* spp., so far the potential of entomopathogenic fungi as control agents of leafhoppers has received little attention.

Both dose rates and insect developmental stage significantly affected mean mortality in *E. decipiens*, with first instar nymphs being less sensitive than older ones. However, 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. 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 molts is short (Vey & Fargues, 1977; Gindin *et al.*, 2001; Alavo *et al.,* 2002), as it is in the case of younger instars *E. decipiens* (Raupach *et al.,* 2002). *Paecilomyces fumosoroseus* has been reported to be highly virulent against fourth instar of the silverleaf whitefly *Bemisia argentifolii* (Bellows & Perring) (Homoptera: Aleyrodidae) (Vidal *et al.*, 1997) while Wraight *et al.* (1998) recorded significantly higher mortality against the second instar nymphs of the same species. Similarly, western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), larvae have been reported to be less susceptible to *V. lecaniicillium* and *M. anisopliae* than the adult and pupal stages (Vestergaard *et al.*, 1995).

Generally virulence of entomopathogenic fungi may depend on the strain, host insect species and the mode of application (Ferron, 1978; De La Rosa *et al.*, 2002). *Paecilomyces fumosoroseus* isolate Pfr12 and *M. anisopliae* isolate 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 of *P. fumosoroseus* (Wraight *et al.*, 1998), as observed in our study 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 molts of the younger nymphal instars by entering the host before the removal of the previous cuticle. According to Castillo *et al.* (2000) differences in virulence among entomopathogenic fungal strains are common, corroborating results of this study. 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. lecaniicillium*, *P. fumosoroseus* and *M. flavoviride* Gams & Rozsypal strains have been reported (Gindin *et al.*, 2001). Similarly, germination speed of *M. anisopliae* (Jackson *et al.*, 1985), *V. lecaniicillium* (Chandler *et al.*, 1993; Altre *et al.*, 1999) was found to be positively correlated with infectivity of the fungi against mosquito larvae, adult aphids and diamondback moths, respectively.

Under the experimental conditions in this 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. Hyphal growth from ventral parts of the thorax and abdomen of dead *Empoasca* spp., attacked by *Zoophtora radicans* (Brefeld) Batko (Zygomycetes: Entomophthorales) has been reported by Ben-Ze'ev and Kenneth (1981). 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 coloration, 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).

The here presented results coupled with our previous investigations (Tounou *et al*., 2003), confirmed that entomopathogenic fungi can be promising agents for control of the green leafhopper *E. decipiens*. In both laboratory and greenhouse experiments, the strains Ma43 and Pf12, have resulted in up to 97% adult mortality 7 days after application and a 100% infection rate at a dosage of $1x10^7$ conidia/ml. Moreover experiments on potential side effects of the entomopathogenic fungi on *Anagrus atomus* L. (Hymenoptera: Mymaridae), an egg parasitoid of *E. decipiens*, showed that the tested isolates had no influence on adult emergence and longevity (Tounou *et al.*, 2003). Additional studies are however needed to confirm isolate performance under field conditions. Moreover, other fungal characteristics such as spore production, germination and hyphal growth rates, and effects of varying environmental conditions that influence persistence must also be evaluated to fully evaluate the biological control potential of the pest using entompathogenic fungi.

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Table 3: Concentration-mortality response of first, third and fifth instar nymphs of *E. decipiens* exposed to *B. assiana* (Bba113), *M. anisopliae* (Ma43) and *P. fumosoroseus* (Pfr12).

Fungal isolate	No. of insects[a]	Slope ± SE [log10 (dose)]	LC_{50}[b] (95% CI)[c] x 10^5	χ^{2d}	*P*-value
***B. bassiana* (Bba113)**					
1st instar nymphs	1120	0.31 ± 0.05	3.21 (8.94 - 49.37)a	38.10	<0.0001
3rd instar nymphs	1120	0.38 ± 0.05	0.34 (0.18 - 0.65)b	60.52	< 0.0001
5th instar nymphs	1120	0.56 ± 0.10	0.12 (0.01 - 1.04)b	31.56	< 0.0001
***M. anisopliae* (Ma43)**					
1st instar nymphs	1120	0.46 ± 0.14	0.14 (0.12 - 0.25)a	11.62	0.0007
3rd instar nymphs	1120	0.42 ± 0.17	0.07 (0.04 - 0.10)b	5.88	0.0153
5th instar nymphs	1120	0.67 ± 0.06	0.05 (0.03 - 0.08)b	131.45	< 0.0001
***P. fumosoroseus* (Pfr12)**					
1st instar nymphs	1120	0.63 ± 0.06	0.14 (0.09 - 0.21)a	131.41	<0.0001
3rd instar nymphs	1120	0.48 ± 0.05	0.08 (0.03 - 0.12)ab	84.14	< 0.0001
5th instar nymphs	1120	0.60 ± 0.06	0.06 (0.03 - 0.09)b	117.11	< 0.0001

[a] 70 insects per replicate, 4 replicates per concentration, 4 concentrations per isolate; [b] Conidia per milliliter; [c] for each strain, LC_{50} followed by a same letter are not significantly different based on confidence intervals; [d] Pearson chi square of the slope