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Pathogenicity, Virulence and the Interaction of *Metarhizium anisopliae* and *Beauveria bassiana* against *Phyllophaga vetula* (Coleoptera: Melolonthidae)

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***Phyllophaga* spp. cause severe damage to maize, sorghum, wheat, sugarcane, bean, amaranth and peanut in Mexico, Central America and the USA. Control measures for white grubs have depended mainly on persistent chemicals. An ecologically safe strategy is the use of entomopathogens in combating soil pests, which is based on the identification of a complex of pest species and their native pathogens and to subsequently select the microorganism with the greatest potential for this purpose. The objective of this study was to determine the pathogenicity, virulence and the interaction between native strains of *Metarhizium anisopliae* and *Beauveria bassiana* from Morelos State against *P. vetula*. The fungal isolates of *M. anisopliae* and *B. bassiana* showed differential pathogenicity against *P. vetula*. The *M. anisopliae* isolates were more pathogenic than those of *B. bassiana*. *M. anisopliae* isolates from a *Phyllophaga* sp. host were more pathogenic (46.66 to 73.33%) than those from an insect tramp, *G. mellonella* (00.00 to 20%). The mortality caused by the most highly pathogenic isolate of *M. anisopliae*, HI-019, (86.06%) decreased significantly (p: 0.05) when the inoculation was simultaneous with *B. bassiana* HI-113 (61.06%), but the mortality was statistically the same as that when the grubs were inoculated with only *B. bassiana* (52.73%). The estimated LC_{50} values of *M. anisopliae* isolates Ma17 and Ma19 against *P. vetula* larvae were 4.749×10^7 conidia/mL and 7.684×10^8 conidia/mL, respectively, which are statistically equivalent.**

Keywords: white grub, bioassay, entomopathogenic fungi, lethal concentration.

Melolonthidae larvae have slightly to strongly curved, C-shaped bodies, distinctive legs, and hardened head capsules, and they are referred to as white grubs. Mexico is a centre of diversity for the Melolonthidae[1], and a large number of species of the genus *Phyllophaga* have been recorded (386 in Mexico). Only relatively few species cause economic damage[2]; these include *Phyllophaga obsoleta* (Blanchard), *P. ravida* (Blanchard), and *P. vetula* Horn, which are distributed throughout the Mexican highlands[3].

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and the USA. Historically, control measures for white grubs have depended mainly on persistent chemicals, but because of concerns regarding safety and environmental contamination, other forms of control such as biological control have been proposed[3].

Due to their underground habitat during development, the grubs are susceptible to infection by microorganisms such as viruses, bacteria, protists, fungi and nematodes[3,4], with the latter having a high potential for use in the control of microbial growth[5]. An ecologically safe strategy in combating soil pests, the use of entomopathogens, is based on the identification of a complex of pest species and their native pathogens and to subsequently select the microorganism with the greatest potential for this purpose, taking as benchmarks the virulence, mobility, persistence, specificity and production costs of the pathogen[6].

Moist conditions, a relatively stable temperature, and protection against ultraviolet light from the soil favour the infection of larval melolonthids by entomopathogenic fungi[7], providing them with a high potential to act as control agents against rhizophagous larvae. Previous work involving bioassays with larval melolonthids has been inconsistent, largely because these bioassays have been performed using larvae collected in the field because breeding these species is difficult as a result of their annual cycles and underground habit. The objective of this study was to determine the pathogenicity, virulence and interactions between native Morelos state strains of *Metarhizium anisopliae* and *Beauveria bassiana* against *P. vetula*.

MATERIALS AND METHODS

Fungal isolates

Seven isolates of *M. anisopliae* and 20 of *B. bassiana* that were previously obtained in a survey conducted in Morelos State to collect native isolates of entomopathogenic fungi from infected white grubs or an insect tramp (*Galleria mellonella*) in a maize field were used[8]. The fungi were grown on Sabouraud dextrose agar (SDA) that included 5 g/l of mild peptone, 5 g/l casein peptone, 40 g/l dextrose, and 1.5 g/l agar. The culture was adjusted to pH 5.6 ± 0.2, was incubated in a dark room at 27 ± 1 °C for 15 d to induce sporulation, and was then preserved at 4 ± 2 °C. Conidia were recovered from the Petri dishes using distilled water (with 0.05% Tween 80) in a laminar flux chamber (CFLV-80; Aparatos de Laboratorio BG, Mexico). The conidia were counted in a Neubauer chamber.

P. vetula larvae

A large number of insects were required to perform the bioassays. For the pathogenicity and interaction experiment, a total of 1000 third-instar larvae of *P. vetula* were collected in the field in Villa de Ayala, Morelos, Mexico, placed individually in 30-ml plastic cups covered with plastic lids, transported to the laboratory, and maintained at 25±1°C for 7d before the bioassay. The collected larvae were separated based on the presence of palidia that were almost parallel in the last abdominal segment (raster) and 23-30 pali[9]. A small piece of carrot was added to each cup for food.

For the LC_{50} bioassays, a total of 600 first-instar larvae of *P. vetula* were field-collected at the same site but were maintained at 25±1°C up to the third instar.

Bioassays

The procedure to inoculate each isolate was the same for all of the experiments. For the analysis of pathogenicity, 27 treatments (seven isolates of *M. anisopliae* and 20 of *B. bassiana*) with 15 larvae each were tested. Field-collected larvae were surface-sterilized with a 2% NaCl solution, washed with distilled water, and placed on paper towels to eliminate excess water. The bioassay followed a modified "maximum challenge test" methodology, which is useful for separating virulent from non-virulent isolates at the early stages of entomopathogen screening programmes[10]. Because of the great variability in isolates[11], various conidial densities were used in the bioassay rather than sporulating strains.

For the interaction bioassay, the conidia of *M. anisopliae* (HI-019) and *B. bassiana* (HI-113) were evaluated alone and in combined doses of 1×10^8 con/ml, and distilled water (with 0.05% Tween 80) was used as a control. A completely randomized design was used, and each experimental unit was composed of 15 third-instar larvae of *P. vetula*. Each treatment was applied in the same way as in the pathogenicity bioassays. All bioassays were carried out in triplicate. The

mortality of white grubs was determined at 8 and 12 d.

After treatment, the larvae were placed individually into 30-ml cups with a piece of carrot as a food source. The larvae were maintained at 25±1°C, and mortality was evaluates by touching the grub on the thoracic segments with a probe.

Virulence bioassays (LC_{50})

Virulence was determined at four concentrations, and distilled water (with 0.05% Tween 80) was used as a control. Four *M. anisopliae* isolate Ma17 conidial densities were evaluated: 1×10^4, 1×10^5, 1×10^6, and 1×10^8 conidia/ml, and four conidial densities of isolate Ma19 were evaluated: 1×10^4, 1×10^5, 1×10^7, and 1×10^8 conidia/ml. Each treatment was applied in the same way as in the pathogenicity bioassays. All bioassays were carried out in triplicate. The mortality was determined at 30 d. A total of 45 third-

Table 1. Mortality of third-instar larvae of *P. vetula* caused by the conidia from seven isolates of *Metarhizium anisopliae* up to 30 d after inoculation. The conidial concentration was 1×10^8 c/mL (n=15). All isolates obtained for this study were from locations within Morelos, Mexico.

Isolate	Geographical origin	Host	% mortality
HI-010	Ocuituco	*Galleria mellonella*	20.00
HI-011	Yecapixtla	*Galleria mellonella*	13.33
HI-014	Chalcatzingo	*Galleria mellonella*	0.00
HI-016	Tlayca	*Anomala* sp.2 L2	26.66
HI-017	Tetela del Volcán	*Phyllophaga* sp. L3	60.00
HI-019	San Andrés de la Cal	*Phyllophaga* sp.3 L2	73.33
HI-020	San Andrés de la Cal	*Phyllophaga* sp.5 L3	46.66

Table 2. Mortality of third-instar larvae of *P. vetula* caused by the conidia from seven isolates of *Beauveria bassiana* up to 30 d after inoculation. The conidial concentration was 1×10^8 c/mL (n=15). All isolates obtained for this study were from locations within Morelos, Mexico.

Isolate	Geographical origin	Host	% mortality
HI-113	Yecapixtla	*Galleria mellonella*	53.33
HI-114	Totolapan	*Galleria mellonella*	0.00
HI-115	Ocuituco	*Galleria mellonella*	40.00
HI-116	Ocuituco	*Galleria mellonella*	26.66
HI-118	Jumiltepec	*Galleria mellonella*	26.66
HI-119	Campus UAEM	*Lygus* sp.	40.00
HI-120	Campus UAEM	*Lygus* sp.	13.33
HI-122	Campus UAEM	*Lygus* sp.	20.00
HI-123	Campus UAEM	*Lygus* sp.	6.66
HI-124	Campus UAEM	*Lygus* sp.	40.00
HI-125	Campus UAEM	*Lygus* sp.	13.33
HI-126	Campus UAEM	*Lygus* sp.	13.33
HI-127	Campus UAEM	*Lygus* sp.	6.66
HI-128	Campus UAEM	*Lygus* sp.	20.00
HI-129	Yautepec	*Galleria mellonella*	26.66
HI-133	Jonacatepec	*Galleria mellonella*	6.66
HI-134	Temoac	*Galleria mellonella*	33.33
HI-135	Temoac	*Galleria mellonella*	6.66
HI-136	Tenextepango	*Galleria mellonella*	33.33
HI-137	Tenextepango	*Galleria mellonella*	33.33

Table 3. Mortality caused by isolates of M. anisopliae (HI-019) and B. bassiana (HI-113) alone and in combination at conidial densities of conidia/mL against third-instar larvae of *P. vetula*

Treatment	8 d (DE)	12 d (DE)
Ma	52.74(±17.33) a	86.06(±9.58) a
Bb	44.4(±4.84) a	52.73(±9.64) ab
Ma + Bb	38.86(±9.64) ab	61.06(±17.37) b
Control	13.86(±9.64) b	22.2(±4.84) c

Table 4. Lethal concentration 50 (LC_{50}) of conidia of *Metarhizium anisopliae* isolates Ma17 and Ma19 against third-instar larvae of *P. vetula*

Strain	LC_{50} (c/mL)	95% CI LC_{50} Lower limit	95% CI LC_{50} Upper limit
Ma17	4.749×10^7	4.735×10^6	3.303×10^9
Ma19	7.684×10^8	3.721×10^8	1.389×10^9

instar larvae of *P. vetula* were used per treatment, and 225 were used per bioassay.

Statistical analysis

The percentage mortality data were arcsine transformed for statistical analysis. After processing the data, we performed analysis of variance (ANOVA) and Tukey's multiple comparisons of means at a significance level of 0.05 using the statistical package SAS 9.1 (2003). Probit analysis was performed to estimate the mean lethal concentration 50 (LC_{50}), and confidence intervals (CIs) were generated using the statistical package Polo Plus[12].

RESULTS AND DISCUSSION

Pathogenicity is a qualitative measure of the ability of a pathogen or parasite to cause disease in a host (5). The fungal isolates of *M. anisopliae* and *B. bassiana* showed different pathogenicity against *P. vetula*. In general, the *M. anisopliae* isolates (Table 1) were more pathogenic than those of *B. bassiana* (Table 2), corroborating other studies with *Phyllophaga* spp[13,14]. *M. anisopliae* isolates from a *Phyllophaga* sp. host were more pathogenic (46.66 to 73.33%) than those from an insect tramp, *G. mellonella* (00.00 to 20%). In this way, differential susceptibility of *Phyllophaga* spp. to fungal infection has been reported elsewhere[15, 16], and in *P. polyphylla*, larval infection never exceeded 30% for *B. bassiana* or *M. anisopliae*[17].

The mortality caused by the highly pathogenic isolate of *M. anisopliae* HI-019 (86.06%) decreased significantly (P Â 0.05) when the inoculation was simultaneous with *B. bassiana* HI-113 (61.06%), but the mortality was statistically the same as when a grub was inoculated with only *B. bassiana* (52.73%) (Table 3). In the biocontrol of insect pests, the efficacy of treatment with multiple pathogens has not been frequently investigated but may have some potential in effective management efforts. Co-infection in the field is not commonly reported; however, co-infection by *Entomophthora aulicae* and *Paecilomyces canadensis* was reported for *Lymantria dispar* in field observations of epizootic disease in a gypsy moth population in Japan[18]. In the rhizosphere, *Phyllophaga* spp. are frequently subject to co-infection by pathogens of distinct species[5]. From the experiments presented here, no beneficial effect was apparent in using the two fungi together. Similar results have been reported for other insect hosts[19]. Recent information about the antimicrobial activity of secondary metabolites isolated from *B. bassiana* and *M. anisopliae* has identified potentially bioactive substances with antimicrobial activity[20], which can cause one fungal infection to outcompete another.

The estimated LC_{50} values of *M. anisopliae* isolates Ma17 and Ma19 against third-instar larvae of *P. vetula* were 4.749×10^7 conidia/mL and 7.684×10^8 conidia/mL, respectively, which are statistically equivalent. Thus, additional studies must be conducted to further evaluate these isolates against white grubs under greenhouse and/or field conditions[10, 14]. Similarly, the more virulent strains can be considered as candidates for sustainable agriculture based on a strategy of conservation biological control[21].

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