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Pathogenicity, formulation and storage of insect pathogenic hyphomycetous fungi tested against *Diabrotica speciosa*

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Abstract. Studies were conducted to search for fungal strains with potential pathogenicity against *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae). Among sixteen fungal isolates screened the most virulent was a *Beauveria bassiana* (Balsamo) Vuillemin isolate (FHD13) that caused 70% mortality of *D. speciosa* third instar larvae. The LC_{50} value of *B. bassiana* isolate FHD13 was 3.48×10^{10} conidia/ml. Different temperatures (4, 17 and 26 °C) and vegetable oils (corn, sunflower and canola) used for storage did not significantly affect viability of conidia. A pathogenicity trial against *D. speciosa* larvae performed with the corn oil formulation (1×10^8 conidia/ml of oil) caused 65% of mortality.

Key words: *Beauveria bassiana*, conidia formulation, *Diabrotica speciosa*, *Metarhizium anisopliae*, microbial control, storage

Introduction

Corn rootworms, *Diabrotica* spp., are among the most important pests in agriculture. In Argentina and neighboring countries, *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae) is a problematic species because its wide distribution coincides with crops of high economic value. Both adults and larvae cause significant damage; while larvae feed on roots and tubers, adults eat the tassels in corn, flowers and young fruits in cucurbits, and cause heavy defoliation in beans (Defagó et al., 2000). The environmental hazard associated with chemical insecticide treatments for the control of this pest is a major additional problem (Ávila and Nakano, 2000). Importantly, alternative pest control strategies could result from releasing natural enemies, and may be considered in the future. Several species of phytophagous insects are attacked by more than 700 species of fungi from approximately 90 genera (Wraight and Roberts, 1987). They frequently decimate insect populations in spectacular epizootics (Roberts et al., 1991) and are important for coleopteran control since viral and

bacterial diseases from most beetle pests are unknown (St. Leger et al., 1992). Most entomogenous fungi belong to the Deuteromycotina and Zygomycotina (Samson et al., 1988). Two deuteromycetes with a pan-global distribution as members of the natural soil flora (Zimmerman, 1993), *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin, have been reported to regulate *D. speciosa* populations (Sosa-Gómez et al., 1994; Heineck-Leonel and Salles, 1997). The use of microbial control is a potentially valuable alternative in comparison with chemical insecticides because of its benefits concerning human safety, selectivity towards target organisms, and environmental preservation (Sosa-Gomez and Moscardi, 1994).

Conidia of entomopathogenic Deuteromycetes may be formulated and applied in a way similar to chemical pesticides (Prior and Greathead, 1989). The maintenance of conidial viability in formulations during storage is crucial for obtaining effective insect control in the field. Thus, the major issues in successful use of fungi for biological control are infectivity and persistence of the inoculum in the environment. It is well documented that effectiveness is increased with the addition of oils (Moore and Prior, 1993), which probably prevents conidial desiccation, helping adhesion and spreading the inoculum on the host body (Vimala Devi and Prasad, 1996).

The aim of this work was to search for novel isolates of fungi pathogenic to larvae of *D. speciosa* with the potential to be used in microbial control. Additionally we have investigated the effects of temperature and time of storage on the viability and virulence of conidial formulations.

Material and methods

Pathogenicity of fungal isolates against D. speciosa

The sixteen fungal isolates used in this study, which are listed in Table 1, were maintained on complete agar medium (Napolitano and Juárez, 1997) at 4 °C and subcultured monthly. Each isolate was tested for their pathogenicity against *D. speciosa* third instar larvae that were obtained from USDA-ARS South American Biological Control Laboratory (Hurlingham, Argentina). Conidia from 15-day-old sporulating cultures were harvested in 0.01% Tween 80, and their final concentration was adjusted to 1×10^8 conidia/ml. Larvae were infected by exposing batches of 20 third-instar larvae to corn sprouts (1 cm length) which had been previously dipped into the conidial suspension for 5 s. Larvae were placed individually in plastic 24-well cell culture plates (2 cm^2 $\times$ well) containing autoclaved sand. After 48 h fresh non-inoculated corn sprouts were supplied every two days. The assay was performed at 28 $\pm$

Table 1. Identification of fungal isolates and mortality of third-instar *D. speciosa* larvae treated with conidial suspensions (1×10^8conidia/ml)

Isolate	Sample origin	Fungal species	Mortality (%)
FHD 1	Soil	*M. anisopliae*	10
FHD 2	Soil	*M. anisopliae*	30
FHD 3	Soil	*M. anisopliae*	25
FHD 4	Soil	*M. anisopliae*	45
FHD 5	Soil	*B. bassiana*	0
FHD 6	Soil	*B. bassiana*	30
FHD 7	Soil	*B. bassiana*	25
FHD 8	*M. bridarolli**	*B. bassiana*	50
FHD 9	*M. bridarolli*	*B. bassiana*	35
FHD 10	*M. bridarolli*	*B. bassiana*	55
FHD 11	*M. bridarolli*	*B. bassiana*	30
FHD 12	*M. bridarolli*	*B. bassiana*	35
FHD 13	*M. bridarolli*	*B. bassiana*	70
FHD 14	*D. speciosa*	*M. anisopliae*	10
FHD 15	*D. speciosa*	*M. anisopliae*	0
FHD 16	*D. speciosa*	*M. anisopliae*	5

**Maecolaspis bridarolli* (Coleoptera: Chrysomelidae)

2 °C, 70% RH with a photoperiod of 14:10 (L:D) h for 10 days; numbers of dead insects were recorded daily. Dead larvae were transferred to moist chambers to determine the proportion of cadavers with resulting fungal emergence and sporulation. Control larvae were fed with corn sprouts dipped into 0.01% Tween 80. Each experiment was replicated twice.

The LC_{50} for strain FHD13 was estimated by Probit analysis (Finney, 1971). Groups of twenty larvae were individually fed with corn sprouts dipped into different concentrations of conidial suspensions, ranging from 10^5 to 10^{10} conidia/ml. The assays were performed as described above. At least six replicates were conducted, each on a different day. Larval mortality was monitored daily during 10 days. The LC_{50} values were calculated using GW-Basic software (Microsoft, 1985) after correction of natural mortality by Abbott's formula (1925). Differences in virulence between fungal species was tested through a Man-Whitney test using STATISTICA software 1999.

Conidial formulation, storage and pathogenicity

The isolate FHD 13 was selected to prepare conidial formulations. The effects of temperature, time of storage and virulence were evaluated for each

formulation. Conidia from 2-week-old cultures grown on complete medium (Napolitano and Juárez, 1997) were collected and suspended in 0.01% Tween 80. Conidia were produced on parboiled rice grains (100 g) by inoculating 1 ml of 1×10^7 conidia/ml suspension (Magalhães and Frazão, 1996) and incubating the flasks at 26 °C for 10 days. Kerosene (100 ml) was then added to the grains which were scraped with a metal spatula; the suspension was stirred for 5 min by hand and filtered through a 500 μm sieve to remove mycelial fragments. Mixtures containing 10^8 conidia/ml in 95% vegetable oils (corn, sunflower or canola) and 5% kerosene were stored at 4, 17 and 26 °C for 4 weeks. Viability and germination of conidia were evaluated by adding 100 μl of conidial formulation on complete agar plates, which were then incubated at 28 °C. A conidium was considered viable when the germ tube length was at least equal to its width. The number of viable or non-viable conidia (>300/plate) were assessed upon microscopical examination (×550) of the plates after 6, 8, 10, 12, 14, 16 and 18 h of incubation. The conidial viability was recorded every 7 days, during 28 days. The effects upon viability during storage of conidia in different oil formulations at several temperatures were analyzed using contingency analyses. Chi-square test ($P < 0.05$) was used to test significant differences by using STATISTICA software 1999.

The virulence of FHD13 conidia formulated in corn oil plus 5% kerosene was tested against groups of 20 larvae at the third instar, topically inoculated with 1 μl of the formulation in the pleural region. The assay was done in 4 replicates on separate dates. Mortality was monitored daily during ten days.

Results

Pathogenicity of fungal isolates against D. speciosa

A high percentage of the isolates (87%) were pathogenic for *D. speciosa* third instar larvae. FHD 13 was the most virulent isolate killing 70% of the larvae at a concentration of 1.0×10^8 conidia/ml (Table 1). The average LC_{50} value was 3.48×10^{10} conidia/ml (Table 2).

Conidial formulation, storage and pathogenicity

The viability of FHD 13 conidial formulation kept at 4 °C was close to 100% and stable for 28 days. There were no significant differences in conidial viability in relation to the type of oil and temperature of storage (Figure 1). A FHD13 conidial formulation in corn oil at 1×10^8 conidia/ml caused a 65% mortality in *D. speciosa* larvae.

Table 2. Probit analysis of four bioassays of *B. bassiana* FHD 13 against *D. speciosa* larvae

Replicate number	Total number of larvae [a]	Slope ± SE	LC_{50} (conidia/ml)	χ^2
1	140	0.39 ± 0.10	2.5×10^9	3.21
2	140	0.11 ± 0.07	6.6×10^{10}	1.08
3	140	0.16 ± 0.07	3.1×10^{10}	0.76
4	140	0.18 ± 0.08	4×10^{10}	1.43

[a]Total number of third instar larvae of *D. speciosa* tested: 20 larvae per dosage, per six dosages from 10^5 to 10^{10} conidia/ml of FHD 13 isolate of *B. bassiana* plus 20 control larvae.

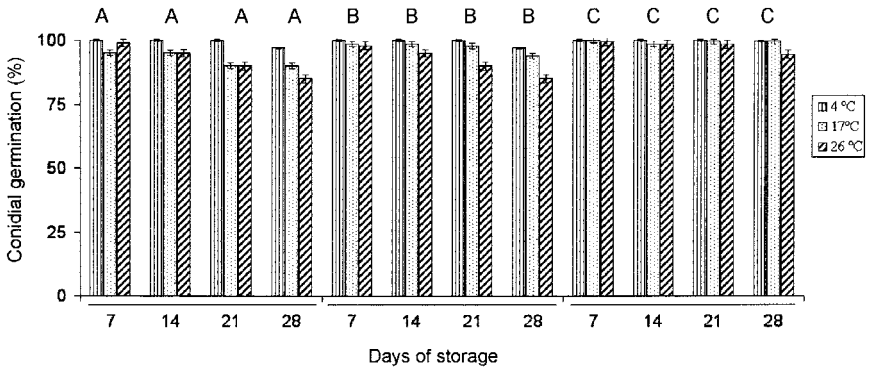

Figure 1. The effects on conidial viability of FHD 13 formulations in different oils (A – canola, B – sunflower and C – corn) stored for 7–28 days at 4 °C, 17 °C and 26 °C.

Discussion

M. anisopliae and *B. bassiana* are currently being produced as commercial biocontrol agents. However, the introduction of exotic strains of fungi has been a controversial point of discussion (Carruthers and Onsager, 1993). Environmental safety and ecosystem stability considerations lead to the conclusion that the use of native isolates in a microbial control program is more convenient (Lookwood, 1993). Most of the isolates tested in this study (87%) caused mortality in *D. speciosa* larvae, *B. bassiana* isolates being more virulent than *M. anisopliae* isolates ($P < 0.05$). This is in accordance with the reported higher control of *D. speciosa* by *B. bassiana* than by *M. anisopliae* in nature (Pianoski et al., 1990; Heineck-Leonel and Salles, 1997). It is interesting to note that isolate FHD 13, although originally from *Maecolaspis*

bridarolli (Coleoptera: Chrysomelidae) was more virulent to *D. speciosa* than isolates from *D. speciosa* as host.

In the preliminary assay, *B. bassiana* isolate FHD13 showed the highest virulence against *D. speciosa* larvae (Table 1), at a lower spore dosage than the LC_{50} value (3.48×10^{10} conidia/ml) determined in the bioassay (Table 2). This difference could be due to a loss of virulence of the isolate after succesive subcultures. A passage of the fungus through a susceptible host and reisolation has been suggested to restaure virulence in such circumstances (Butt et al., 1992; Varela and Morales, 1996). In addition, the LC_{50} value for the FHD 13 isolate is comparable with field concentrations used for the control of other coleopteran species, such as *Leptinotarsa decemlineata* (Say) and *Hypothenemus hampei* (Ferrari) (Anderson et al., 1988; Reithinger et al., 1997).

Conidia of FHD13 isolate germinated at similar percentages when they were formulated in different vegetable oils with kerosene, which was included to improve the miscibility of the oil and to obtain a more uniform conidial suspension (Goettel et al., 1995). Furthermore, the virulence of FHD13 formulation was not affected in a wide range of temperatures during four-week storage. FHD 13 might be a promising isolate for use against *D. speciosa* in large scale applications.

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