

COMPATIBILITY OF *METARHIZIUM ANISOPLIAE* (ASCOMYCOTA: HYPOCREALES) WITH SEVERAL INSECTISIDES

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Abstract: The entomopathogenic fungus conidia viability and sporulation may be affected by different environmental factors or by bio-pesticides and chemical products used to protect cultivated plants. In this research, we investigated the compatibility of *Metarhizium anisopliae* DEMI 001 isolates with three insecticides: fipronil, pyriproxyfen and hexaflumuron. The effect of fipronil, pyriproxyfen and hexaflumuron on sporulation, vegetative growth and conidial germination of the fungus was studied based on measuring the vegetative growth and sporulation. SDA medium was mixed with fipronil (10, 40 and 120 ppm), pyriproxyfen (10, 500 and 1 000 ppm), hexaflumuron (20, 50 and 120 ppm). SAD medium excluding insecticides was designated as blank. To determine the sporulation rate, spores was counted on surface area and vegetative growth was measured on the basis of colony diameter. The results showed that hexaflumuron at the concentration of 120 ppm reduced the vegetative growth to 0% showing the highest reduction effect compared to pyriproxyfen (24.59%) and fipronil (24.31%). All three insecticides reduced drastically the conidial germination at the highest concentrations (0–15%). Hexaflumuron treatment with 0% germination at all three concentrations and fipronil and pyriproxyfen with 32.36% and 9.7% germination at 200 ppm were not significantly different. Regarding the highest negative effect of hexaflumuron on germination at all three concentrations, this insecticide should not be applied together with the entomopathogenic fungus.

Key words: entomopathogenic fungi, *Metarhizium anisopliae*, pesticide, compatibility

INTRODUCTION

Both the conidium and blastospore viability and sporulation entomopathogenic fungi may be affected by different environmental factors (Furlong and Pell 1997) or by bio-pesticides and chemical products used to protect cultivated plants (Anderson and Roberts 1983; Loria *et al.* 1983; Alves and Lecuona 1998). Conidia of entomopathogenic fungi are strongly hydrophobic and difficult to suspend in water. This feature prevents the suspension formation in water (Behle *et al.* 2006). The success of a pest control program with the use of entomopathogenic fungi, mainly depends on conidia, survival in the field environment (Benz 1987). The entomogenous fungus *Metarhizium anisopliae* was studied extensively for the control of some pest species (Vestergaard *et al.* 1995; Ekesi and Maniania 2000; Butt *et al.* 2001; Azaizeh *et al.* 2002; Maniania *et al.* 2003; Ansari *et al.* 2007). The knowledge of the compatibility between entomopathogenic fungi and pesticides may facilitate the choice of proper products for integrated pest management (IPM) program considering the fungus as an important pest control agent (Neves *et al.* 2001). Combined utilization of selective insecticides in association with fungus pathogens can increase the effi-

ciency of control by reduction of the amount of applied insecticides, minimizing environmental contamination hazards and pest resistance (Moino and Alves 1998; Quintela and McCoy 1998). Neves *et al.* (2001) emphasized the importance of a formula and suggested that the viability should be considered in order to calculate compatibility value, especially taking into account biological agent the fact that a pathogen infects an insect through conidial germination and also that survival of the entomopathogenic fungus inoculum in the field depends primarily on conidia, responsible for the first foci of a disease. To use of incompatible pesticides with entomopathogenic fungi propagules and products may inhibit the development and reproduction of biocontrol agents, and this negatively effect the efficacy IPM programme (Anderson and Roberts 1983; Duarte *et al.* 1992; Malo 1993). The interaction between *Beauveria bassiana* and mineral oil was evaluated by Batista Filho *et al.* (1995) in order to control the banana plant borer, *Cosmopolites sordidus* (Germ.). These authors revealed an additive effect of mineral oil combination, resulted in high level of adult insect mortality (98%) as compared to the fungus applied alone (70%) and mineral oil used alone (33%). Alves *et al.* (1998) con-

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cluded that triadimenol/disulfoton and *B. bassiana* could be an alternative for using in the integrated management of coffee pests and diseases. Roberts and Campbell (1977) and Osborne and Boucias (1985) published an extensive review on the effect of pesticides on entomopathogenic fungi. According to Anderson and Roberts (1983), spores germination is an important criterion to evaluate compatibility of pesticides with entomopathogenic fungi *in vitro*. Todorova *et al.* (1998) reinforced the importance of pesticide effect on conidial germination, since those fungal structures are responsible for the appearance of the first insect disease foci in the field. In the authors opinion germination inhibition decreases the efficiency of pathogen as biocontrol agent, either of those already occurring in the agro-ecosystem or those applied by overflow, associated or not to pesticide products. De Olivera and Neves (2004) evaluated compatibility of *B. bassiana* with 12 acaricides and showed that Avermectin and the Pyrethroids were more compatible with *B. bassiana* than the others. This knowledge should facilitate the choice of chemicals with or less harmful to naturally occurring or artificially inoculated beneficial fungi. The aim of presented study was to evaluate the *in vitro* effect of fipronil, pyriproxyfen and hexaflumuron on different developmental stages of *M. anisopliae* and to determine their compatibility with the fungus.

MATERIALS AND METHODS

Fungal Isolates

Isolate *M. anisopliae* DEMI 001 (isolated from *Rhynchophorus ferrugineus*) were used in this study. The fungus was cultured on Sabouraud's dextrose agar (Merck, Darmstadt, Germany) with 1% yeast extract (SDAY) in Petri dishes (9 cm in diameter), and incubated for 2–3 weeks at 25±1°C, under a 16 : 8 h (L : D) photoperiod and 60±5% RH. Harvested conidia were used for the evaluation of conidial germination, vegetative growth and sporulation.

Pesticides

The pesticides selected for the experiments are used in table 1. For compatibility tests, the pesticides were used in three different concentrations, mean concentration (MC), half MC and twice the MC (De Olivera and Neves 2004).

Conidial germination

Each pesticide of the above mentioned concentrations (Table 1) was added to 50 ml of cooling Malt agar medium. The experimented medium was inoculated with 2 ml of conidial suspension containing 10⁷ spores/ml. The control group was treated with an aqueous solution of 0.05% Tween 80. All treatments were kept in an incubator (25±1°C; 12 h photophase) for 24 h. After 24 hours germinated and non germinated spores were counted per 100 spores in order to determine the thier viability.

Vegetative growth and spore production

Standard SDA medium was autoclaved at 115°C and 1.5 atm for 21 min, cooled to 40±5°C and amended with 0.8 g/l streptomycin. Pesticides were filtered with milipore filters (0.2 µm) to remove contaminant microorganisms. Then pesticides, at the pre-established concentrations were added. Approximately 50 ml of each one of these amended media was poured into three 9 cm culture plates. The same amount of streptomycin-amended medium without the pesticides was used as a control (De Olivera and Neves 2004). After the medium was solidified, the fungi were inoculated in one point per plate, (three dishes/treatment). The dishes were then incubated at 25±1°C and 12 h photophase. After nine days, the colony areas were measured (Neves *et al.* 2001) and 3 central colony disks (2,84 cm²) were drawn from each treatment to quantify the conidial reproduction. A standard sample colony area in relation to all colony area was chosen for conidia production quantification. Each disk was placed in a glass tube and the conidia were suspended in 10 ml of water containing 0.05% Tween 80. Conidial concentration was estimated using a hemocytometer.

Table 1. Compounds and their concentrations used in compatibility tests

Trade name	Insecticides	Formulatin	Chemical group	Concentration of active substance ¹ : [ppm] ³	Concentration of active substance ³ : [ppm]
Consult	hexaflumuron	EC ⁴ 10%	IGR ⁵	20 ,50, 120	50, 100, 200
Admiral	pyriproxyfen	EC 10%	IGR	10, 500, 1 000	50, 100, 200
Agenda	fipronil	EC 2.5%	phenylpyrazole	10, 40, 120	50, 100, 200

¹ concentrations used in vegetative growth and spore production tests

² concentrations used in germination tests

³ active substance

⁴ EC – Emulsifiable Concentration

⁵ IGR – Insect Growth Regulators

Statistical analysis

Computations for this experiment were done using the statistical software package SYSTAT version 12 (SYSTAT 2005). Data were analyzed using PROC General Linear Model. Duncan multiple range test was used for pairwise comparisons of means among treatments ($p < 0.05$). This study was carried out as factorial experiments based on Completely Randomized Design (CRD).

RESULTS

Conidial germination

The pesticides tested in this experiment caused different levels of inhibition of germination, vegetative growth and sporulation of *M. anisopliae* depending on chemical nature of used compounds. The effect of the used pesticides on germination of *M. anisopliae* showed to be independent on their concentration (Table 2). Among all tested pesticides, hexaflumuron showed absolutely complete inhibition of *M. anisopliae* conidial germination, whereas pyriproxyfen

and fipronil showed relatively little fungal inhibition and at concentrations of 50 and 100 ppm, The obtained results significantly different from the control. The formulation with hexaflumuron, at all three concentrations (50, 100 and 200 ppm), pyriproxyfen and fipronil at highest concentration (200 ppm) induced the high reduction of germination. Considering the group of tested pesticides and their concentrations of hexaflumuron, fipronil and pyriproxyfen caused 100%, 28.2% and 3.31% reduction of conidial germination at the concentration of 50 ppm, respectively. The result showed a significantly diferen between the used concentration 100 ppm and 200 ppm for the formolations fipronil and pyriproxyfen (Table 2).

Vegetative growth and spore production

Effects of the pesticides on *M. anisopliae* vegetative growth showed that almost all tested formulations significantly inhibited the fungal development (Table 3).The vegetative growth inhibition induced by hexaflumuron and pyriproxyfen formulation, respectively at 20 and

Table 2. Compatibility of the entomopathogenic fungi *M. anisopliae* with selected insecticides- reduction (mean±SE), of conidial germination

Treatments	Concentration of active substance [ppm]	Germination reduction [%]
		mean±SE
Pyriproxyfen	50	3.31±2.7 a
Pyriproxyfen	100	5.5±2.8 a
Pyriproxyfen	200	91.2±8.5 b
Hexaflumuron	50	100±0.0 b
Hexaflumuron	100	100±0.0 b
Hexaflumuron	200	100±0.0 b
Fipronil	50	28.2±16.1 a
Fipronil	100	31.4±15.2 a
Fipronil	200	68.6±11.9 b
Control	0	0 a

Table 3. Compatibility of the entomopathogenic fungi *M. anisopliae* with selected insecticides – reduction (mean±SE), vegetative growth and sporulation

Treatments	Concentration of active substance [ppm]	Vegetative growth [mm] [%] reduction	Sporulation [$\times 10^7$ spores/ml] [%] reduction
		mean±SE	mean±SE
Pyriproxyfen	10	12.8±1.3 a	40.7±11.5 ab
Pyriproxyfen	500	60.0±1.5 c	32.0±20.8 a
Pyriproxyfen	1 000	76.4±4.8 d	85.5±7.7 cb
Hexaflumuron	20	18.7±4.5 a	14.0±19.4 a
Hexaflumuron	50	73.9±5.0 cd	43.7±30.9 ab
Hexaflumuron	120	100±0.0 e	100± 0.0 c
Fipronil	10	41.3±5.4 b	95.5±4.4 c
Fipronil	40	68.1±4.6 cd	98.6±1.3 c
Fipronil	120	76.6±6.6 d	98.2±1.7 c
Control	0	0 a	0 a

10 ppm concentrations was not significantly different from the control treatment. However, the formulation with hexaflumuron, fipronil and pyriproxyfen (at two highest concentrations) induced fungal growth inhibition more than 60%. All tested pesticides: hexaflumuron, fipronil and pyriproxyfen caused, respectively 100%, 76.6% and 76.4% reduction of vegetative growth then applied at the highest concentrations. The insecticides hexaflumuron, fipronil and pyriproxyfen (at the highest concentrations), reduced the sporulation level more than 85%, whereas the insecticides fipronil totally prevented sporulation (95–98% reduction) at three concentrations used. However, only data on pyriproxyfen at 500 ppm concentration and hexaflumuron at 20 ppm concentration were not significantly different from the control treatment. The treatment with hexaflumuron induced to the highest level the inhibition of germination, vegetative growth and sporulation of the fungus *M. anisopliae*.

DISCUSSION

The result of carried out experiment indicated a significant reduction in cases of spore germination, vegetative growth of *M. anisopliae* treated by hexaflumuron whereas pyriproxyfen and fipronil showed relatively little fungal inhibition at the concentrations of 50 and 100 ppm, similarly to the control. All tested insecticides at the highest concentration significantly reduced the fungus sporulation. Fungal germination is an important factor of pesticides compatibility evaluation with entomopathogenic fungi considering the pest management, because the beginning of epizootics is conditioned by the capacity of conidia to germinate on the host (Anderson and Roberts 1983; Alizadeh *et al.* 2007). The use of incompatible pesticides may lead to inhibition of the development and reproduction of entomopathogens such as *M. anisopliae* and restrict their application in IPM strategies (Anderson and Roberts 1983; Duarte *et al.* 1992; Malo 1993). The insecticides caused different levels of inhibition of germination, vegetative growth and sporulation of *M. anisopliae* depending on chemical nature as well as concentrations of the active compounds. In this study hexaflumuron resulted in maximally negative effect, on spore germination and spore vegetative growth and spore sporulation of *M. anisopliae*, probably due to the presence of organic material (phenyl, urea, carbonyl, and difluorobenzamide) in the product. Studying the effects of organic acids on *M. anisopliae*, Li and Holdom (1995) concluded that the acetic and malic acids reduced vegetative growth and conidiogenesis. Another possible explanation for inhibition of conidia germination is the accumulation of chemical compounds against a concentration gradient, starting from diluted solutions, as described by Kimati (1995) for phytopathogenic fungi. In this process the compound molecules get in contact with conidia and slowly diffuse to their inner cytoplasm allowing fungal germination before achieving a lethal dose. Conidial germination is a very important step in pest management involving fungi, because the beginning of epizootics is conditioned by the capacity of spores to germinate on a host. The entomopathogenic fungus success, however, depends on

conidial viability (Batista Filho *et al.* 1998; De Olivera and Neves 2004). Inhibition of vegetative growth is not necessarily an indication of reduction of sporulation or conidial viability and vice versa (Zimmerman 1975). Under the field conditions, vegetative growth inhibition may not be considered as a good indication of fungicidal effect such as spore viability (Loria *et al.* 1983). For field studies the inhibition of conidial germination should be the key factor to be considered (Neves *et al.* 2001). Boucias and Pendland (1988) used similar methodology to demonstrate that the treatment of entomopathogenic fungus conidia with chemical products, either ionic or molecular ones, may neutralize the electrostatic charge of the surface and/or remove the mucous layer covering conidia, thus affecting the substrate recognition process and the transduction of the signal that initiates germination. St. Leger and Cooper (1987) and St. Leger *et al.* (1991) demonstrated the role of this coat removal on substrate recognition and on transduction of the signal that triggers germ tube formation in *M. anisopliae*. Metabolic blockage in phytopathogenic fungi conidia due to ions accumulation on the surface of the cellular membrane was also described by Ghini and Kimati (2000). The authors reported that molecules, analogous to prosthetic groups, diffuse to the cytoplasm where they bind to specific receptors affecting membrane permeability and enzymatic synthesis, consequently affecting metabolic processes. The same authors also emphasize that organophosphate compounds directly interfere on cell wall formation due to inhibition of the enzyme that converts phosphatidylethanolamine into chitin. The same mechanism of inhibition was probably responsible for the drastic reduction of *M. anisopliae* conidial germination, vegetative growth and sporulation observed in this study. When the insecticide is compatible *in vitro*, there are strong evidences about its selectivity under field conditions (Alves *et al.* 1998). Likewise, a high toxicity *in vitro* does not always mean that the same phenomena will occur in the field, but it indicated such possibility (Alves *et al.* 1998). On the other hand, for compatibility in the field we should consider the effect of product on conidial germination as one of the most important factors (Anderson and Roberts 1983; Malo 1993). In conclusion, hexaflumuron and fipronil induced the highest and the lowest level of inhibition on the germination, vegetative growth and sporulation of *M. anisopliae* *in vitro*, respectively. There is need of information about compatibility of entomopathogenic fungi with products used in organic agriculture, like fertilizers and insecticides. Laboratory and field studies on combined application of products with entomopathogenic fungi and their compatibility might provide valuable data useful for the development of strategies for handling plagues in organic agriculture.

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POLISH SUMMARY

WPLYW WYBRANYCH INSEKTYCYDÓW NA GRZYBA *METARHIZIUM ANISOPLIAE* (ASCOMYTA: HYPOCCREALES)

Zarówno czynniki środowiskowe jak też biopestycydy i chemiczne środki stosowane w ochronie roślin uprawnych mogą wpływać na zarodnikowanie i żywotność konidiów entomopatogenicznych grzybów. W pracy przedstawiono wyniki badań nad kompatybilnością izolatu DEMI 001 grzyba *Metarhizium anisopliae* z trzema insektycydami: fipronil, pyriproxen i hexaflumuron. Ocenę wpływu insektycydów na wzrost grzybni, zarodnikowanie i kiełkowanie konidiów dokonano w oparciu o pomiary kolonii grzyba i zarodnikowania. Testowane insektycydy: fipronil (w koncentracji 10, 40, 120 ppm), pyriproxifen (10, 500, 1 000 ppm) oraz hexaflumuron (20, 50, 120 ppm) dodawano do pożywki agarowej Sabouraud'a. Ta sama pożywka bez dodatku insektycydów stanowiła obiekt kontrolny. W celu określenia zarodnikowania

grzyba, liczone zarodniki wytworzone na powierzchni agarowej pożywki, a wzrost grzybni określano w oparciu o pomiary średnicy kolonii grzyba.

Wyniki przeprowadzonych badań wykazały, że insektycyd heksaflumuron zastosowany w koncentracji 120 ppm całkowicie zahamował wzrost kolonii grzyba (0%), natomiast pozostałe insektycydy – pyriproxifen i fipronil, charakteryzowały się słabszym działaniem, a uzyskane wielkości procentowe wynosiły odpowiednio 24,59 i 24,31%. Wszystkie testowane insektycydy zastosowane w najwyższych koncentracjach zdecydowanie ograniczyły kiełkowanie zarodników konidialnych (0–15%). Nie stwierdzono istotnych różnic pomiędzy kombinacją z insektycydem hexaflumuron (0% kiełkujących zarodników), a kombinacjami z insektycydami fipronil (32,36%) i pyriproxifen (9,7%) zastosowanymi w koncentracji 200 ppm. Biorąc pod uwagę zdecydowanie negatywny wpływ insektycydu hexaflumuron (wszystkie testowane koncentracje) na kiełkowanie zarodników *M. anisopliae*, nie należy stosować go łącznie z grzybami entomopatogenicznymi.