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EFFECT OF SYNTHETIC PESTICIDES ON CONIDIAL GERMINATION AND ENDOPHYTIC ACTIVITY OF *Beauveria bassiana* AND *Metarhizium anisopliae* IN COMMON BEAN PLANTS

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is an important legume crop worldwide. Endophytic fungi have proven effective as biocontrol agents in several crops, but they can be affected by pesticides. The aim of this study was to evaluate the effect of synthetic pesticides on the conidial germination and endophytic activity of *Beauveria bassiana* and *Metarhizium anisopliae* in *P. vulgaris*. Three fungicides (Mancozeb, Tebuconazole and Zinc Ethylene-bis), three insecticides (Abamectin, Diazinon and Spirotetramat) and one herbicide (Glufosinate) were evaluated. Each pesticide was applied at the application rate recommended by the manufacturer and also using one-half of the application rate on the commercial strains *B. bassiana* Bb-18 and *M. anisopliae* Ma-30. Tebuconazole, Mancozeb and Glufosinate totally inhibited conidial germination. Spirotetramat and Diazinon resulted in a less pronounced negative effect on *B. bassiana* and *M. anisopliae* germination. However, Spirotetramat has the lowest impact on the entomopathogenic fungi, allowing for 86 and 74% germination of *B. bassiana* and *M. anisopliae*, respectively. Tebuconazole and Mancozeb affected the endophytic activity of the entomopathogenic fungi studied. At one-half of the recommended concentration, Spirotetramat and Zinc Ethylene-bis allowed for the total colonization of *B. bassiana* in roots of common bean, whereas *M. anisopliae* reached a colonization of 75%. These results indicate that Spirotetramat, Zinc Ethylene-bis and Diazinon can be used in combination with *B. bassiana* Ba-18 and *M. anisopliae* Ma-30. However, Tebuconazole, Mancozeb and Glufosinate should not be combined with these entomopathogenic fungi in Integrated Pest Management programs.

Keywords: agrochemicals, entomopathogenic fungi, Integrated Pest Management, *Phaseolus vulgaris*

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INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important staple crop in countries from Eastern and Southern Africa, being regarded as a good protein source in Central and South America, (Deshpande, 1992; Zaugg et al., 2013). In fact, it is considered as one of the most important food sources in Cuba due to cultural aspects and nutritional value (Quintero et al., 2000). Agricultural practices are aimed at reducing pests, diseases, and weeds, including different programs and techniques such as the use of entomopathogenic fungi.

Entomopathogenic fungi can infect a wide range of arthropods and have proven effective as biocontrol agents in various cropping systems (Lacey et al., 2015). Several species of entomopathogenic fungi are used as mycopesticides in Integrated Pest Management (IPM) programs, because they can proliferate on cheap substrates and have good product stability during storage (Khetan, 2000; De Faria and Wraight, 2007).

Entomopathogenic fungi are found in several habitats, particularly in the rhizosphere associated with plant roots (Guesmi-Jouini et al., 2013), but they can be also found as endophytes, colonizing plant tissues (stems, leaves, and fruits) (Vega, 2008). The endophytic habit of the entomopathogenic fungi can provide benefits to both plants and fungi themselves. The fungus benefits through protection against environmental stress, while the plant can benefit through the reduction of damage caused by herbivorous insects (Clement et al., 2011). However, entomopathogenic fungi applied in agroecosystems can be affected by biotic (soil microbiota) and abiotic factors, i.e., farming practices and organic and synthetic pesticides (Hummel et al., 2002; Klingen and Haukeland, 2006).

Synthetic pesticides can inhibit germination, vegetative growth, conidiogenisis and sporulation (Alves et al., 1998; Inglis et al., 2001). Some herbicides have shown a negative impact on entomopathogenic fungi in the soil, even at low rates, and therefore are incompatible with these agents (Kos and Celar, 2013). Moreover, fungicides can reduce infection rates and delay epizootics of entomopathogenic fungi (Sosa-Gomez et al., 2003).

Several studies have demonstrated the compatibility of synthetic pesticides and entomopathogenic fungi *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Schumacher and Poehling, 2012; Kos and Celar, 2013; Clifton et al., 2015). However, there is little information about the effect of synthetic pesticides used in common bean on the endophytic association of entomopathogenic fungi. In addition, to the best of the authors' knowledge, there are no previous studies that analyze the impact of synthetic pesticides on the conidial production and germination of *B. bassiana* and *M. anisopliae* in common bean. Therefore, the aim of this study was to evaluate the effect of synthetic pesticides on the conidial germination and endophytic activity of *B. bassiana* and *M. anisopliae* in common bean plants.

MATERIALS AND METHODS

Fungal cultures

The commercial strains of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30 were supplied by the Reproduction Center of Entomopathogenic fungi, Sancti Spíritus province, Cuba. Strains of *B. bassiana* Bb-18 were isolated from the coffee berry borer, *Hypotenemus hampei* (Ferrari), while strains of *M. anisopliae* Ma-30 were obtained from soil samples collected from an agroecological bean field. The strains were maintained in the Centro de Investigaciones Agropecuarias collection and belonged to Universidad Central "Marta Abreu" de Las Villas, Cuba. The entomopathogenic fungi *B. bassiana* and *M. anisopliae* were cultivated on Potato Dextrose Agar (PDA) culture medium (BioCen, La Habana) in Petri dishes (ø 9 cm) and incubated in a climatic chamber (TP RTOP-310D, China) at 25°C, 75% relative humidity (RH) in the dark for 14 days.

Synthetic pesticide treatments

Seven pesticides, which are commonly used in bean, were used. The commercial products, active ingredients, recommended concentrations (RC), and manufacturers of each synthetic pesticide are presented in Table 1. The fungicides Mancozeb and Zineb were wettable powder formulations; the fungicide Silvacur, the insecticides Abaco, Diazinon and Movento, as well as the herbicide Finale were liquid formulations. All synthetic pesticides were dissolved and homogenized in distilled water into a laminar flow hood (FasterBio, Belgium) and the concentration of active ingredients was adjusted according to the application rate recommended by the manufacturer. The mixtures were used once they were prepared.

Germination and sporulation tests

Both entomopathogenic fungi, *B. bassiana* B-18 and *M. anisopliae* Ma-30, were vortexed with the synthetic pesticides (previously prepared

Table 1. Specifications of the synthetic pesticides used.

Commercial name	Active ingredient	Type of pesticide	Chemical groupe	Recommended concentration per L*
Mancozeb	Mancozeb	Fungicide	Dithiocarbamate	0.1 g
Silvacur	Tebuconazole	Fungicide	Triazol	0.039 mL
Zineb	Zinc Ethylene-bis	Fungicide	Dithiocarbamate	0.1 g
Finale	Glufosinate	Herbicide	Phosforic	0.16 mL
Abaco	Abamectin	Insecticide	Abamectin	0.036 mL
Diazinon	Diazinon	Insecticide	Organophosphate	0.075 mL
Movento	Spirotetramat	Insecticide	Pyrethroid	4.0 mL

*All recommended concentrations were obtained from the product label.

according to the application rate recommended by the manufacturer and also using one-half of the application rate), and then inoculated on Potato Dextrose Agar (PDA) culture medium (BioCen, La Habana) in Petri dishes (ø 9 cm). Sterile distilled water was applied as control. The entomopathogenic fungi were incubated in a climatic chamber (TP RTOP-310D, China) at 25°C, 75% relative humidity (RH) in the dark for 14 days. Subsequently, four pieces (1 cm^2) were cut out of the agar of each Petri dish and diluted in 5-mL sterile distilled water with 0.05% Triton X-100 (Sigma Adrich, USA) and then observed under a compound microscope (400x, Speed fair, China) to determine the sporulation of each fungal species. Conidia were counted using a Neubauer chamber (Brand, Germany), and sporulation was expressed as the number of conidia per mL. Germination was evaluated at 14 days after pesticide applications, while criteria to evaluate germination were spore formation and growth of the germ tube. Viable conidia were those that had germ tubes longer than their diameters (Oliveira et al., 2015). Each treatment and the whole assay were replicated four times.

Semi-field assay

The common bean cultivar ICA Pijao (seed coat black) was sown in an Inceptisol soil (USDA Soil Taxonomy). The soil was sterilized three times in an electric stove at 170°C for two hours. The sterilization quality was checked by the inoculation of 1 mL of the diluted soil on Potato Dextrose Agar Yeast (PDAY) culture medium. The soil was placed in a plastic tray (22 cm large x 3.5 cm height) and then the entomopathogenic fungi *B. bassiana* Bb-18 and *M. anisopliae* Ma-30 plus 0.05% Triton X-100® (Sigma-Aldrich, USA) were separately sprayed in the soil at a concentration of $1x10^8$ conidia/mL at a total rate of 50 mL per tray. The spraying was carried out using a backpack sprayer (Hudson, USA; 10 L capacity). The backpack sprayer was disinfected with tap water, ethanol 70%, acetone 99% and washed three times with distilled water between treatments to avoid contamination among treatments (Castro et al. 2016).

Common bean seeds were sown three days after fungal inoculation in the soil, whereas all the synthetic pesticides were sprayed on the soil three days after sowing. Sterile distilled water was applied as control and the backpack sprayer was disinfected between treatments as described above. Once the plants reached the growth stage BBCH 22 (Meier, 2001) (second side shoot visible), they were transferred to the Microbiology Laboratory of the Universidad Central "Marta Abreu" de Las Villas to determine the effect of pesticides on the endophytic activity of the entomopathogenic fungi *B. bassiana* Bb-18 and *M. anisopliae* Ma-30.

Entomopathogenic fungi live in the organic matter of the soil, and they are directly associated with the rhizosphere where they have a close relationship with the roots. Therefore, roots could be the first scenario for endophytic fungi establishment. Taking this into consideration, roots from bean plants were surface sterilized by dipping for 3 min in 1.5% sodium hypochlorite, 2 min in 70% ethanol and then rinsed three times in sterile distilled water. The efficacy of the sterilization was evaluated by inoculating 100 µl of the last rinse water on PDA (Parsa et al., 2013). Each root was cut into six pieces (1 cm) in a laminar flow hood and then placed in sterile Petri dishes with PDA containing chloramphenicol (250 mg/l w/v) (Ramos et al., 2017). The bean root tissues were placed at 25 ± 1°C and 90% HR for 14 days in the dark for emergence of fungal colonies. Fungal outgrowths from roots were isolated onto PDA with chloramphenicol (250 mg/l w/v) and pure cultures were grown on PDA at 25 °C, 90% HR in the dark. Finally, the fungal isolates were morphologically identified under

a microscope (Motic, USA, 400x magnification) according to the morphological characteristics described by Humber (2012) to confirm that the *B. bassiana* and *M. anisopliae* recovered from the bean roots were those initially inoculated in the soil. The percentage of colonization for each entomopathogenic fungus was determined by the number of fungal outgrowths from roots with respect to the total number of roots. Each treatment and the whole assay were replicated four times.

Statistical analyses

Analyses of variance (ANOVA) were applied to determine the effect of the synthetic pesticides on the sporulation, germination, and endophytic activity of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30. Means were separated using Tukey's Honestly Significant Difference (HSD) test. ANOVA and HSD were run using STATGRAPHICS Plus 5.1 (Manugistics Inc, 1992) with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Effect of synthetic pesticides on the conidial production of *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30

The number of conidia of the entomopathogenic fungi *B. bassiana* obtained after growth and sporulation in contact with synthetic pesticides was statistically different between treatments ($F = 16.72$; $df = 7$; $P = 0.0001$) (Table 2). The conidial production of *B. bassiana* was negatively affected by all the pesticide treatments compared with the control, except for Diazinon (RC/2). The application of the RC and RC/2 of Spirotetramat, Abamectin and Zinc Ethylene-bis also resulted in a reduced formation of conidia compared with the control treatment. Tebuconazole (RC and RC/2), Glufosinate (RC) and Mancozeb (RC) totally inhibited the number of conidia produced by *B. bassiana* (Table 2).

Statistical differences were observed in the number of conidia of *M. anisopliae* produced in directed contact with the synthetic pesticides and the control treatment ($F = 21.35$; $df = 7$; $P = 0.0013$) (Table 2). The insecticide Diazinon (RC and RC/2) did not affect the number of conidia of this entomopathogenic fungus. However, Spirotetramat, Abamectin and Zinc Ethylene-bis allowed for conidia formation, but this occurred in lower concentrations than in the control treatment. Furthermore, the fungicides Tebuconazole and Mancozeb as well as the herbicide Glufosinate completely inhibited the sporulation of *M. anisopliae* (Table 2).

The results obtained by Celar and Kos (2016) demonstrated that Mancozeb caused a total inhibition of 61% on the conidial production of *B. bassiana*, being listed in class 4 according to Ambethgar et al. (2009).

Effect of synthetic pesticides on the conidial germination of *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30

The effect of the evaluated synthetic pesticides on the conidial germination of *B. bassiana* Bb-18 was statistical different between treatments ($F = 18.20$; $df = 7$; $P = 0.0001$) (Table 3). Diazinon, Abamectin and Zinc Ethylen-bis at RC caused a reduction of the conidial germination of *B.*

Table 2. Effect of synthetic pesticides on the conidial production (Mean± standard error (SE)) of *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30. Pesticides were inoculated on PDA at the recommended concentration (RC) and at one-half of the recommended concentration (RC/2) before fungal inoculation. Comparison criteria are based on the effect of pesticide type at the same concentration.

	Mean of conidia ± SE *			
	B. bassiana Bb-18		*M. anisopliae* Ma-30	
Treatments	**RC**	**RC/2**	**RC**	**RC/2**
Control	6.50 ± 0.02 a		6.50 ± 0.02 a	
Glufosinate	0.00	1.50 ± 0.04 d	0.00	0.00
Spirotetramat	2.5 ± 0.03 b	3.1 ± 0.03 c	3.5 ± 0.03 b	4.6 ± 0.01 b
Diazinon	2.3 ± 0.03 b	6.5 ± 0.02 a	6.3 ± 0.02 a	6.5 ± 0.02 a
Tebuconazole	0.00	0.00	0.00	0.00
Mancozeb	0.00	4.5 ± 0.01 b	0.00	0.00
Abamectin	2.5 ± 0.03 b	3 ± 0.03 c	2.9 ± 0.04 bc	5 ± 0.01 ab
Zinc Ethylene-bis	2.9 ± 0.03 b	5 ± 0.01 ab	2.9 ± 0.04 bc	3.8 ± 0.03 c

* Different letters indicate significant differences according to Tukey's HSD test ($P < 0.05$).

Table 3. Effect of synthetic pesticides on the percentage of conidial germination (± standard error (SE) of the commercial strains *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30. Pesticides were inoculated on PDA at the recommended concentration (RC) and at one-half of the recommended concentration (RC/2) before fungal inoculation. Comparison criteria are based on the effect of pesticide type at the same concentration.

Treatments	Conidial germination (%) ± SE *			
	Beauveria bassiana Bb-18 (%)		*Metarhizium anisopliae* Ma-30 (%)	
	RC	RC/2	RC	RC/2
Control	98.35 ± 0.02 a	99.50 ± 0.01 a	97.42 ± 0.09 a	99.20 ± 0.02 a
Glufosinate	0.00	0.00	0.00	0.00
Spirotetramat	86.54 ± 0.04 ab	90.53 ± 0.03 ab	74.25 ± 0.12 b	89.13 ± 0.08 b
Diazinon	61.53 ± 0.07 c	83.10 ± 0.06 abc	69.71 ± 0.21 b	81.10 ± 0.12 bc
Tebuconazole	0.00	0.00	0.00	0.00
Mancozeb	0.00	0.00	0.00	0.00
Abamectin	45.33 ± 0.16 cd	52.16 ± 0.25 de	58.34 ± 0.33 c	65.18 ± 0.27 c
Zinc Ethylene-bis	52.10 ± 0.18 cde	63.80 ± 0.22 d	60.15 ± 0.29 c	68.28 ± 0.23 d

*Means ± SE with different letters between the rows denote significant differences according to Tukey's HSD test of Tukey (P <0.05).

bassiana Bb-18 compared with the effect reached by Spirotetramat and the control treatment. However, no significant differences were observed between Diazinon and Spirotetramat when applied at RC/2.

The conidial germination of *M. anisopliae* Ma-30 was affected by some pesticides in compared with the control treatment (F = 17.45; df = 7; P = 0.0001) (Table 3). A detrimental effect on conidial germination was also observed when Abamectin and Zinc Ethylene-bis were applied on *M. anisopliae* Ma-30 with significant differences compared with the control treatment, which allowed for conidial germination. No conidial germination of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30 was observed with the fungicides Tebuconazole and Mancozeb or the herbicide Glufosinate (Table 3).

A study conducted by Gardner and Storey (1985) revealed the inhibitory effect of 20 herbicides (even at a low concentration rate) on germination and growth of *B. bassiana.* In addition, it has been reported that the herbicides Isoxaflutole, Fluazifop-P-butyl, Flurochloridone, Foramsulfuron, Pendimethalin and Prosulfocarb completely inhibit sporulation and germination of *B. bassiana* (Clear and Kos, 2016).

Our results suggest that the combination of Tebuconazole, Mancozeb and Glufosinate with *B. bassiana* and *M. anisopliae* is not recommended for Integrated Pest Management programs.

Effect of synthetic pesticides on the endophytic activity of *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30

The fungicides Tebuconazole and Mancozeb completely affected the endophytic activity of the commercial strains *B. bassiana* Bb-18 and *M. anisopliae* Ma-30 on common bean roots. However, both the RC and RC/2 of the fungicide Zinc Ethylene-bis allowed for the colonization of *B. bassiana* B-18, whereas RC/2 on *M. anisopliae* Ma-30 induced 75% of colonization on common bean plants. The herbicide Glufosinate also inhibited the colonization of both entomopathogenic fungi inside the root tissues of common bean. The insecticides Spirotetramat and Diazinon at RC/2 allowed a higher colonization of *B. bassiana* Ba-18 (F = 12.85; df = 7; P = 0.0023) and *M. anisopliae* Ma-30 (F = 10.21; df = 7; P = 0.0001) in the common bean roots than the RC. However, both concentrations of the insecticide Abamectin showed no differences in the endophytic activity of *B. bassiana* Bb-18 (Table 4).

Entomopathogenic fungi can establish themselves as endophytes in a specific part of the plant, but they can also move within the plant (Jaber and Araj, 2018). Adverse conditions could produce a transient colonization of these microorganism inside plants (Schulz and Boyle, 2005) or even affect conidial production.

Our results reveal that the endophytic activity of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30 on roots of common bean was suppressed by the effect of Tebuconazole, Mancozeb and Glufosinate. Similar results were obtained by Win et al. (2021) who demonstrated that the endophytic colonization of fungi in different tea plant tissues obtained from a plantation treated with agrochemicals was lower than that in untreated plants. These

Table 4. Effect of synthetic pesticides on the root colonization of the commercial strains *Beauveria bassiana* Bb-18 and *Metarhizium anisopliae* Ma-30 in common bean. Pesticides were sprayed at the recommended concentration (RC) and at one-half of the recommended concentration (RC/2). Comparison criteria are based on the effect of pesticide type at the same concentration.

	Root colonization (%)*			
	B. bassiana Bb-18		*M. anisopliae* Ma-30	
Treatments	**RC**	**RC/2**	**RC**	**RC/2**
Control	100 a	100 a	100 a	100 a
Glufosinate	0 c	0 c	0 d	0 c
Spirotetramat	50 b	100 a	50 b	75 b
Diazinon	50 b	70 b	25 c	75 b
Tebuconazole	0 c	0 d	0 d	0 c
Mancozeb	0 c	0 d	0 d	0 c
Abamectin	50 b	50 c	50 b	75 b
Zinc Ethylene-bis	100 a	100 a	50 b	75 b

*Means with different letters between the rows denote significant differences according to Tukey's HSD test of (P <0.05)

results are consistent with those obtained in the laboratory bioassay where it was demonstrated that the action of Tebuconazole, Mancozeb and Glufosinate applied at the RC by the manufacturer completely inhibited the production and germination of conidia of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30.

The action of the insecticides Diazinon, Abamectin and Spirotetramat at the RC allowed between 25 and 50% of fungal colonization of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30. The effects of other synthetic insecticides were tested on two *M. anisopliae* strains (MA-7 and MA-K) and the results revealed that a dosage of Fipronil over 40 and 1.6 ppm produced a lower number of conidia than the control, respectively. However, Permethrinat 0.32 ppm allowed for the conidial production of the strain MA-7 (Schumacher and Poehling, 2012). Our results agree with those obtained by these authors since both Diazinon (CR/2) and Spirotetramat (CR/2) allowed 70 and 100% of *B. bassiana* Bb-18 colonization, respectively, whereas colonization of *M. anisopliae* reached 75% with the same insecticide concentrations. Therefore, these synthetic insecticides applied at one-half of the recommended concentration can be combined with *B. bassiana* Bb-18 and *M. anisopliae* Ma-30.

CONCLUSIONS

Our results suggest that Tebuconazole, Mancozeb and Glufosinate affect the conidial germination and endophytic activity of *B. bassiana* Bb-18 and *M. anisopliae* Ma-30, indicating that their combined application can be used in Integrated Pest Management. However, Zinc Ethylene-bis could be combined with *B. bassiana* Bb-18, whereas Diazinon and Spirotetramat at one-half of the recommended concentration can be applied together with *B. bassiana* Bb-18 and *M. anisopliae* Ma-30. These results contribute to a better understanding of the combination of biological and synthetic controls in Integrated Pest Management programs. However, further research under field conditions is required for in-depth knowledge about the action mode of synthetic pesticides on the conidial germination and endophytic activity of entomopathogenic fungi.

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