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Effect of the Insecticide Fenpropathrin on Exoenzyme Production in *Verticillium lecanii* (Zimm.) Viégas

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Studies on the collateral effects of chemical insecticides on entomopathogenic fungi have consisted mainly in the description of their effects on fungal growth and sporulation.[1] The pesticide´s action on the enzymatic activity of fungi is a scarcely studied aspect. Considering that the virulence of these natural bioregulators of pests depends, to a great extent, on their ability to synthesize enzymes that degrade the cuticle of insects,[2,8] it is important to know the effect of chemical insecticides, widely used in agriculture, on the enzymatic activity of entomopathogenic fungi. This study was aimed at analyzing the effect of a pyrethroid insecticide, fenpropathrin, used for chemical control of the whitefly (Homoptera: Aleyrodidae), on the enzymatic activity of strain C of *Verticillium lecanii*, used as a bioinsecticide for the whitefly. This strain was isolated in Mexico, its virulence was demonstrated in the laboratory based on the 92% mortality on nymphs of *Trialeurodes vaporariorum* whitefly,[5] its safety tested by infectivity in mammals,[6] and its efficiency as a bioinsecticide was demonstrated in an open field bean crop, where it produced a mortality of 72.7 to 84.3% in different-stage nymphs of *T. vaporariorum* (Mier, personal communication).

Strain C of *V. lecanii* was preserved in H medium (0.5% dextrose, 1.0% sacarose, 0.5% yeast extract, 0.05% peptone, 1.5% agar) and also as a spore suspension in distilled water, both at 4°C, in the Microbiology Laboratory, Department El Hombre y su Ambiente, Universidad Autónoma Metropolitana-Xochimilco. A suspension of 10^8 conidia/ml was prepared from a *V. lecanii* culture (medium H), incubated for 12 days at 26°C, and used for all assays. For enzymatic determination, the fungus was grown in liquid culture in Gupta medium[4] (0.06% $MgSO_4$, 0.05% NaCl, 1.5% KH_2PO_4 , 0.001% $FeSO_4$, 0.001% $ZnSO_4$, pH 6.0 and, supplemented with 1.0% gelatine, 1.0% glucose, 0.6% NO_3Na) on a rotary shaker at 150 rpm and 26°C for 12 days. The liquid medium was supplemented with Herald 375 (fenpropathrin: (RS)-α-cyano-3 phenoxybenzyl-2,2,3,3-tetramethyl cyclopropancarboxylate, equivalent to 375 g of a.i./l, Valent de México, S.A. de C.V.) at a final concentration of 0.2%. The supernatant was obtained by centrifugation at 2000 *g*, 10 min. Exoenzymes were determined in the supernatant using the API ZYM test kit system (bioMérieux México, S.A.), a semi-quantitative micromethod designed for the research of enzymatic activities.[3,7,9] Briefly, test strips were inoculated with 65 µl culture fluid in each well, and incubated for 4 h at 26°C. Enzyme activity was determined by color reactions after adding the API ZYM reagents A and B, and according to the sensitivity of the method indicated in the kit included color chart, where the approximate number of free nanomoles (nmol), may be known from the color strength: 1 corresponds to the liberation of 5 nmol, 2 to 10 nmol, 3 to 20 nmol, 4 to 30 nmol and 5 to 40 or more nmol. Results were compared with those obtained with the fungus incubated in the same culture conditions without insecticide (control). Assays were performed in triplicate and the experiment was performed on three separate occasions in order to investigate the reproducibility of the results.

All exoenzymes tested are depicted on Table 1, revealing inhibition of enzimatic activities when the fungus was incubated with fenpropathrin, in all the enzymes detected in the fungus. The highest color intensity detected in *V. lecanii* supernatant without insecticide (control) was for leucine arylamidase (protease) and N-acetyl-β-glucosaminidase (chitinase). These two enzymes showed the greatest inhibition when the fungus was incubated with fenpropathrin. Results showed that *V. lecanii* contact with the pyrethroid influences protease and chitinase activities, enzymes involved on the degradation of the epicuticle of insects.[2,8] Furthermore, St. Leger[8] suggested that proteases initiate insect cuticle degradation, and recent studies of aphid invasion by *V. lecanii*[2] provided further evidence of chitinase activity during cuticle colonization by this fungus. Variations in pathogenicity of *V. lecanii* related, among other characteristics, to enzymatic activities evidence the probable repercussions of this chemical insecticide on natural bioregulators. This is the first report in Mexico on the effect of a chemical insecticide on the enzymatic activities of an entomopathogenic fungus.

demand in selecting and introducing alternative methods to control insect pests.[19] Although microbial products currently constitute only 2% of the world pesticide market, this percentage is expected to increase sharply in the future.[22]

The compatibility of entomopathogenic fungi with selective pesticides is needed in modern integrated pest management control programs to minimize their negative side effects on important natural enemies.[25] Anderson & Roberts[2] have shown that in *Beauveria bassiana*-insecticide tank mixes, most inhibitory effects occurred within the first few hours, and by separate applications of each control agent the inhibition was minimized. Furthermore, Anderson *et al.*[3] demonstrated that combinations of sublethal concentration of an insecticide were clearly compatible with *B. bassiana,* hence use of mixtures might have advantages by minimizing the danger of pesticide contamination on non-target sites and organisms, as well as by delaying the expression of insecticide resistance in insects.

The working group on Pesticides and Beneficial Organisms of the International Organization for Biological Control (IOBC) has recently claimed that selective pesticides suitable for use in integrated control programs and the development of standard methods to test the side effects of pesticides on most natural enemies are urgently needed.[8] *V. lecanii* effectiveness as a mycoinsecticide for whiteflies has been demonstrated In Mexico, this fungus has been isolated from whiteflies[14] and coffee rust,[5] and its compatibility tested against two chemical pesticides used for coffee rust.[4] In most compatibility studies, the effects of pesticides have been examined *in vitro* by germination and sporulation inhibition on agar plates.[1,16,18,20,28] In this study, two strains, one isolated in Cuba and another in México, were tested *in vitro* to determine if lambda-cyhalotrin, permethrin, and methamidophos, commonly used in this country for whiteflies chemical control, and benomyl used in vegetable crops to control fungal diseases caused by phytopathogenic fungi, are harmful to *V. lecanii.*

MATERIALS AND METHODS

Fungi. Two strains of *V. lecanii* were used. Strain A was isolated in Cuba from *Coccus viridis* (Green) (Homoptera: *Coccidae*) in grapefruit (Cabrera, personal communication). Strain C was isolated in México from *Trialeurodes vaporariorum* (West.) (Homoptera: *Aleyrodidae*) by Mier *et al.*[14] from beans. The strains are maintained in Sabouraud agar at 4°C.

Conidia were produced by growing mycelia on mycological agar (Bioxón, México) with 0.25% yeast extract (Bioxón). Plates were incubated at 26°C for 12 days. Conidia were prepared for testing, by suspending them in sterile water and thoroughly mixing with a Vortex mixer (Fisher Scientific, New York). Hemocytometer counts were made to achieve a 1 x 10^6 conidia/ml suspension.

Pesticides. Assays were performed with one fungicide: Benomyl, methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate (Benomilo, WP, 500 g AI/Kg, Promotora Técnica Industrial, México), two piretroid insecticides: lambda-cyhalotrin, (S)-α-cyano-3-phenoxybenzyl-(1S + 1R)-*cis*-3-(Z-2-chloro-=3,3,3-trifluoropropenyl)-2,2-=dimethylcyclopropanecarboxylate (Karate SL, 70 g AI/l, ICI Agroquímicos, México) and permethrin, 3-=phenoxybenzyl (1RS)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (Ambush SL, 50 g AI/l, ICI Agroquímicos), and an organophosphorus insecticide: methamidophos, *O,S*-dimethyl phosphoramidothioate (Tamaron 600 SL, 600 g AI/l, Bayer, Mexico). Each pesticide was tested at three concentrations: the recommended one for field use (II), and two additional ones, a lower (I) and a higher (III). Pesticide concentrations were: 0.05, 0.1, and 0.2 mg/l for benomyl; 0.5, 1.3, and 2.3 ml/l for lambda-cyhalotrin; 0.5, 1.4, and 2.4 ml/l for permethrin, and 3.13, 4.13, and 5.13 ml/l for methamidophos.

Compatibility assay. The inhibition percentage was determined as an inverse measure of fungal viability, using a modification of the Anderson & Roberts[2] method. Briefly, each treatment (strain, pesticide, and concentration) consisted of 1 ml of an aqueous suspension of 1 x 10^6 conidia/ml prepared as mentioned above, mixed with 9 ml of the pesticide concentration. Three replicates per treatment were processed in time, as well as a control for each treatment, without any pesticide. The *V. lecanii*-pesticide combinations were shaken for 15 h at 26°C, as tank mix. The colony forming units (CFU) were determined by dilutions in mycological agar plates, and the average values for each treatment were compared with those obtained for the control. The percent inhibition (%I) was calculated by the following equation:

$$\%I = \frac{\text{(CFU control - CFU treatment)}}{\text{CFU control}} \times 100$$

Experimental results were subjected to a factorial variance analysis using a SAS program.[11] The strains, pesticides, and concentrations were taken as the independent variables and the percent inhibition as the dependent variable. When triple interaction in factorial analysis of variance was statistically significant ($P<0.05$), a Tukey test was applied.

Colonial morphology assay. A sample of 0.3 ml of an aqueous conidial suspension from each strain, prepared as mentioned above, was plated on a mycological agar plate and incubated at 26°C for 7 days. Five to 6 mm mycelial fragments were taken from the middle portion of the colony and transferred to another mycological agar plate previously impregnated with each pesticide concentration. Care was taken to turn the fungal fragments upside down, so that the mycelia would come in contact with the pesticide impregnated medium. The same procedure was performed with the controls in pesticide free agar plates.

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Three replicates in time were done for each treatment and plates incubated at 26°C for 2 to 3 weeks. Alterations in the colony morphology were noted, microscopic samples stained with 1% acid fuchsin, and observed under a bright field microscope.

RESULTS

The four pesticides assayed showed viability inhibition on both strains of *V. lecanii* as showed in Table 1.

Both strains of *V. lecanii* developed the typical fungal morphology in agar plates without pesticides, forming white colonies of moderate growth, ca. 3 cm in diameter after 10-12 days of incubation at 26°C, turning yellowish with aging, but without diffusible pigment to their reverse (Fig. 1). The somatic hyphae were hyaline and the conidiophores were erect, with verticillate branches along their axes, bearing solitary or clustery phialides, usually divergent, with mucilaginous conidial heads at their tips; conidia were cylindrical or ellipsoidal, with rounded ends, 2.3-10.0 X 1.0-2.5 µm (Fig. 2). In pesticide impregnated agar plates, *V. lecanii* morphology was altered. Benomyl completely inhibited growth of both fungal strains. The three insecticides used, at all concentrations tested, caused a change in colony color, from the characteristic white to a light cinnamon hue, and in colony consistency, from cottony to a more compact, hardened, sclerotioid mass of mycelia, without conidia, and with hyphae united in synnemata-like structures that lacked phialides (Figs. 3-4). After 20-25 days, when these altered cultures were transferred to mycological agar, containing 0.25% yeast extract but no pesticides, morphology reverted to typical *V. lecanii* colonies.

DISCUSSION

The results of this study showed that pesticides commonly used in the field in Mexico for the chemical control of whiteflies and the fungicide are toxic for the natural bioregulator *V. lecanii*. The difference of compatibility shown by the two studied strains leads to consider the relevance and convenience of testing chemical pesticide compatibility with strains of different origins.[13,15,23,27] A point of discussion in pest control management programs should be the selection of fungal strains compatible with chemical pesticides when applied in combination with entomopathogenic fungi.

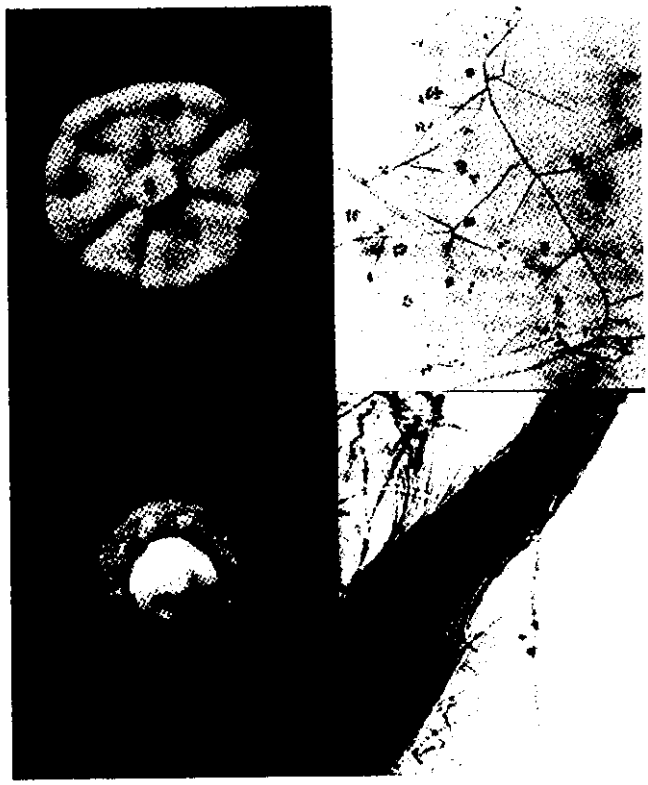
Lambda cyhalotrin and benomyl were the most toxic for both strains of this fungus, even in a lower concentration than that recommended for field use. Olmert & Kenneth[16] also showed 100% growth inhibition of *V. lecanni* isolates from Israel, by a poisoned-bait method, at the fungicides recommended field dose plus two more 1/10 dilutions. Carrión *et al.*[4] showed that two fungicides (triadimephon and copper oxychloride) used for coffee rust control in Mexico were toxic for *V. lecanii*. Benomyl incompatibility with other entomopathogenic fungi has also been demonstrated.[10,21,26] Based on these reports and the results of this study, with two strains of *V. lecanii* isolated in Latin America, carefull surveillance of the applications of this chemical for phytopathogenic fungi control should be performed. Its effect upon both entomopathogenic natural bioregulators and soil fungi is particularly harmful.

According to these results lambda-cyhalotrin had the same deleterious effect on the *V. lecanii* strains tested as benomyl, reaching near 100% inhibition with the three tested concentrations. Therefore, care should be taken to avoid its use when this fungus is naturally present, or if it

Table 1. Viability inhibition percentages by pesticides on strains A and C of *Verticillium lecanii*.

Pesticides	Viability inhibition percentages		
	Conc I X ± SD[a]	Conc II X ± SD	Conc III X ± SD
Strain A			
Benomyl	88.50 ± 4.83ab[b]	95.90 ± 5.68ab	97.90 ± 2.85a
Lambda-cyhalothrin	99.20 ± 0.58a	99.90 ± 0.02a	100a
Permethrin	17.70 ± 8.46d	44.80 ± 2.64cd	76.90 ± 20.56abc
Methamidophos	50.00 ± 23.10bcd	47.60 ± 20.87cd	59.80 ± 20.52bc
Strain C			
Benomyl	86.00 ± 5.05A	96.00 ± 1.57A	99.50 ± 0.64A
Lambda-cyhalothrin	99.00 ± 0.61A	100A	100A
Permethrin	25.40 ± 7.58C	78.50 ± 6.45AB	90.70 ± 9.17A
Methamidophos	28.90 ± 12.23C	29.70 ± 13.15C	58.10 ± 12.06B

[a] X ± SD = Mean ± standard deviation. [b]Means with the same letter are not significantly different (Tukey's, P<0.05). Lower-case letters for strain A and upper-case letters for strain C.



Figs. 1-4. Morphological characteristics of *V. lecanii* with and without methamidophos (the least toxic insecticide). 1. Fungal morphology in a mycological agar plate without the insecticide, showing a white cottony colony. 2. Typical fungal microscopic morphology from the agar plate without insecticide, showing the characteristic hyaline hyphae, erect conidiophores with verticillate branches along their axes, bearing phialides with mucilaginous conidial heads and conidia (X 342). 3. The fungus with the insecticide impregnated medium showing a compact, hard colony. 4. Microscopic morphology from the fungus grown in insecticide impregnated medium showing hyphae united in synnemata-like structures without phialides (X 342).

is introduced by spraying on crops or ornamentals attacked by whiteflies. Furthermore, this insecticide's DT_{50} in water-sediment mixtures in sunlight is 20 days, and in soil 22-82 days,[29] which would suggest negative side-effects for naturally-ocurring *V. lecanii* in agroecosystems.

Among the four pesticides tested, permethrin and methamidophos showed the least inhibition effect, on both strains. At the recommended field concentration there is a difference in strain sensitivity to permethrin and methamidophos. This has been also shown by three strains of *V. lecanii* with different fungicides and insecticides.[16] In México, methamidophos is widely used in chemical control of whiteflies. Although its toxicity for the fungus is not as high as lambda-cyhalotrin, its mammalian toxicity according to the acute oral LD_{50} in rats[29] is the highest of the four pesticides assayed.

The morphological alterations observed in the pesticides impregnated agar plates were reverted when the fungus was grown on media without the pesticides, suggesting that the morphological abnormalities observed were physiological adaptations, and that no inherited damage was present, at least in the short term of this study. It is interesting to note that Carrión *et al.*[4] observed that this fungus produced sclerotia, which are fungal structures developed during unfavorable conditions, when *V. lecanii* was grown in fungicide impregnated agar plates.

It is a matter of concern that toxic chemical pesticide users in Latin America are not fully instructed in the correct concentration and management of these chemicals, causing severe damage to humans as well as a negative ecological impact. Pest management programs should include the correct application techniques to all users. Fi-

nally, the convenience of laboratory and field studies concerning the influence of these chemicals upon soil mycobiota and natural fungal bioregulators is strongly suggested.

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