Relationship between protease and chitinase activity and the virulence of *Paecilomyces fumosoroseus* in *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)

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Relación de la actividad de proteasa y quitinasa con la virulencia de *Paecilomyces fumosoroseus* en *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae)

Resumen. Se evaluó la actividad de proteasa y quitinasa y la virulencia en ninfas de mosquita blanca de aislados del hongo entomopatógeno *Paecilomyces fumosoroseus*. La producción de enzimas se ensayó con l8 aislados fúngicos, en un medio sintético líquido adicionado de cutícula de camarón coloidal y de quitina coloidal teñida con azul brillante de remazol como substratos para proteasa y quitinasa, respectivamente. Los bioensayos de virulencia se realizaron en ninfas de mosquita blanca (*Trialeurodes vaporariorum)* de segundo estadio, con 9 cultivos monospóricos. La virulencia fue determinada a partir de la concentración letal media (CL_{50}) en mosquita blanca. Los aislados EH-506/3 y EH-511/3 fueron los más virulentos con una CL_{50} de 1.1×10^3 y 6.9×10^3 conidios/mL, respectivamente, con la más alta actividad de proteasa (39.8 y 14.9 PU/mL, respectivamente) a las 120 h del experimento. Las actividades de quitinasa alcanzaron sus valores más altos al final (264 y 312 h) del experimento. Se presenta por primera vez, en una muestra representativa de cepas de *P. fumosoroseus* aisladas en México, una gran variabilidad intraespecífica en las actividades de proteasas y quitinasas y en la virulencia. Se sugiere que la actividad de proteasas podría ser utilizada en la selección de cepas de este hongo para el control biológico de mosquita blanca.
Palabras clave: control biológico, factores de virulencia, hongos entomopatógenos.

Abstract. The production of protease and chitinase activities of isolates of *Paecilomyces fumosoroseus* and their virulence in whitefly nymphs were evaluated. Enzyme production was tested in l8 fungal isolates, on synthetic liquid medium with colloidal shrimp cuticle and remazol bright blue stained colloidal chitin as substrates for protease and chitinase activity, respectively. The virulence bioassay was carried out in second stage whitefly (*Trialeurodes vaporariorum*) nymphs in 9 monospore cultures of selected isolates. The virulence was determined by the median lethal concentration (LC_{50}) in whiteflies. Isolates EH-506/3 and EH-511/3 with the highest virulence (LC_{50} of 1.1×10^3 and 6.9×10^3, respectively) also showed the highest protease activity (PU/mL of 39.8 and 14.9, respectively) at 120 h. Chitinases reached maximum activities at the end (264 and 312 h) of the *in vitro* enzymatic assay. This is the first time that an ample intraspecific variability in protease and chitinase activities as well as in virulence has been reported in a representative sample of *P. fumosoroseus* strains from Mexico, which suggest that protease activities could be used in the selection of candidates for whiteflies biological control.
Key words: biological control, virulence factors, entomopathogenic fungus,.

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Introduction

Unlike most other pathogens that need to be ingested, fungi usually infect insects by active penetration through the cuticle, being able to control insects independent of their feeding habits (Smith and Grula, 1983). Insect cuticle is the first barrier that fungi encounter, therefore a wide variety of extracellular enzymes are synthesized for the enzymatic processes involved in the degradation of protein, chitin and lipids, principal cuticle components (Charnley and St. Leger, 1991; St. Leger *et al.*, 1986a; St. Leger *et al.*, 1986b; St. Leger *et al*., 1996). Different studies suggest proteases and chitinases as major determinants of fungal virulence in the complex and multifactorial phenomenon insect host/pathogen relationship (Fang *et al*., 2007; Gillespie *et al*., 1998; Gillespie *et al*., 2000; Gupta *et al*., 1994; Paterson, 1994; St. Leger, 1995; Yang *et al*., 2007). Recently, Castellanos-Moguel *et al*. (2007) have pointed subtilisine-like (Pr1) protease as a phenotypic marker of virulence for *Paecilomyces fumosoroseus* (Wize) Brown & Smith.

Paecilomyces fumosoroseus, a mitosporic ascomycete, has been successfully used as a biocontrol agent of whiteflies (Hemiptera: Aleyrodidae) and other insect pests (Butt *et al*., 2001; Shah and Pell, 2003). In Mexico, the whitefly is considered an important pest of vegetable crops, and of ornamental plants (Mier *et al*., 1991; Ramírez-Villapudua, 1996). Due to the relevance of enzymatic activities for fungal penetration in the pathogenic mechanism to attack the host and the importance of virulent strains for an efficient biological control, in this study, protease and chitinase activities was measured in 18 isolates of *P. fumosoroseus* from whiteflies in Mexico, as well as their relationship in fungal virulence, to be able to select the most suitable strains for biological control of whiteflies adapted to Mexican environmental conditions.

Materials and methods

Fungal isolates

All *Paecilomyces fumosoroseus* strains were isolated from whiteflies and obtained from the National Center for Biological Control, Mexico (Centro Nacional de Referencia de Control Biológico-CNRCB, SAGARPA, Tecomán, Colima) (Table 1). Single spore cultures were prepared as initially described by Goettel and Inglis (1997) and later modified by Cavallazzi *et al*. (2001). Monospore cultures (MCs) were preserved in sterile water, mineral oil, and in liquid nitrogen cryopreservation at –196 °C and deposited in the fungal collection of the Basic Mycology Laboratory, Microbiology and Parasitology Department, Faculty of Medicine, National Autonomous University of Mexico (UNAM) and in the Mycology Laboratory, Depto. El Hombre y su Ambiente, División de Ciencias Biológicas y de la Salud, Metropolitan Autonomous University-Xochimilco (UAM-X). Isolates were maintained on Sabouraud dextrose (SAB, Bioxon, Mexico) slants until used.

Polyspore original cultures were used for the determination of enzyme activities. Nine MC were selected for whitefly bioassays based on high, medium and low protease and chitinase activity (Castellanos-Moguel *et al*., 2001) to investigate their relationship with virulence. Lethal median concentration (LC_{50}) of three of these nine MC were previously reported (Castellanos-Moguel *et al.*, 2007).

Shrimp cuticle and chitin substrates

Colloidal shrimp cuticle and remazol bright blue stained colloidal chitin were used as substrates for protease and chitinase activity production, respectively. Both, shrimp cuticle and commercial chitin were treated with phosphoric acid (10 g/100 mL) overnight. Then, several water washings and filtrations were performed until a pH 7 was reached. Substrates were then sterilized and kept at 4 °C (Chávez-Camarillo y Cruz-Camarillo, 1984). Remazol bright blue stained chitin was prepared by the addition of 0.23 g of bright blue remazol diluted in water to 7 g of colloidal chitin, mixed and heated for 1 h, then filtrated and washed. After, 5 g of stained colloidal chitin with 0.075 g of sodium dichromate and 0.075 g of sodium-potassium tartrate was boiled (10 min), washed again, and sterilized by autoclave at 121°C for 15 min (Gómez-Ramírez, 2000).

Table 1. Identification and origin of *Paecilomyces fumosoroseus* isolated from whiteflies (Homoptera: Aleyrodidae) in Mexico

Isolate identification CNRCB[1]	Isolate identification UNAM[2]	Crop	Origin State of Mexico
MBP	EH-503	Cucumber	Yucatán
MBP1	EH-504	Cucumber	Yucatán
MBCH	EH-505	Habanero chilli	Yucatán
PFCAM	EH-506	Habanero chilli	Campeche
MBPO1N	EH-507	Poinsettia	Colima
MBPO2N	EH-508	Poinsettia	Colima
MBPO3A	EH-509	Poinsettia	Colima
MBNS1	EH-510	Watermelon	Colima
AMBAS2	EH-511	Watermelon	Colima
AMBAS3	EH-512	Watermelon	Colima
AMBAS4	EH-513	Watermelon	Colima
AMBAS5	EH-514	Watermelon	Colima
AMBASG	EH-515	Watermelon	Colima
MBPO4	EH-516	Poinsettia	Colima
MBTH	EH-517	NK	Sinaloa
MBMAC	EH-518	NK	Sinaloa
MBBRT	EH-519	Eggplant	Colima
PSMB	EH-520	Tomato	Nayarit

[1]Original isolates of the "Centro Nacional de Referencia de Control Biológico" (CNRCB), SAGARPA, Tecomán, Colima, México.
[2] Culture code of Laboratorio de Micología Básica, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), México D.F., México.
NK = Not known

Enzyme activity assays

For all shake-flask enzyme activity assays, cultures of *P. fumosoroseus* were grown in 125-mL Erlenmeyer flasks containing 25 mL of synthetic liquid medium (per liter: 0.375 g of KH_2PO_4; 0.375 g of Na_2CO_3; 0.250 g of NaCl; 0.275 g of $MgSO_4$) supplemented with 14% colloidal shrimp cuticle or 1% of remazol bright blue stained colloidal chitin as substrates, for protease or chitinase production, respectively. Inocula for both enzyme activities consisted of conidia from 10-day old slant SAB medium cultures with a final density of 1 x 10^6 conidia/mL. Flasks were incubated with orbital shaking (150 rpm) at 28 °C, for 312 h (13 days). Control flasks of liquid medium without inocula were maintained under the same experimental conditions. All experiments were reproduced at least three times.

At 24, 72, 120, 168, 216, 264 and 312 h, 3-ml aliquots of cultures for protease and chitinase activities and controls were collected. Samples were centrifuged (5,000 *g*, 10 min) and cell-free supernatants used immediately for chitinase activity determination or maintained at -20 °C until protease activity was determined.

A non-specific proteolytic assay with azocasein was performed for cell-free supernatants and controls of synthetic liquid medium supplemented with colloidal shrimp cuticle, according to Sarath *et al*. (1989), and described in Castellanos *et al*. (2007). Briefly, 2% azocasein was dissolved in phosphate buffer pH 7.0. 150 µL of cell-free supernatant

(enzyme) and 250 µL of substrate were incubated at 25°C for 1 h. Reaction was stopped with 1.2 mL of 10% trichloroacetic acid for 15 min. The reaction mixture was centrifuged at 10,000 rpm for 5 min, NaOH was added to develop color and absorbance read at 440 nm. One unit of protease activity (PU) was defined as the amount of enzyme that produced a change of 0.010 in optical density at 440 nm.

Chitinase activity was determined for cell-free supernatants and controls of synthetic liquid medium supplemented with remazol bright blue stained colloidal chitin, by the color development from the dye liberation by chitinase, and absorbance was read at 595 nm in a Beckman DU650 spectrophotometer (Beckman Instruments, CA, USA). One unit of chitinase activity (CU) was defined as the amount of enzyme that produced a change of 0.010 in optical density at 595 nm.

Insects

The whitefly nymphs (*Trialeurodes vaporariorum)* used for bioassays originated from colonies maintained at the greenhouse and experimental field of the Center for Biotechnology Research, Autonomous University of the State of Morelos (CEIB-UAEM), Mexico. Whiteflies were reared on "flor de mayo" bean (*Phaseolus vulgaris*) leaves.

Bioassay procedure

The method used was according to Vidal *et al*. (1997), with minor modifications as described in Castellanos *et al.* (2007). Fungal conidia were produced in SAB medium cultures, and five doses ranging from 4.7 x 10^2 to 4.7 x 10^6 conidia/mL in 0.05% sterile Tween 80 (v/v) were used and only 0.05% sterile Tween 80 for controls.

Experiments were conducted during a total of 10 days. Individual selected nymphs were monitored for mortality at day 10. Emerged whiteflies (empty pupal cases) were considered non-infected. A total of 75 nymphs were used, 25 for each replicate bioassay, and another 25 nymphs as control, for each conidial concentration tested. The number of nymphs of second instar that did not change to the next developmental stage was recorded (dead nymphs). All nymphs were then removed from the leaf surface, placed on sterile water agar dishes, and incubated at 24 ºC for 5-7 days to determine the percentage of mortality caused by mycosis, based on fungal sporulation on the insects. Nymphs that remained in second instar, were considered dead, and lethal median concentration (LC_{50}) was calculated using second instar data.

Chemicals

Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich Química (Toluca, Mexico).

Statistical analysis

Mortality of second stage nymphs was subjected to Probit analysis (Finney, 1971), generating a concentration-mortality relationship for the estimates of LC_{50} and its 95% confidence intervals for each of the MC tested, using POLO-PC program (1996). The data of protease and chitinase activities were analyzed using ANOVA ($\alpha = 0.05$) and a Tukey multiple mean comparison test (Dowdy and Wearden, 1983). Statistical analyses were performed using the SPSS Program, version 10, 2003.

Results

Enzymatic activity

Protease activity, in culture supernatants of synthetic liquid medium supplemented with colloidal shrimp cuticle, was detected at 72 h in almost all the isolates tested, except for EH-515 (data not shown). During the course time of the experiment (0-312 h), the highest protease activities were observed at 120 ($F_{17,53}$=190.2; P<0.05), 168 ($F_{17,53}$=120.6; P<0.05), 264 ($F_{17,53}$=67.7; P<0.05) or 312 h ($F_{17,53}$=129.3; P<0.05) for all isolates (Table 2). In six of 18 isolates (EH-506, EH-511, EH-512, EH-514, EH-517, EH-518) a peak of maximum activity was reached at 120 h. In the rest of the isolates, levels of maximum activity varied at 168, 264 and 312 h of the experiment. The highest significant ($P < 0.05$) activity was registered by EH-506, which exhibited 39.8, 23.6, 23.5 and 25.0 PU/mL at 120, 168, 264 and 312 h, respectively (Table 2).

Chitinase activity in culture supernatants of synthethic liquid medium supplemented with remazol bright blue stained colloidal chitin was detected since 72 h but in lower levels than protease activity (data not shown). Chitinase activities were detected at 120 ($F_{17,53}$= 26.8; P<0.05), 168 ($F_{17,53}$=52.6; P<0.05), 264 ($F_{17,53}$ = 89.1; P < 0.05) and 312 h ($F_{17,53}$= 39.9; P<0.05). In general, the highest activities were reached at the end of the experiment, specifically at 264 or 312 h. Only one isolate, EH-511, had the maximum activity at 168 h (Table 3). The highest activity was exhibited by EH-503 with 28.1, 47.2 and 90.4 CU/mL at 120, 168 and 264 h respectively, and by EH-518 (62.3 CU/mL) at 312 h.

Nine isolates (EH-503, EH-504, EH-506, EH-509, EH-511, EH-516, EH-518, EH-519, EH-520) were selected according to a high, medium and low protease and chitinase activities to obtain monospore cultures (see Table 4) and perform the virulence bioassay with second instar whitefly nymphs.

Virulence bioassay

The results of LC_{50} of monospore isolates EH-506/3 (high virulence), EH-503/3 (medium virulence) and EH-520/3 (low virulence) were reported previously in Castellanos *et al*. (2007), but for a better understanding of enzyme activities in our present results we have included them with the other six monospore isolates of this work.

All MCs infected second instar nymphs exhibited different virulences (Table 4). The mortality criterion of

Table 2. Protease activity of *Paecilomyces fumosoroseus* isolates at different times of incubation in synthetic liquid medium supplemented with colloidal shrimp cuticle as substrate

	Protease Units (PU/mL)			
Isolates	120 h	168 h	264 h	312 h
EH-503	3.17 ^{h}	6.1 ^{ef}	3.1 ^{g}	2.8 ^{i}
EH-504	8.67 ^{ef}	8.7 ^{de}	11.2 ^{abc}	9.5 ^{bcd}
EH-505	8.13 ^{fg}	12.2 ^{cd}	10.2 ^{abc}	5.8 ^{def}
EH-506	39.8 ^{a}	23.6 ^{a}	23.5 ^{a}	25 ^{a}
EH-507	12.5^{d}	17.9 ^{ab}	18.8 ^{ab}	19.9 ^{ab}
EH-508	7.1 ^{fg}	8.8 ^{de}	4.9 ^{ef}	5.1 ^{gh}
EH-509	0.4 ^{i}	2.3 ^{f}	4.7 ^{f}	3.3 ^{h}
EH-510	2.7 ^{h}	4.2 ^{f}	1.1 ^{h}	5.2 ^{gf}
EH-511	14.9 ^{cd}	13.9 ^{bc}	8.5 ^{bcde}	8.2 ^{bcde}
EH-512	9.17 ^{de}	0.43 ^{g}	2.3 ^{g}	1.3 ^{j}
EH-513	4.6 ^{gh}	3.7 ^{f}	6.1 ^{def}	4.2 ^{h}
EH-514	18.1 ^{bc}	17.8 ^{ab}	9.3 ^{abcd}	11.1 ^{bc}
EH-515	8.6 ^{ef}	3.3 ^{f}	4.8 ^{ef}	9.1 ^{bcd}
EH-516	2.7 ^{h}	3.2 ^{f}	9.9 ^{abcd}	17.6 ^{ab}
EH-517	18 ^{bc}	9.9 ^{c,d,e}	10.4 ^{abc}	8.1 ^{cde}
EH-518	20.1 ^{ab}	13.9 ^{bc}	18.6 ^{ab}	13.4 ^{abc}
EH-519	7.6 ^{fg}	11 ^{cd}	8.8 ^{abcd}	9.3 ^{bcd}
EH-520	12.5 ^{d}	12.8 ^{cd}	8 ^{c,d,e,f}	5.6 ^{efg}

Average of three independent experiments.
Values in the same column marked with the same letter did not differ significantly according to the Tukey´s test at a significance level of 5%.

Table 3. Chitinase activity of *Paecilomyces fumosoroseus* isolates at different times of incubation in synthetic liquid medium supplemented with remazol bright blue stained colloidal chitin as substrate

Isolates	Chitinase Units (CU/mL) 120 h	168 h	264 h	312 h
EH-503	28.1 ^{a}	47.2 ^{a}	90.4 ^{a}	58 ^{abc}
EH-504	13.1 ^{cd}	16 ^{ef}	41.9 ^{bcde}	47.9 ^{bcde}
EH-505	18.9 ^{abc}	27 ^{cd}	31.6 ^{fgh}	44.3 ^{cde}
EH-506	9.1 ^{e}	12.4 ^{g}	26.1 ^{hi}	37 ^{fgh}
EH-507	11.4 ^{de}	11.2 ^{g}	28.8 ^{gh}	31.6 ^{ghij}
EH-508	16.3 ^{c}	12.4 ^{g}	18.7 ^{j}	37.1 ^{efg}
EH-509	24.4 ^{ab}	22.3 ^{d}	38.7 ^{cdef}	52.8 ^{abcd}
EH-510	24.4 ^{ab}	22.3 ^{d}	38.7 ^{cdef}	52.8 ^{abcd}
EH-511	8.1 ^{e}	39.1 ^{ab}	25.5 ^{hij}	28.6 ^{hij}
EH-512	9.4 ^{e}	11.5 ^{g}	37.6 ^{defg}	24.9 ^{j}
EH-513	16.3 ^{c}	13.3 ^{g}	49.1 ^{abc}	58.4 ^{ab}
EH-514	18.7 ^{abc}	16.5 ^{ef}	21.1 ^{ij}	41.2 ^{def}
EH-515	14.3 ^{cd}	35.4 ^{abc}	54.5 ^{ab}	31.6 ^{ghij}
EH-516	18 ^{bc}	13.5 ^{f}	20.6 ^{ij}	24.5 ^{j}
EH-517	16.1 ^{c}	11 ^{g}	27.1 ^{hi}	42.6 ^{cde}
EH-518	16.4 ^{c}	27.8 ^{bcd}	35.4 ^{e,f,g,h}	62.3 ^{a}
EH-519	11.8 ^{de}	20.7 ^{de}	45.1 ^{bcd}	47.9 ^{bcde}
EH-520	13 ^{cd}	14.2 ^{f}	15.5 ^{k}	26.9 ^{ij}

Numbers are average of three replicates.
Values in the same column marked with the same letter did not differ significantly according to Tukey´s test at a significance level of 5%.

whiteflies was instar change, because nymphs are immobile, and at day 10, nymphs that had not shown change from second to third instar, were considered dead. At the end of the experiment (day 10), selected nymphs were transferred to water-agar and all of them showed mycelial growth, suggesting an insect mycosis and considered killed by the fungus. Control nymphs reached the adult stage in the same time period of the bioassay. Considering high virulence isolates those with the lower conidia concentration required to kill 50% of insects, LC_{50} calculated showed high virulent isolates as EH-506/3 and EH-511/3; medium virulence isolates as EH-518/6, EH-503/3, EH-509/3; and low virulence isolates as EH-519/2, EH-520/3 and EH-516/2 (Table 4). Isolate EH-504/1 exhibited a very low virulence because the LC_{50} could not be calculated with the five conidial concentrations used in the bioassay (Table 4).

Insect instar percentage change was recorded for all isolates, the percentage of nymphs that remained in second instar corresponded mainly to nymphs infected with the higher concentrations, 4.7×10^{5} and 4.7×10^{6} conidia/mL. For each MC the higher conidial concentrations stopped instar change, while lower conidial concentrations permitted the changes. The most virulent isolates (EH-506/3 and EH-511/3) showed that more than 50% of second instar nymphs remained in this instar, even with low conidial concentrations (4.7×10^{3}), while medium and low virulence isolates, less than 50% of nymphs remained in second instar. In comparison, the percentage of nymphs that reached fourth instar, before changing to adults, are shown in Figure 1, and the high virulence isolates (EH-506/3 and EH-511/3) exhibited very low percentages (< 20%) that reached fourth instar. The conidial concentrations that allowed nymphs to reach fourth instar were 4.7×10^{2}, 4.7×10^{3} and 4.7×10^{4} conidia/mL (Figure 1).

The maximum activity time and PU or CU/mL of protease and chitinase activity, respectively, with virulence

Table 4. Maximum activity time for protease and chitinase and virulence expressed as median lethal concentration (LC_{50}) of *Paecilomyces fumosoroseus*

Original isolates	Maximum activity time for enzyme production: Protease (PU/mL)	Chitinase (CU/mL)	Monospore isolates	Virulence (LC_{50}) Conidia/mL
EH-503	168 h 6.1	264 h 90.4	EH-503/3	$2.0 \times 10^{4*}$ (medium)**
EH-504	120 h 8.6	312 h 47.9	EH-504/1	> 7.6×10^{4}*** (low)
EH-506	120 h 39.8	312 h 37.0	EH-506/3	$1.1 \times 10^{3*}$ (high)
EH-509	264 h 4.7	264 h 38.7	EH-509/3	2.7×10^{4} (medium)
EH-511	120 h 14.9	168 h 39.1	EH-511/3	6.9×10^{3} (high)
EH-516	312 h 17.6	312 h 24.5	EH-516/2	1.1×10^{5} (low)
EH-518	120 h 20.1	312 h 62.3	EH-518/6	1.4×10^{4} (medium)
EH-519	168 h 11.0	312 h 47.9	EH-519/2	7.4×10^{4} (low)
EH-520	268 h 12.8	312 h 26.9	EH-520/3	$7.6 \times 10^{4*}$ (low)

*These LC_{50} data were reported in Castellanos *et al.* (2007).
**Data of low, medium and high virulence for each monospore isolate were included for a better understanding.
*** The LC_{50} of this isolate could not be calculated with the five *P. fumosoroseus* conidial concentrations assayed and used for the other isolates.

LC_{50} is shown in Table 4. Protease production (39.8 and 14.9 PU/mL) of high virulence isolates (EH-506/3 and EH-511/3, respectively) was reached early in the experiment (120 h). In medium and low virulence isolates, with the exception of EH-518/6 and EH-504/1, protease was produced later in the experiment (168, 264 and 312 h). High chitinase production of all isolates, with exception of the high virulence isolate EH-511/3, was observed at the end of the experiment at 264 and 312 h.

Discussion

The whitefly life cycle is approximately between 5 and 11 days depending on the insect species and environmental conditions (Byrne and Bellows, 1991). At this time (120 h) *in vitro* protease activity was the highest for the two most virulent isolates of our study and agrees with St. Leger *et al*. (1996) that report an early protease cuticular degradation allowing a faster colonization of the host. The tendency of the protease and chitinase activities in submerged cultures of the

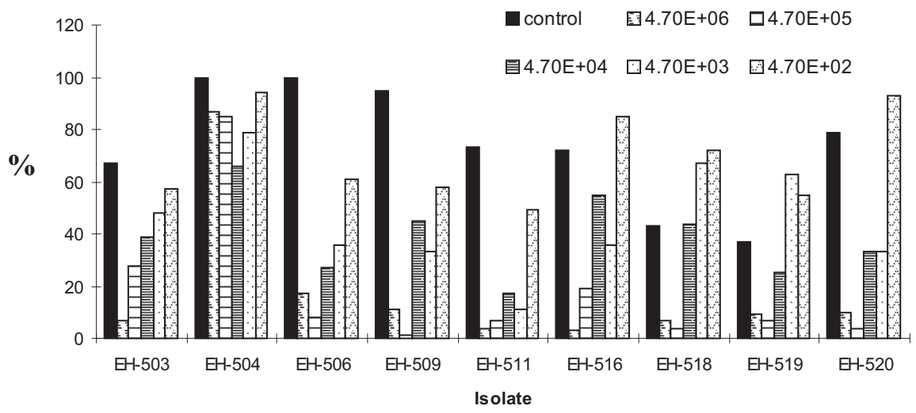

Figure 1. Percentage of whitefly nymphs that reached fourth instar after inoculation with different conidial concentrations of monospore isolates of *Paecilomyces fumosoroseus*.

isolates, reflect a starting protease followed by the chitinase activity. The most virulent isolates (EH-506/3 and EH-511/3) had an early high production of protease (Table 4).

Concerning the other enzymatic activity, high chitinase appeared, in almost all isolates, at the end of the experiment (264 and 312 h). In other entomopathogens, i.e., *Metarhizium anisopliae*, high chitinase activities have been observed at the end of the experimenting cultures when the fungus was grown with chitin as the sole source of carbon and nitrogen (Chul-Kang *et al*., 1999).

The LC_{50} of isolate EH-504/1 could not be calculated with the five *P. fumosoroseus* conidial concentrations assayed and used for the other isolates. This fact points out to the very low virulence of this isolate, that would probably be in need of higher conidial concentrations to achieve the LC_{50}.

The different concentrations and appearance of both relevant enzymes (protease and chitinase) for fungal pathogenesis of insects (St. Leger *et al*., 1986a; 1986b; 1987) in liquid cultures with the specific substrates shows the variability among different isolates of the same fungus. The same was observed concerning the virulence of the studied isolates.

Fungal pathogenesis of insects involves many different factors among host, parasite and environment. In the initial contact between fungus and insect, after the adhesion process, the penetration of the fungal pathogen into the cuticle depends relevantly on the action of the enzymatic activities (St. Leger *et al*., 1986a; 1986b; 1987). Under the experimental conditions of this study, the results suggest a relationship between a high protease activity and the high virulence isolates, EH-506/3 and EH-511/3. Protease and chitinase activities in entomopathogenic fungi are important due to the great amount of protein and chitin in insect cuticle (St. Leger *et al*., 1986a).

The development of mycoinsectides depends largely on the selection of a virulent strain for the insect pest, among other characteristics. This virulence trait must be well conserved in the selected strain, thus different authors have shown that monospore cultures are capable of decreasing the danger of a potential variation because of its homogenous genetic fungal population (Veen, 1967; Samsinakova y Kalalova, 1983). In a recent study, Cortez-Madrigal *et al*. (2003) also showed differences with the median germination time and conidial production between monospore and multispore cultures of *Lecanicillium lecanii*, monospore cultures exceeding their multispore isolates. The abovemetioned facts support the use of monospore cultures in order to attain a homogenous genetic fungal population in candidate strains for biological control.

In addition, the total protease and chitinase activities determined in the original multispore isolates are representative of the enzymatic potential of the monospore cultures due to their homogenous genetic population.

Broad-spectrum proteases are the main enzymes produced by entomopathogens during infection and degradation of the insect cuticle (St. Leger, 1995), and this protease activity has been related to virulence (Bidochka *et al*., 1999; Jackson *et al*., 1985; St Leger *et al*., 1986a; 1986b; 1987). However, this is the first time that an ample intraspecific variability in protease and chitinase activities as well as in virulence has been reported in a representative sample of *P. fumosoroseus* strains from Mexico, which suggest that protease activities could be used in the selection of candidates for whiteflies biological control.

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References

Butt, T.M., C. Jackson, N. Magan, 2001. Fungi as Biocontrol Agents, Progress, Problems and Potential. CABI, New York.

Byrne, D.N., T.S. Belows, 1991. Whitefly biology. Annual Review of Entomology 36: 431-457.

Bidochka, M.J., R.J. St. Leger, A. Stuart, K. Gowanlock, 1999. Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and plant virulence, extracellular proteases and carbohydrases. Microbiology 145: 955-963.

Castellanos-Moguel, J., M. González-Barajas, T. Mier, M.R Reyes Montes, E. Aranda, C. Toriello, 2007. Virulence testing and extracellular subtilisin-like (Pr1) and trypsin-like (Pr2) activity during propagule production of *Paecilomyces fumosoroseus* isolates from whiteflies (Homoptera: Aleyrodidae). Revista Iberoamericana de Micología 24: 62-68.

Castellanos-Moguel J., R. Cruz-Camarillo, E. Aranda, C. Toriello, 2001. Selección de aislados de *Paecilomyces fumosoroseus* (Wize) Brown y Smith, con base en sus niveles de proteasa y quitinasa. Memorias del IX Congreso Nacional de Biotecnología y Bioingeniería; XIII Congreso Nacional de Ingeniería Bioquímica; II Congreso Internacional de Ingeniería Bioquímica, Veracruz, México, septiembre 10-14, CIV-33.

Cavallazzi-Vargas, G., A. Pérez-Mejía, A. Berlanga Padilla, V. Hernández-Velázquez, C. Toriello, 2001. Selección de cultivos monospóricos de *Paecilomyces fumosoroseus* con base en sus características fenotípicas. *In*: Nevárez-Morillón G.V., G. Sánchez-Martínez, L.N. Muñoz-Castellanos (eds.), Memorias del XXIV Congreso Nacional de Control Biológico. Chihuahua, México, agosto 9-10, pp. 112-115.

Charnley, A.K., R. J. St. Leger, 1991. The role of cuticle degrading enzymes in fungal pathogenesis in insects. *In*: Cole, G.T., H. C. Hoch (ed.), The Fungal Spore and Disease Initiation in Plants and Animals. Plenum Press, New York. pp. 267-286.

Chávez-Camarillo, G., R. Cruz-Camarillo, 1984. El sistema quitinolítico de *Serratia marcescens.* Revista Latinoamericana de Microbiología 26: 203-216.

Chul-Kang, S., S. Park, D. Gyu-Lee, 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus *Metarhizium anisopliae*. Journal of Invertebrate Pathology 73: 276-281.

Cortez-Madrigal, H., R. Alatorre-Rosas, G. Mora-Aguilera, H. Bravo-Mojica, C.F. Ortíz-García, L. A. Aceves-Navarro, 2003. Characterization of multisporic and monosporic isolates of *Lecanicillium* (= *Verticillium*) *lecanii* for the management of *Toxoptera aurantii* in cocoa. BioControl 48: 321-334.

Dowdy, S., S. Wearden, 1983. Statistics for Research. John Wiley & Sons, New York.

Fang, W.G., S.J. Gou, X.Q. Pei, Y.J. Zhang, Y.H. Xiao, D.M. Li, K. Jin, M.J. Bidochka, Y. Pei, 2007. Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase. Applied and Environmental Microbiology 73: 295-302.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London.

Gillespie, J., P.R. Bateman, A.K. Charnley, 1998. Role of cuticule degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. Journal of Invertebrate Pathology 71: 128-137.

Gillespie, J., P.C. Burnett, A.K. Charnley, 2000. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. Journal of General Microbiology 135: 2233-2239.

Goettel, M.S., G.D. Inglis, 1997. Fungi: Hyphomycetes. *In*: Lacey, L.A. (ed), Manual of Techniques in Insect Pathology. Academic Press, London. pp. 213-247.

Gómez-Ramírez, M., 2000. Selección y caracterización de una cepa quitinolítica de *Bacillus thuringiensis.* Tesis de Maestría, Escuela Nacional de Ciencias Biológicas, IPN, México, D.F.

Gupta, S. C., T. D. Leathers, G. N. El-Sayed, C. M. Ignoffo, 1994. Relationships among enzyme activities and virulence parameters in *Beauveria bassiana* infections of *Galleria mellonella* and *Trichoplusia ni.* Journal of Invertebrate Pathology 64: 13-17.

Jackson, C.W., J.B. Heale, R.A. Hall, 1985. Traits associated with virulence to the aphid *Macrosiphoniella sanbornii* in eighteen isolates of *Verticillium lecanii*. Annals of Applied Biology 106: 39-48.

Mier, T., F. Rivera, J.C. Bermúdez, Y. Domínguez, C. Benavides, M. Ulloa, 1991. Primer reporte en México del aislamiento de *Verticillium lecanii* a partir de la mosquita blanca y pruebas de patogenicidad *in vitro* sobre este insecto. Revista Mexicana de Micología 7: 149-156.

Paterson, I.C., A.K. Charnley, R.M. Cooper, J.M. Clarkson, 1994. Specific induction of a cuticle degrading protease of the insect pathogenic fungus *Metarhizium anisopliae*. Microbiology 140: 3153-3159.

Ramírez-Villapudua J., 1996. Manejo integrado de la mosquita blanca de la hoja plateada. Universidad Autónoma de Sinaloa, Sinaloa, México.

Samsinakova, A, S. Kalalova, 1983. The influence of a single-spore isolate and repeated subculturing on the pathogenicity of conidia of the entomophagous fungus *Beauveria bassiana*. Journal of Invertebrate Pathology 42: 156-161.

Sarath, G., R.S. De la Motte, F.W. Wagner, 1989. Protease assay methods. In: R.J. Benyon, J.S. Bond (eds.), Proteolytic Enzymes, a Practical Approach. IRN, Oxford. pp. 25-55.

Shah, P.A., J.K. Pell, 2003. Entomopathogenic fungi as biological control agents. Applied Microbiology Biotechnology 61: 413-423.

Smith, R.J., E.A. Grula, 1983. Chitinase is an inducible enzyme in *Beauveria bassiana*. Journal of Invertebrate Pathology 42: 319-326.

St. Leger, R.J., 1995. The role of cuticle degrading proteases in fungal pathogenesis of insects. Canadian Journal of Botany 73: 1119-1125.

St. Leger, R.J., R.M. Cooper, A.K. Charnley, 1986a. Cuticle degrading enzymes of entomopatogenic fungi: cuticle degrading *in vitro* by enzymes from entomopathogens. Journal of Invertebrate Pathology 47: 167-177.

St. Leger, R.J., A.K. Charnley, M. Cooper, 1986b. Cuticle degrading enzymes of entomopathogenic fungi: mechanisms of interaction between pathogen enzymes and insect cuticle. Journal of Invertebrate Pathology 47: 295-302.

St. Leger, R.J., M. Cooper, A.K. Charnley, 1987. Production of cuticle-degrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. Journal of General Microbiology 133: 1371-1382.

St. Leger, R. J., L. Joshi, M.J. Bidochka, D. W. Roberts, 1996. Biochemical characterization and ultrastructural localization of two extracelular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Applied and Environmental Microbiology 64: 1257-1264.

Veen, K.H., 1967. A technique for monospore cultures and the determination of nucleus numbers in *Metarhizium anisopliae*. Journal of Invertebrate Pathology 9: 276-278.

Vidal, C., L.A. Lacey, J. Fargues, 1997. Pathogenicity *of Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay method. Journal of Economic Entomology 90: 765-770.

Yang, J.K., N. Tao, L.M. Liang, Q.L. Mi, J. Li, K.Q. Zhang, 2007. Cloning of the gene *Lecanicillium psalliotae* chitinase Lpchil and identification of its potencial role in the biocontrol of root-knot nematode *Meloidogyne incognita*. Applied Microbiology and Biotechnology 76: 1309-1317.