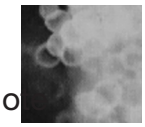

Virulence testing and extracellular subtilisin-like (Pr1) and trypsin-like (Pr2) activity during propagule production of *Paecilomyces fumosoroseus* isolates from whiteflies (Homoptera: Aleyrodidae)

Judith Castellanos-Moguel[1], Margarita González-Barajas[1], Teresa Mier[1], María del Rocío Reyes-Montes[2], Eduardo Aranda[3]† and Conchita Toriello[2]

[1]Departamento el Hombre y su Ambiente, Universidad Autónoma Metropolitana-Xochimilco, México D. F. 04960, Mexico; [2]Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, México D. F. 04510, Mexico; [3]Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, 62250, Mexico

Summary

To properly characterize several isolates of *Paecilomyces fumosoroseus,* a fungal entomopathogen of whiteflies (Homoptera: Aleyrodidae) and other insect pests for biocontrol purposes, virulence towards *Trialeurodes vaporariorum*, and subtilisin-like (Pr1) and trypsin-like (Pr2) protease activity during propagule production were investigated in monospore cultures (MCs). The virulence of three MCs towards second instar whiteflies was measured and expressed as lethal median concentration (LC_{50}). Number and width–length ratio of propagules (blastospores, hyphal bodies, short hyphae, submerged conidia) and extracellular proteolytic activity was determined simultaneously in liquid medium. Total protease activity was assayed with azocasein, Pr1 and Pr2 activity was determined with the substrates N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and N-Benzoyl-Phe-Val-Arg-p-nitroanilide, respectively. Natural variability in virulence, propagule production and cuticle-degrading proteases among isolates was observed. Bioassays showed a LC_{50} of 1.1 x 10^3, 2.5 x 10^4 and 7.6 x 10^4 conidia/ml for MCs EH-506/3, EH-503/3 and EH-520/3, respectively, EH-506/3 being the most virulent isolate. Isolate EH-503/3 produced the highest yield of propagules (7.7 x 10^7 propagules/ml), followed by EH-520/3 with 6.4 x 10^7 and EH-506/3 with 1.0 x 10^7 propagules/ml. Subtilisin-like (Pr1) and trypsin-like (Pr2) activity was present in the three MCs. Subtilisin-like (Pr1) activity was highest (745.7 UPr1/ml at 120 h) in the most virulent isolate, EH-506/3, pointing at Pr1 as a phenotypic marker of virulence for *P. fumosoroseus*. EH-506/3 appears to be a good candidate for whitefly biocontrol due to its high virulence, Pr1 concentration and rapid transition to blastospores in submerged liquid medium.

Key words

Paecilomyces fumosoroseus, Whitefly, *Trialeurodes vaporariorum*, Subtilisin-like (Pr1), Trypsin-like (Pr2), Biocontrol, Virulence, Blastospores

Corresponding Author:
Dra. Conchita Toriello
Departamento de Microbiología y Parasitología
Facultad de Medicina, Universidad Nacional Autónoma de México
México D.F. 04510, Mexico
Tel./Fax: (52 55) 5623-2461
E-mail: toriello@servidor.unam.mx

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Pruebas de virulencia y determinación de actividad extracelular de proteasas tipo subtilisina (Pr1) y tipo tripsina (Pr2) durante la producción de propágulos de aislados de *Paecilomyces fumosoroseus* de mosquita blanca (Homoptera: Aleyrodidae)

Resumen

Con el objetivo de caracterizar aislamientos para el control biológico de mosquita blanca (Homoptera: Aleyrodidae), se estudiaron tres cultivos monospóricos (CMs) de *Paecilomyces fumosoroseus*, hongo entomopatógeno de la mosquita blanca (Homoptera: Aleyrodidae). Se determinó la virulencia en ninfas de segundo estadio de la mosquita blanca expresada como concentración letal media (CL_{50}). Se determinó la producción de propágulos fúngicos (blastosporas, cuerpos hifales, hifas cortas, conidios sumergidos) en medio líquido, se midió la relación de largo y ancho de los propágulos, y la actividad proteolítica (total; tipo subtilisina, Pr1 y tipo tripsina, Pr2) simultáneamente con la producción de propágulos. La proteasa total se determinó con azocaseina, y las actividades de Pr1 y Pr2 con N-Succinil-Ala-Ala-Pro-Phe-p-nitroanilida y N-Benzoil-Phe-Val-Arg-p-nitroanilida como sustratos específicos, respectivamente. Se observó variabilidad entre los tres CMs en cuanto a la virulencia, producción de propágulos y proteasas. Los bioensayos mostraron una CL_{50} de 1,1 x 10^3, 2,5 x 10^4 y 7,6 x 10^4 conidios/ml para los CMs EH-506/3, EH-503/3 y EH-520/3, respectivamente.
El CM donde se observó el mayor número de propágulos en las condiciones ensayadas fue EH-503/3 (7,7 x 10^7), luego EH-520/3 con 6,4 x 10^7 y EH-506/3 con 1,0 x 10^7 propágulos/ml. Las actividades enzimáticas de Pr1 y Pr2 se demostraron en los tres CMs. La actividad tipo subtilisina (Pr1) fue mayor en el aislado más virulento (EH-506/3) con 745,7 UPr1/ml a las 120 h, y señala a Pr1 como un marcador fenotípico de virulencia para *P. fumosoroseus.*
EH-506/3 es un buen candidato para el control biológico de la mosquita blanca en México, por su alta virulencia, elevada concentración de Pr1 y su rápida transición a blastosporas en el medio líquido ensayado.

Palabras clave

Paecilomyces fumosoroseus, Mosquita blanca, *Trialeurodes vaporariorum,* Proteasa tipo subtilisina (Pr1), Proteasa tipo tripsina (Pr2), Control biológico, Virulencia, Blastosporas

Whiteflies (Homoptera: Aleyrodidae) are polyphagous insects that live on vegetable, staple and ornamental crops, and on many other cultivated plant species, both in greenhouses and in the open field worldwide. In Mexico, the whitefly is considered an important pest of vegetable crops, and of ornamental plants [30].

Paecilomyces fumosoroseus, a fungal entomopathogen of whiteflies, has been successfully used as a biocontrol agent of this and other insect pests [8,36]. The successful use of mycoinsecticides for biocontrol in the field depends, among many factors, on their virulence toward target insects. Proteases are primary enzymes in insect cuticle degradation that play a key role in various aspects of fungal biology and have been related to virulence [12,13]. St. Leger et al. [39] isolated a general protease from *Metarhizium anisopliae* capable of degrading a wide variety of proteins. The serine proteases, Pr1 and Pr2, have been identified in *M. anisopliae*, *Metarhizium flavoviride*, *Beauveria bassiana, Verticillium lecanii,* and *Nomuraea rileyi* [3,24,38,40]. Pinto et al. [28] described Pr1 and Pr2 activity in *M. flavoviride* growing in a mineral medium supplemented with either cuticle from the locust *Rhammatocerus schistocercoides* or casein. Recently, eleven protease isoforms and one metalloprotease related to the mechanism of fungal penetration have been identified and cloned from *M. anisopliae* var. *anisopliae* and var. *acridum* [16]. Activity of these enzymes has been reported in other entomopathogenic fungi, particularly *Metarhizium* isolates [39,41], in the mycelial phase, but to the best of our knowledge not in *P. fumosoroseus* during propagule production in submerged cultures. In these cultures, entomopathogenic fungi usually form asexual spores as single cells by schizolytic separation at septa, or by mechanical fragmentation of hyphae, or they can also be produced from hyphae by yeast-like budding [31]. These structures are described by several authors as blastospores or hyphal bodies [21,31,32,45]. Blastospores have been shown to be at least as infective as conidia in assays using topically applied propagules against several insects [1]. These structures are similar to those found when the fungus grows in the insect haemolymph [19]. Furthermore, submerged culture processes have advantages over conidial production in solid-state fermentation, such as reducing the time of propagule production, and a faster germination rate of blastospores on the insect cuticle [1,43,45].

To select a strain for biocontrol purposes, it is necessary to assay the virulence toward the target insect of different isolates to asses their potential as biocontrol agents, in addition to other fungal characteristics. In this study, virulence of three monospore cultures (MCs) of *P. fumosoroseus* isolated in Mexico was tested on whiteflies, regarding virulence and blastospore production in submerged cultures, total proteases, and Pr1 and Pr2 activity during propagule production to select a suitable candidate to be used as a microbial agent for whitefly control.

Materials and methods

Fungal isolates and growth conditions. All *P. fumosoroseus* PFCAM, MBP, and PSMB1 strains were isolated from whiteflies and obtained from the National Center for Biological Control, Mexico (Centro Nacional de Referencia de Control Biológico-CNRCB). Single spore cultures labeled EH-506/3, EH-503/3 and EH-520/3 from PFCAM, MBP, and PSMB1, respectively, were prepared as initially described by Goettel and Inglis [17] and later modified by Cavallazzi et al. [11]. 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). Isolates were maintained on culture medium slants containing (in grams per liter): sucrose, 10; glucose, 5; peptone, 0.5; yeast extract, 5; agar, 23, (SGPYE medium), until used.

The MC was selected based on total protease activity, showing high, low, and intermediate activity [9,10].

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" (*Phaseolus vulgaris*) bean leaves.

Bioassay procedure. The method used was according to Vidal et al. [46], with minor modifications. Fungal conidia were produced in SGPYE medium cultures, and five doses ranging from 4.7 x 10^2 to 4.7 x 10^6 conidia/ml were used. *Phaseolus vulgaris* leaf disks were outlined with a plastic cap (3.5 cm of diameter) and cut with a sterile scalpel. In all bioassays, second instar nymphs were identified by marking leaves with a permanent ink pen near the selected insects. Nymphs were disinfected in a laminar flow hood by soaking them in the following solutions: 70% alcohol for 5 s, sterile distilled water for 40 s, 5% sodium hypochlorite for 20 s, followed by rinsing in 3 changes of sterile distilled water for a total of 120 s. Leaves were air-dried on sterile filter paper. Nymphs were infected by floating the disks downwards on the fungal suspension so that the side infested with the whitefly nymphs touched the suspension, on each of the five selected conidia concentrations (4.7 x 10^2 to 4.7 x 10^6 conidia/ml). Disks were transferred to 3.5 cm Petri dishes containing sterile KNOP medium (in grams per liter: phosphate nitrate, 0.125; calcium nitrate, 0.500; manganese sulfate, 0.125; potassium phosphate, 0.125; agar, 23). The top side of the leaf disks was placed against the agar so that the side of the nymphs faced upward. Control disks were used with 0.05% Tween 80. The lids of the Petri dishes were sealed with strips of parafilm to maintain saturated humidity and placed in an incubator at 24 °C and a photoperiod of 16:8 h light:dark, (L:D). After incubation for 24 h under high humidity, the lids were replaced with others that had a paper-filter covered 1-cm hole for aeration, and to prevent condensation as described in Vidal et al. [46]. Petri dishes were maintained in the incubator at 26 °C (± 1 °C), 60% RH, and a photoperiod of 16:8 (L:D) h, during a total of 10 d. Individual selected nymphs were monitored for mortality at 10 d. 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 °C for 5-7 d 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.

Propagule production, and enzyme activity assays. For all shake-flask blastospore production studies, cultures of *P. fumosoroseus* were grown in 250-ml Erlenmeyer flasks containing 70 ml of SGPYE liquid medium supplemented with 1% casein (w/v) to ensure a high yield of blastospores and, simultaneously, produce enzymes. Inocula consisted of conidia from 10-day old slant SGPYE medium cultures with a final cell density of 1 x 10^6 conidia/ml. All experiments were reproduced at least three times. Conidia and blastospore concentrations were determined microscopically with a haemocytometer. The length and width of 30 submerged randomly chosen propagules (blastospores, hyphal bodies, short hyphae, and submerged conidia) of each isolate were measured using an Olympus BX-40 microscope with a calibrated objective at 40 x. Micrographs were made with an Olympus PM-C35 camera and an Olympus PM20 exposure control unit. Fungal transformation was registered according to the classification scheme of Bidochka et al. [4], with slight modifications. This scheme comprises six developmental stages (DS): (I) Unswollen conidia. (II) Swollen conidia. (III) Emergence of the germ tube. (IV) Elongation of the germ tube and formation of the first septum. (V) Polar and bipolar elongation (growth) of the resulting mycelium and initiation of a blastospore; and (VI) Secession of that blastospore. Flasks were incubated with orbital shaking (150 rpm) at 28 °C, for 312 h. Control flasks of liquid medium without inocula were maintained under the same experimental conditions. At 6, 12, 18, 24, 48, 72, 96, 120, 168, 216, 240, and 312 h, 3-ml aliquots of cultures and controls were collected. After propagule measurements, the remaining samples were centrifuged (5,000 *g*, 10 min), and cell-free supernatants were maintained at -20 °C until enzymatic activity was determined.

A non-specific proteolytic assay with azocasein was performed for both, cell-free supernatants and controls, according to Sarath et al. [35]. Briefly, 2% azocasein in 0.2 M sodium phosphate buffer, pH 7.0, was equilibrated at 25 °C. Reactions were performed in Eppendorf tubes containing 250 µl of azocasein and 150 µl of crude enzymatic extract. After 1 h of incubation at 25 °C, reactions were stopped by the addition of 1.2 ml of 10% trichloroacetic acid (Baker, Mexico). After 10 min at 4 °C, samples were centrifuged (5,000 *g*, 10 min), 1.2 ml of supernatant was placed in test tubes, mixed with 1.4 ml of 1.0 M NaOH for color development and absorbance was read at 440 nm in a Beckman DU650 spectrophotometer (Beckman Instruments, CA, USA). One unit of protease activity (UP) was defined as the amount of enzyme that produced a change of 0.010 in optical density at 440 nm.

Pr1 and Pr2 activity was determined using N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and N-Benzoyl-Phe-Val-Arg-p-nitroanilide as substrates, respectively, essentially as described by St. Leger et al. [39], except that 0.1 M Tris/HCl buffer, pH 8.0 was used. Briefly, 1 mM substrate diluted in this last buffer and the enzyme fractions were pre-warmed at 23 °C prior to mixing. Control absorbance values were read at 410 nm, before adding the substrate. Then, triplicate reaction mixtures, containing 50 µl of crude enzyme extract, 50 µl of substrate (for Pr1 and Pr2), and 900 µl of Tris buffer, pH 8.0, were incubated at 23 °C. After 1 min, absorbance was read at 410 nm in the spectrophotometer. One enzyme unit was defined as

the amount of Pr1 (UPr1) or Pr2 (UPr2) that produced a change of 0.001 in the optical density at 410 nm under the experimental conditions described above. Control samples were also incubated with specific substrates for Pr1 and Pr2.

Experiments were replicated three times with different aerial conidia inocula and samples were taken from three shake-flasks for each MC tested.

Protein determination. Protein content was measured using Coomassie Brilliant Blue G-250 [7].

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

Statistical analysis. Mortality of second stage nymphs was subjected to Probit analysis [15], generating a concentration-mortality relationship for the estimates of LC_{50} and its 95% confidence intervals for each of the MCs tested, using POLO-PC program (1996). Analysis of variance (ANOVA $\alpha = 0.05$) was calculated for the length to width ratio of propagules at the different assay times, total protease, and Pr1 and Pr2 activity among the three MCs selected, followed by a Tukey multiple mean comparison test [14]. Statistical analyses were performed using the SPSS Program, version 10, 2003.

Results and discussion

Bioassays. Fungal MCs varied in their ability to infect *T. vaporariorum* nymphs. The criterion for mortality status of whitefly nymphs was instar change, because nymphs are immobile, and at day 10, nymphs that had not shown change from 2nd to 3rd instar, were considered dead. At day 10, all marked nymphs, independently from the instar stage were transferred to water-agar and all of them showed mycelial growth, suggesting an insect mycosis, and were considered killed by the fungus. In all cases, mycoses were above 95 % of the treated nymphs (data not shown). Control nymphs reached the 4th stage or adult stage in the same time period of the bioassay. The MCs impact on nymph mortalities largely depended on the conidial concentrations tested. Change of nymphs to the next developmental stage tended to decrease with increasing concentrations of conidia, showing a concentration-mortality response in all MCs tested.

All EH-506/3 concentrations were highly toxic for 2nd instar whiteflies, in contrast with a lower mortality observed for EH-520/3 at the same concentration, for the same instar stage (data not shown). This last MC (EH-520/3) showed a marked concentration-response, mycelial growth from nymphs placed on water-agar showed that insects were infected but infection signals were observed late (7 days) in the experimental time-course. Conidial concentrations of the other MC tested, EH-503/3, were also virulent, however, this isolate infected nymphs in the 2nd instar, but killed them late in the time-course experiment. The same conidial concentration-response has been observed for whiteflies treated with *Verticillium lecanii* [25], and mites treated with several isolates of *P. fumosoroseus* [37]. In contrast, James et al., [23] observed no differences among the dose ranges of *P. fumosoroseus* tested in whitefly (*Bemisia argentifolii*) nymphs.

MC EH-506/3 was considered the most virulent isolate because 1.1×10^3 conidia/ml were enough to kill half of the tested insects in the experimental period. In contrast, EH-503/3 showed a LC_{50} of 2.5×10^4, and EH-520/3 of 7.6×10^4 conidia/ml, suggesting the latter as the isolate with the lowest virulence. All LC_{50} observed in this study (from 1.1×10^3 to 7.6×10^4 conidia/ml) were lower than those mentioned by Vidal et al. [46] (from 1.79×10^8 to 9.9×10^8 conidia/ml) for *P. fumosoroseus* treated whiteflies.

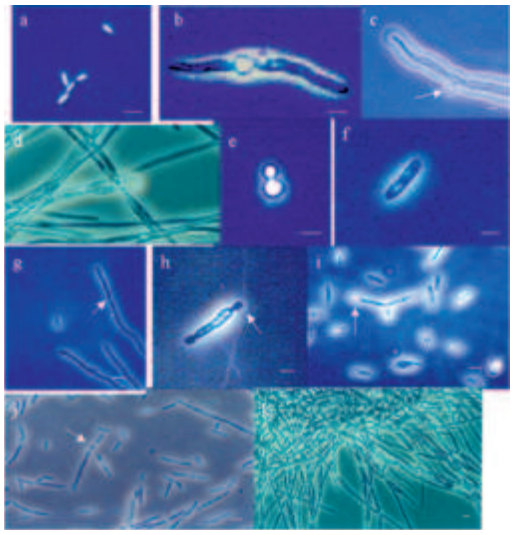

Figure 1. *Paecilomyces fumosoroseus* propagules produced in submerged culture. a) aerial conidia of EH-503/3. Developmental stage I: unswollen conidia. b) EH-503/3 swollen conidia with two germ tubes at 6 h. Developmental stage III: emergence of the germ tube. c) EH-520/3 blastospore originated from hyphal septum at 36 h. Developmental stage V: polar and bipolar elongation of the resulting mycelium and initiation of a blastospore. d) EH-520/3 blastospore originated from hyphal tip at 24 h. e) EH-503/3 budding yeast at 24 h. Developmental stage V. f) EH-506/3 hyphal body at 24 h. Developmental stage VI: secession of the blastospore. g) EH-503/3 short hyphae at 24 h. h) EH-503/3 blastospores at the hyphal body tips at 36 h. i) EH-503/3 submerged conidia at 60h, j) EH-503/3 large hyphal bodies at 72 h of incubation. k) EH-520/3 hyphal pellets after 240 h of incubation. Scale bar: 5 µm.

Propagule production. The three MCs exhibited transition from conidia (DS: I) (Figure 1a) to different propagules at different times. First, the conidium swelled (DS: II) and began producing one or more germ tubes (Figure 1b) (DS: III). Early during the experimental time, differential development of MCs was observed: at 6 h, EH-506/3 already produced germ tubes (DS: III), while EH-503/3 started to germinate between 6 and 12 h, and EH-520/3, until 12 h (Table 1). At 36 h, the three isolates produced primary blastospores (DS: V), originated from hyphal septa (Figure 1c) and tips (Figure 1d); budding blastospores (DS: V) (Figure 1e); and hyphal bodies (DS: VI) (Figure 1f). Similar yeast-like structures have been described in several liquid media [1, 22,43,44,45]. After 48-96 h, oblong narrow structures (Figure 1g) similar to short hyphae were formed, but they produced both blastospores (DS: V) (Figure 1h), or submerged conidia (Figure 1i). From 96 h on, pellets were more evident, and at 196 h larger and more hyphal bodies were observed (Figure 1j). At 240 h, blastospores showed well-defined vacuoles, and produced germ tubes. After 240 h, single propagules were less evident, and mainly hyphal pellets were observed (Figure 1k).

Assays showed different propagules (blastospores, hyphal bodies, short hyphae, and submerged conidia) for the three MCs tested during the first 96 h of the experiment, as reported by other authors [1,22,43,45].

Significant differences in propagule concentrations were observed between EH-506/3, the most virulent MC, and the other two isolates. Maximal propagule (blastospores, hyphal bodies) concentrations were observed at different times for each isolate. At 96 h, 1.0×10^7 propagules/ml for EH-506/3; at 60 h, 7.7×10^7 conidia/ml for EH-503/3; and 6.4×10^7 conidia/ml for EH-520/3. Other authors [34]

found a similar amount of blastospores (5 x 10^7 at 72 h) with the *P. fumosoroseus* strain 612 in a mineral medium supplemented with glucose, peptone, and yeast-extract.

Blastospores were obtained in a rich medium, with sucrose and glucose, and several nitrogen sources, such as casein, peptone, and yeast extract. A medium with peptone has been pointed out by Prenerová [29] as the optimal germination medium for *P. farinosus*. Jackson et al. [22] obtained high yields (6.0 x 10^8) of desiccation-tolerant *P. fumosoroseus* blastospores in a fermentor with a medium containing casein. Issaly et al. [18] reported high yields of *M. flavoviride* blastospores (1-5.4 x 10^8 blastospores/ml) using a medium with a high rate of nitrogen and low rate of carbon in flask cultures.

The size of the aerial conidia used for initial inoculation, and measured after incubation for 10 d at 28 °C on SGPYE solid medium, was 5 ± 0 µm length and 2.5 ± 0 µm width. The propagule size was determined by the ratio of length to width at each sampling time (Table 1). At 12 h, MC EH-503/3 showed a ratio of 16 that corresponds to short hyphae (conidia producing one or more germ tubes), but longer than the two other MCs. From 24 h on, all submerged propagules showed higher ratios than those of aerial inoculated conidia (DS: I). This has been cited for *P. fumosoroseus* and other entomopathogens [21]. Significant differences ($p < 0.05$) of propagule size among the three MCs were observed (Table 1) at almost all times tested, with the exception of 18 and 96 h. Ratio values of propagules from MC EH-506/3 and EH-503/3 between 36 and 96 h, and between 48 and 96 h for EH-520/3, were similar to the modal diameters obtained by Vidal et al. [45], using Goral medium (4.28 µm) at 96 h, and Jackson, Catroux and Paris (6.07 µm) medium, at 48 h. In our study, MC EH-506/3 which exhibited the highest virulence showed the fastest transition from conidia (DS: I) to blastospore (DS: VI) (Table 1). This dimorphic transition from conidia to yeast-like propagules (blastospores) has been documented in most entomopathogenic fungi [33]. In liquid culture, MC (EH-506/3) produced 1 x 10^7 propagules/ml; however, on slant or rice cultures, it has shown a very low conidia production (unpublished results). Nevertheless, its fast transition from conidia to blastospore, suggests another characteristic of the more virulent strains.

Enzymatic activity. Dimorphism was evident in SGPYE medium, with several nitrogen and carbon sources, and proteases determined during blastospore production. During the first hours of the experiment, proteolytic activity remained low, but after 96 h of culture incubation, a rise in total proteolytic activity was detected for the three MCs. Total proteases started to rise at 120 and 168 h (data

Table 1. Morphological type and length to width ratio of fungal propagules from *Paecilomyces fumosoroseus*.

Time	Isolate	Length to width ratio	Morphological type (developmental stage)
0 h	EH-506/3	2.0[a]	conidia (I)
	EH-503/3	2.0[a]	conidia (I)
	EH-520/3	2.0[a]	conidia (I)
6 h	EH-506/3	2.1 (± 0.5)[a,b]	germinating conidia (III)
	EH-503/3	2.3 (± 0.4)[b]	germinating conidia (III)
	EH-520/3	1.9 (± 0.4)[a]	swollen conidia (II)
12 h	EH-506/3	8.5 (± 2.57)[b]	hyphal bodies, blastospores (V, VI)
	EH-503/3	16 (± 3.11)[c]	germinating conidia (III)
	EH-520/3	5 (± 5.16)[a]	germinating conidia (III)
18 h	EH-506/3	5.1 (± 2.16)[a]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.5 (± 2.11)[a]	hyphal bodies, blastospores (V, VI)
	EH-520/3	6.9 (± 6.5)[a]	hyphal bodies, blastospores (V, VI)
24h	EH-506/3	4.4 (± 1.5)[a]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.6 (± 1.7)[a]	hyphal bodies, blastospores (V, VI)
	EH-520/3	10.4 (± 7.4)[b]	hyphal bodies, blastospores (V, VI)
36 h	EH-506/3	4.4 (± 1.5)[a]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.6 (± 1.7)[a]	hyphal bodies, blastospores (V, VI)
	EH-520/3	10.4 (± 7.4)[b]	hyphal bodies, blastospores (V, VI)
48 h	EH-506/3	5.4 (± 2.3)[b]	hyphal bodies, blastospores (V, VI)
	EH-503/3	3.2 (± 1.2)[a]	hyphal bodies, blastospores (V, VI)
	EH-520/3	5.1 (± 2.5)[a]	hyphal bodies, blastospores (V, VI)
60 h	EH-506/3	5.5 (± 2.4)[b]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.1 (± 1.4)[a]	hyphal bodies, blastospores (V, VI), submerged conidia
	EH-520/3	4.6 (± 1)[a,b]	hyphal bodies, blastospores, (V, VI), submerged conidia
72 h	EH-506/3	4.8 (± 1.2)[a,b]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.3 (± 0.9)[a]	hyphal bodies, blastospores (V, VI), submerged conidia
	EH-520/3	5.5 (± 1.3)[b]	hyphal bodies, blastospores (V, VI), submerged conidia
96 h	EH-506/3	4.5 (± 0.9)[a]	hyphal bodies, blastospores (V, VI)
	EH-503/3	4.03 (± 0.81)[a]	hyphal bodies, blastospores (V, VI), submerged conidia
	EH-520/3	4.0 (± 1.1)[a]	hyphal bodies, blastospores (V, VI), submerged conidia

Values are representative of three independent experiments run in triplicate.
Values in the same column corresponding to the same time, marked with the same letter did not differ significantly according to Tukey´s test at a significance level of 5%.

Table 2. Total proteases, subtilisin-like (Pr1) and trypsin-like (Pr2) activity of *P. fumosoroseus* isolates with different virulence against whiteflies.

Isolates	Lethal median concentration (conidia/ml)	Total protease activity (UP/ml)			Subtilisin-like Pr1 activity (UPr1/ml)			Trypsin-like Pr2 activity (UPr2/ml)		
		0 h	120 h	216 h	0 h	120 h	216 h	0 h	120 h	216 h
EH-506/3	1.1 x 10^5	3.6[a]	62.4[c]	91[c]	66.0[a]	745.7[b]	620.4[b]	49.3[a]	23.4[a]	251.3[c]
EH-503/3	2.5 x 10^4	3.3[a]	2.7[a]	58.7[b]	150.6[c]	90.1[a]	391.1[a]	138.4[c]	109.0[c]	155.7[b]
EH-520/3	7.6 x 10^4	8[a]	8.7[b]	49[a]	94.0[b]	111.2[a]	347.4[a]	103.0[b]	95.4[b]	92.3[a]

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%.

not shown). A peak of *P. fumosoroseus* protease activity corresponding to 91 UP/ml was reached at 216 h of incubation, by the highly virulent EH-506/3 strain. The medium-virulence isolate (EH-503/3) showed a lower activity (58.7 UP/ml) at 216 h. The low virulence isolate (EH-520/3), on the other hand, exhibited a lower activity (49 UP/ml) at 216 h than the other tested isolates (Table 2). Total extracellular proteases were measured by the azocasein method [35] instead of by absorbance at 280 nm because *P. fumosoroseus* produces beauvericin [26], which contains phenylalanine [27] that interferes with absorbance at this wavelength.

The three MCs tested, expressed Pr1 and Pr2 activity under the experimental conditions used in this study. Some points showed significant differences, and these values are summarized on table 2.

At day 0, the three isolates showed Pr1 and Pr2 activity from 49.3 UPr2/ml to 150.6 UPr1/ml, suggesting that conidia contain proteolytic activity, as reported by Boucias and Pendland [6]. Significant differences were observed ($F_{2,24}$ =127.8, $p < 0.05$ for Pr1 and $F_{2,24}$ = 184.9, $p < 0.05$ for Pr2) at this time among the three MCs. For Pr1, at 120 h of incubation, this difference persisted between the highest-virulence MC (EH-506/3) and the other two MCs, but not between the low and medium virulence isolates ($F_{2,24}$ = 1631; $p < 0.05$), as well as at 216 h ($F_{2,24}$ = 120.4; $p < 0.05$) (Table 2). For Pr2, a significant difference was observed among the three MCs, at 120 ($F_{2,24}$ = 214.1, $p < 0.05$) and 216 h ($F_{2,24}$ = 208, $p < 0.05$).

For the three isolates, Pr1 activity varied between 66 and 192 UPr1/ml up to 96 h of incubation (data not shown). Pr1 activity of EH-506/3 started to rise, reached a major peak (745.7 UPr1/ml) at 120 h and a second, lower one (620.4 UPr1/ml), at 216 h. Pr1 activity of EH-503/3, on the other hand, started to rise after 168 h and reached a value of 496 UPr1/ml at the end of the experimental period (312 h, data not shown). The lowest Pr1 activity was shown by the low virulence EH-520/3 strain with a maximum of 347.4 UPr1/ml at 216 h.

In general, in all three isolates tested, Pr2 showed a much lower activity than Pr1. Broad-spectrum subtilisins are the main proteins produced by *M. anisopliae* and other entomopathogens during infection and degradation of the insect cuticle [38]. Different levels of production are signals of intraspecific variation among isolates, and could be associated with adaptive differences [2,16,39]. The differences observed here among isolates of the same species of *P. fumosoroseus* confirm previous studies with other fungi in which protease activity has been related to virulence [5,20,39,42].

Pr1 and Pr2 activity on the specific substrates used here have been reported in *M. anisoplie* isolates [38,39,41] but not in *P. fumosoroseus* propagules. In this study, Pr2 was induced at lower levels among isolates than Pr1. This phenomenon was also observed in *M. flavoviride* by Pinto et al. [28].

Subtilisins comprise the major protease components in entomopathogenic fungi, such as *Metarhizium* and *Beauveria* [2,39]. The number of Pr1 proteases produced by *M. anisopliae* and the slight differences in their catalytic activity is not only of great interest to the functional pathology but also to the population structure of species of this fungus [3]. This may also be valid for other entomopathogenic fungi as well. Results showed that the virulent MC (EH-506/3), had high Pr1 activity (745.7 UPr1/ml at 120 h; 620.4 UPr1/ml at 216 h), and the low virulence isolate (EH-520/3) had low Pr1 activity (111.2 UPr1/ml at 120 h; 347.4 UPr1/ml at 216 h) when compared with EH-506/3 (Table 2). As Pr1 appears to be a pathogenicity determinant by virtue of its considerable ability to degrade cuticle [41], our findings suggest that Pr1 production of the propagules of *P. fumosoroseus* could be incriminated as a virulence marker, and supernatant concentration measurements considered as a screening test for preliminary selection among several isolates. The selected isolates with high Pr1 activity could be used for pathogenicity bioassays. Studies of the induction-repression mechanism of these enzymes must be done to determine optimal production in conditions of low nutrimental availability, similar to the ones found when infecting the insect cuticle.

The intra-specific variability of virulence towards whitefly nymphs, propagule production, and Pr1 and Pr2 activity found among *P. fumosoroseus* isolates are relevant findings for the characterization and selection of isolates suitable for their use as biocontrol agents. Our results suggest MC EH-506/3 as the best candidate for whitefly control among the three studied MCs, due to its high virulence, Pr1 production (745.7 UPr1/ml at 120 h) of the propagules, and rapid transition to blastospores in submerged culture.

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