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Correlation between *pr1* and *pr2* Gene Content and Virulence in *Metarhizium anisopliae* Strains

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Metarhizium anisopliae is a widely studied model to understand the virulence factors that participate in pathogenicity. Proteases such as subtilisin-like enzymes (Pr1) and trypsin-like enzymes (Pr2) are considered important factors for insect cuticle degradation. In four *M. anisopliae* strains (798, 6342, 6345, and 6347), the presence of *pr1* and *pr2* genes, as well as the enzymatic activity of these genes, was correlated with their virulence against two different insect pests. The 11 *pr1* genes (A, B, C, D, E, F, G, H, I, J, and K) and *pr2* gene were found in all strains. The activity of individual Pr1 and Pr2 proteases exhibited variation in time (24, 48, 72, and 96 h) and in the presence or absence of chitin as the inductor. The highest Pr1 enzymatic activity was shown by strain 798 at 48 h with chitin. The highest Pr2 enzymatic activity was exhibited by the 6342 and 6347 strains, both grown with chitin at 24 and 48 h, respectively. Highest mortality on *S. exigua* was caused by strain 6342 at 48 h, and strains 6342, 6345, and 6347 caused the highest mortality 7 days later. Mortality on *Prosapia* reached 30% without variation. The presence of subtilisin and trypsin genes and the activity of these proteases in *M. anisopliae* strains cannot be associated with the virulence against the two insect pests. Probably, subtilisin and trypsin enzyme production is not a vital factor for pathogenicity, but its contribution is important to the pathogenicity process.

Keywords: Entomopathogen, enzymes, virulence, pathogenicity, protease, infection

Introduction

Metarhizium anisopliae is a deuteromycete that belongs to family Moniliaceae. Owing to the great number of insect species that it attacks, it has become an important entomopathogenic fungus. *M. anisopliae* has been studied widely as a model to understand virulence and pathogenicity processes against insect pests since many years ago. Such studies involve the relation with the host that includes adhesion, germination, appressorium formation, penetration, colonization, and pathogen release. An important part of the study of the initial pathogenic process has been focused on proteins with enzymatic activities that intervene in the degradation of the insect cuticle. It has been considered since past decades that the main *M. anisopliae* virulence factors lie in such proteins. In the penetration process, once the appressorium is formed, enzymes such as proteases, chitinases, lipases, phospholipases, esterases, phosphatases, and others degrade the insect cuticle. Such enzymes lead the way for the fungus to penetrate the insect cuticle and obtain nutrients from the hemolymph, causing an imbalance in the insect due to the production of different metabolites synthesized by the fungus [6, 7]. As insect cuticle contains a chitin filament matrix with lipids embedded in proteins, protease enzymes degrade or weaken the physical barrier between the insect and the environment [16].

Among many enzymes that participate in this process, proteases are considered the most important enzymes for cuticle degradation. Pr1 proteases, coded by *pr1* genes, are subtilisin-like enzymes, and Pr2 proteases, coded by *pr2*, are trypsin-like enzymes. These enzymes are two different types of proteases called isoforms as they can be distinguished

by analytical isoelectric focusing, their substrate specificity, and inhibition patterns. Both proteases are expressed during the first stage of the infection, which begins when conidia contact insect cuticle [16]. Pr1 is an enzyme that adsorbs to the cuticle *via* nonspecific electrostatic bonds through positively charged groups [15], with activity against Suc-Ala-Ala-Pro-Phe-pNa, which makes it more able to degrade cuticles. The other important protease is Pr2, a serine protease with specificity for basic residues. This enzyme cleaves preferentially at the carboxyl side of positively charged amino acids [17] and possesses activity against the substrate Bz-Phe-Val-Arg-Na, exhibiting less ability to degrade insect cuticles. This fact makes Pr1 the most active enzyme; however, both proteases can work synergistically in cuticle degradation [8].

It has been said that pathogenicity is related to the enzyme complex that could destroy the insect cuticle. Such cuticle-degrading enzymes are produced sequentially depending on the sequence of the cuticle components [14]. Moreover, even they can be produced according to the mechanisms of adaptation to hosts [12]. However, several reports indicate that there is no correlation between the ability of enzymes and cuticle degradation process, or between virulence and protease production [3, 4, 8, 13]. According to previous study [8], the entomopathogenic activity of *M. anisopliae* can be modulated in response to cuticle protein components and degree of sclerotization. In this study, several *M. anisopliae* strains were tested against two different insects, the spittlebug and the beet armyworm, in order to understand if the enzymatic activities of proteases Pr1 and Pr2 are related in some way to virulence towards these insect pests. It is noteworthy that fungal strains have been reported to be active against spittlebug, *Aeneolamia* sp., but have not been reported against *S. exigua*. The knowledge about the protease activity might give a certainty in choosing suitable strains to control insect pests efficiently.

Materials and Methods

Fungal Strains

Fungal strains were kindly provided by the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF). *Metarhizium anisopliae* strains isolated from grass spittlebug (*Aeneolamia varia*) corresponded to the following identification numbers: 798, 6342, 6345, and 6347. Strains 3019 and 3345 corresponded to *Beauveria bassiana*, and *Fusarium* sp., respectively. All fungal strains were activated in 250 ml Erlenmeyer flasks containing 50 ml of Sabouraud dextrose broth supplemented with 1% yeast extract and 25 µg/ml streptomycin to avoid bacterial growth. Flasks were incubated in a rotary shaker at 200 rpm and 28°C for 48 h.

Protease Gene Identification

For gene identification in *Metarhizium* strains, primers were designed to identify the *pr1* genes (A, B, C, D, E, F, G, H, I, J, and K) that express subtilisin-like proteins, and the *pr2* gene that expresses trypsin-like protein (Table 1). Strains 3019 and 3345 were used as negative controls. Primer design was done using the program Lasergene 8 DNAStar with the option PrimerSelect, from *M. anisopliae* gene sequences available in the National Center for Biotechnology Information (NCBI). Primers were manufactured by Bio-Synthesis, Inc.

DNA extraction from each fungal strain was carried out according to a previous protocol [9]. DNA (1 µl) was placed in a microcentrifuge tube containing 1.25 U of Go*Taq* DNA polymerase (Promega), 1× buffer Go *Taq*, 0.2 µM each primer, 0.2 mM dNTPs, and 14.8 µl of miliQ H_2O for a final volume of 25 µl. Amplification was accomplished with a thermal cycler (Gen Amp PCR System 9700, Singapore) with an initial denaturation cycle at 96°C for 2 min and a cycle program set for 30 cycles (with a cycle consisting of denaturation at 94°C for 45 sec, annealing at 60°C (for *pr1* genes), 55°C (for *pr2* gene), and 63°C for (*pr1*H and *pr1*I genes) for 1 min, and extension at 72°C for 1 min), and an extra step of extension at 72°C for 7 min. Following amplification, 1 µl of sample of each PCR mixture was mixed with 2 µl of SYBR Gold (Invitrogen) and electrophoresed on a 1.5% agarose gel in 0.5× TBE buffer at 80 V for 1 h. PCR products were visualized with UV light coupled to the Kodak Molecular Imaging Program.

Conidia Recovery

One milliliter of mycelia, taken from a 48 h culture grown in Sabouraud dextrose broth (Sigma-Aldrich) enriched with 1% yeast extract, was inoculated in the bottom of a Petri dish and covered with 20 ml of potato dextrose agar enriched with 1% yeast extract and supplemented with 25 µg/ml streptomycin. Mycelia and culture medium in each Petri dish were mixed by diffusion and incubated at 25°C for 21 days or until sporulation was observed. For conidial recovery, 1 ml of 0.05% Tween 80 was added to each Petri dish. The surface of the culture medium was scraped with a spatula, and the contents were recovered in glass bottles. The concentration from conidial homogeneous suspension was determined using a Neubauer chamber.

Total Protease Activity Determination

Approximately 2.5×10^6 conidia/ml of each strain were inoculated in 150 ml of Reese modified medium (0.02% glucose, 0.05% casein, 0.005% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$) and cultures were incubated at 37°C, 200 rpm. One milliliter of each culture was collected at 72 and 96 h and centrifuged at 14,000 ×*g* for 10 min. Then, 100 µl of supernatant was mixed with 200 µl of 1% casein in 200 mM glycine-NaOH (pH 9.0) and incubated at 37°C for 30 min. Reactions were stopped by adding 700 µl of 4%

Table 1. Primers used for *pr1* and *pr2* gene detection in *Metarhizium anisopliae*.

Gene	Primers	Sequence	Expected size (bp)
pr1A	SubPr1F	5´TGTCTGCCTTCTCACTCTTCTCC3´	1,421
	SubPr1R	5´AGCCAATCGCCGTCTCTTCACTG3´	
pr1B	Pr1BF	5´AGCGCCGGCGAAGGAACA3´	444
	Pr1BR	5´CGCCGCCACCGAGACAAAC3´	
pr1C	PR1CF	5´CACGCCCGATCTCAGCAAGTAT3´	321
	PR1CR	5´CTCAGAGCGCCCGCCAGTAATG3´	
pr1D	PR1DF	5`CAAATATCCGCGCCTCCAATGT 3`	800
	PR1DR	5`ACGCCCGTCCCTCAAACTCC 3`	
pr1E	PR1EF	5´TACCCTCGCGCCTCTTGAACTA3´	563
	PR1ER	5´TCGGAATATCGGTGTCGGTAAT3´	
Pr1F	PR1FF	5´TGCTACCGGGGAGGATGACT3´	446
	PR1FR	5´CTGGGGCAAAAATGGTGACA3´	
pr1G	PR1GF	5´GCCGGCACCGCTGATTATTC3´	472
	PR1GR	5´GGGGGTGCTTACGGGATGTG3´	
pr1H	Pr1H-m1F	5´ GCGGCTTCAAGGGCTATTCC 3´	483
	Pr1H-m1R	5´TGGCCGTTGCCATCCTCATCG 3´	
pr1I	Pr1I-m 1F	5´CCCGACAAGTATATTGTCAAGTA 3´	393
	Pr1I-m 1R	5´ATGTGCCTTCACCGGCACTGTC 3´	
pr1J	PR1JF	5´GACGCAGGCAGCACCAATGA3´	409
	PR1JR	5´CGGCCAGCCCAGCAACA3´	
pr1K	PR1KF	5´TCCCGGCTCCAGTCCTTGTT3´	402
	PR1KR	5´CTCGCCAGCGCTATCATCGTA3´	
pr2	TryPAR2F	5´CGGCTCGCATACCCTACG3´	439
	TryPAR2R	5´CAGCAGCAAAATCTCCTACTCAAA3´	

trichloroacetic acid and centrifuged, and the absorbance of the supernatants containing hydrolyzed casein was measured at 280 nm. A standard curve of tyrosine (0–200 µg/ml) was estimated to determine the protease units (PU). One PU was defined as the amount of enzyme required to release 1 µg of tyrosine/min [2, 10]. Results were submitted to ANOVA and Tukey's tests for mean comparison using the SPSS software ver. 10.0.

Pr1 and Pr2 Activity Determination

A suspension of 1×10^9 conidia/ml was used to inoculate 50 ml of basal medium (0.02% $MgSO_4$, 0.01% K_2HPO_4), and basal medium enriched with 1% shrimp chitin. Flasks were incubated in a rotary shaker, at 180 rpm, at 30ºC for 4 days. To determine protease activity 3 ml samples were taken every 24 h and filtered through a chiffon fabric. Samples were centrifuged at 1,425 ×*g* for 10 min at 4°C. Supernatants were used to determine enzymatic activity. Subtilisin and trypsin activities were assayed using the synthetic substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride, respectively. Each substrate (1 mmol) was mixed with 15 mmol Tris-HCl buffer (pH 8.5) and 0.1 ml of supernatant. The mixture was incubated at 28°C for 1 h, and the reaction was stopped by adding 0.25 ml of 30% acetic acid and placing on ice during 15 min. Absorbance at 410 nm was measured and the concentration of *p*-nitroaniline was determine. A standard curve of each substrate (0.1–0.5 mM) was estimated to determine protease units (PU). One protease unit (PU) was defined as the amount of enzyme required to release 1 mM of *p*-nitro aniline/min [5]. Results were transformed to square root (Y+ ½) and then submitted to ANOVA and Tukey's tests for multiple mean comparisons with $p \leq 0.05$, using the SPSS software ver. 10.0.

Laboratory Bioassays with *Prosapia* sp. Adults

Spittlebug adult individuals (*Prosapia* sp.) were collected from weeds surrounding sorghum crops located at the roadside of the Reynosa-San Fernando highway (50 km), Tamaulipas, Mexico, using a trapping net. All individuals were placed in plastic boxes (56 × 26 × 38 cm) and transported to the laboratory. Grass plants with root (family Poaceae) were extracted from soil with the help of a garden shovel and were placed in 500 ml expanded polystyrene containers. The roots were covered with soil that was then fully hydrated with water. Each plant was placed inside a wooden cage (30 × 30 × 30 cm) for bioassays.

In the laboratory, 6 groups of 10 adult individuals each were formed. Ten adult individuals were taken individually with a camel hair brush and placed inside a 100 ml plastic container with

screw cap. In this way, six containers were prepared for testing against fungal strains. Each group of 10 individuals was covered with 1 ml of a conidial suspension containing 10^9 conidia/ml, for 20 sec. After this, each individual was taken with a camel hair brush and placed on a paper towel to remove excess moisture. Each group was placed on the plant contained in the wooden cages. Bioassays were conducted for 7 days in the dark and at room temperature. At the end, dead insects were taken with dissecting forceps and placed in a humidity chamber consisting of a Petri dish covered at the bottom with filter paper moistened with 800 µl of sterile miliQ water. Humidity chambers were incubated at 25°C for 7 days until the observation of mycosis or emergence of conidia.

Laboratory Bioassays with *S. exigua* Larvae

S. exigua larvae were provided by the insect rearing area of the Environmental Biotechnology Laboratory CBG-IPN. Five milliliters of artificial diet [11] was poured into 30 ml plastic recipients and allowed to solidify. From each strain, 100 µl of a suspension containing 3×10^8 conidia/ml was placed on the surface of the diet and allowed to dry for 1 h. Later, one-first-instar larva was placed in a recipient using a camel hair ultra-thin round brush. For each strain, 25 insects were used, with three replicates, totaling 75 larvae. A control was conducted in the same way without conidial suspension. The bioassays were carried out in the dark and at room temperature, and mortality was recorded 7 days later. Mortality percentage was calculated.

Results

Protease Gene Identification

The 11 *pr1* genes (A, B, C, D, E, F, G, H, I, J, and K) and *pr2* gene were found in the four *M. anisopliae* strains analyzed. As expected, these genes were not found in the *B. bassisana* and *Fusarium* sp. strains, which were used as negative controls. The gene amplification results are shown in Fig. 1.

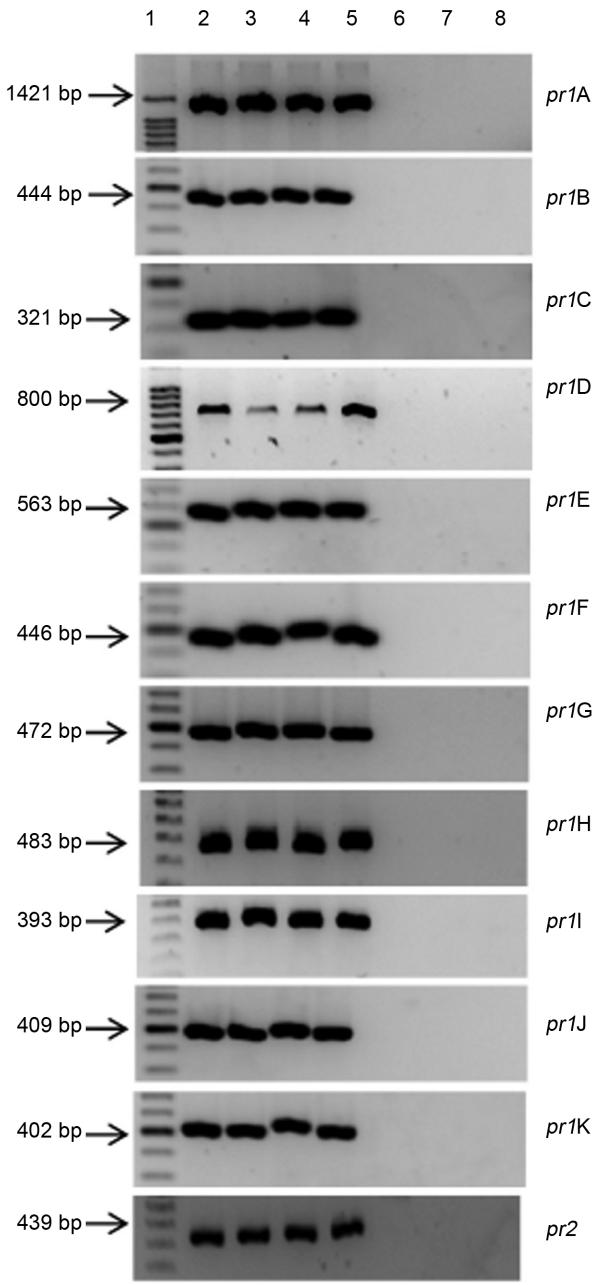

Fig. 1. PCR detection of protease-type subtilisin (*pr1*) and trypsin (*pr2*) genes in *Metarhizium anisopliae* strains.
Lane 1: molecular size marker (100 bp; Promega); lanes 2–5: *M. anisopliae* 798, 6342, 6345, and 6347 strains; lane 6: *Beauveria bassiana* 3019 strain (negative control); lane 7: *Fusarium* sp. 3345 strain (negative control); lane 8: water.

Total Protease Activity Determination

All fungal strains produced variable amount of proteases at the two recorded times. It was observed that protease production was increased at 96 h of fungal growth. The strains 798, 6342, and 6347 produced less protease units than strain 6345. In the first case, protease units were below 50 PU/ml at 72 and 96 h. The strain 6345 produced more than 90 PU/ml after 96 h of growth. Results are shown in Fig. 2.

Pr1 and Pr2 Activity Determination

Results indicated a statistically significant difference among the Pr1 enzymatic activity of the strains ($F = 3{,}160.92$, $df = 39/120$, $p \geq 0.05$) (Table 2). For Pr1, in almost all cases, the presence of chitin in the medium increased the enzymatic

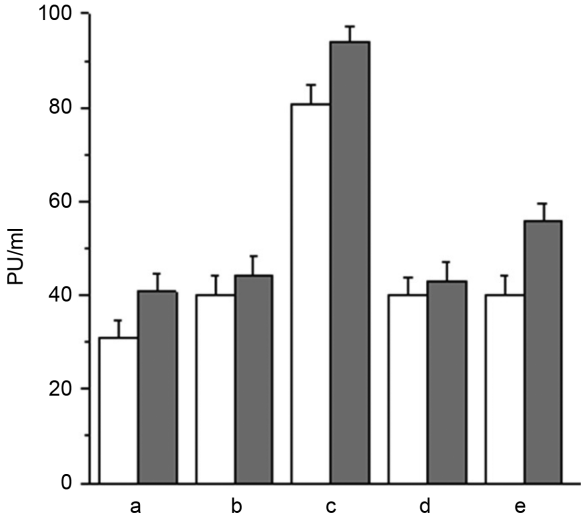

Fig. 2. Determination of total protease activity produced by *Metarizhium anisopliae* strains (a) 798, (b) 6342, (c) 6345, and (d) 634, and by (e) *Beauveria bassiana* 3019, during fungal growth at 72 h (white bars) and at 96 h (gray bars).

activity, but no effect was observed in 6342 strain at 72 h. The 798 strain exhibited the highest enzymatic activity in the presence of chitin at 48 h of fungal growth, followed by strains 6347 and 6345 under the same conditions. The lowest enzymatic activity was exhibited by strains 6342 and 3019 in the absence of chitin at 24 h (Fig. 3).

Pr2 results indicated a statistically significant difference among the enzymatic activities of the strains ($F = 7{,}349.85$, $df = 39/120$, $p \geq 0.05$) (Table 3). The presence of chitin increased the enzymatic activity in all cases. The highest enzymatic activity was exhibited by strains 6342 and 6347, both grown in the presence of chitin at 24 and 48 h, respectively. Lower enzymatic activity was exhibited by strain 798 in the presence of chitin at 48, 72, and 96 h, followed by strain 6345 at 96 h also in presence of chitin. The lowest enzymatic activity was shown by strain 6342 in the absence of chitin at 24 h (Fig. 3).

Laboratory Bioassays

Mortality caused by all *M. anisopliae* strains to adults of *Prosapia* sp. did not exceed 30%. On the other hand, mortality caused by all strains in *S. exigua* larvae was variable. The highest mortality was caused to *S. exigua* by strain 6342 at 48 h, reaching up to 73% mortality. Strain 6347 caused the lowest mortality at 48 h. Mortality above 85% was caused to larvae 7 days after by strains 6342, 6345, and 6347. Results are shown in Table 4.

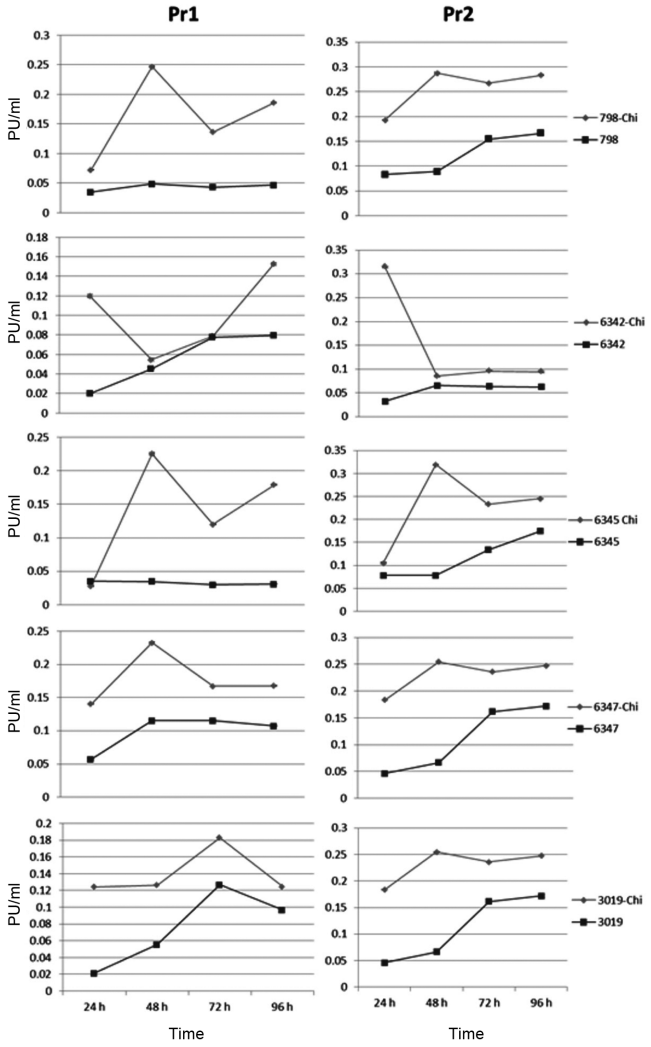

Fig. 3. Enzyme kinetics of Pr1 and Pr2 from *Metarhizium anisopliae* strains grown in basal medium and basal medium with chitin as inductor.

Pr1 enzymes were incubated with *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, and Pr2 enzymes were incubated with *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride for activity determination.

Discussion

This work intended to associate the content of *pr1* and *pr2* proteases genes of four *M. anisopliae* strains with their virulence against two different insect pests. Results indicated that all four strains harbor 11 *pr1* genes. This number of genes is reported so far as the largest existing number of subtilisins in *Metarhizum* [1]. This could represent an advantage in comparison with other strains such as *M. anisopliae* sf. *anisopliae* that possesses eight *pr1* subtilisin genes, and *M. anisopliae* sf. *acridum* that possesses

Table 2. Enzymatic activity of subtilisin-like protease Pr1 from *Metarhizium anisopliae* strains.

		Time (h)			
		24	48	72	96
Strain	CM	Mean ± SE[a]	Mean ± SE[a]	Mean ± SE[a]	Mean ± SE[a]
798	A	0.0348 ± 0.0007 q	0.0482 ± 0.0011 p	0.0429 ± 0.0012 p	0.0463 ± 0.0015 p
	B	0.0723 ± 0.0005 n	0.2465 ± 0.0017 a	0.1359 ± 0.0007 g	0.1856 ± 0.0009 d
6342	A	0.0200 ± 0.0004 s	0.0452 ± 0.0003 p	0.0775 ± 0.0010 mn	0.0794 ± 0.0004 m
	B	0.1196 ± 0.0017 ij	0.0546 ± 0.0014 o	0.0789 ± 0.0011 m	0.1526 ± 0.0014 f
6345	A	0.0349 ± 0.0011 q	0.0345 ± 0.0008 q	0.0300 ± 0.0003 qr	0.0307 ± 0.0008 qr
	B	0.0282 ± 0.0014 r	0.2251 ± 0.0012 c	0.1201 ± 0.0012 ij	0.1791 ± 0.0004 d
6347	A	0.0568 ± 0.0007 o	0.1153 ± 0.0010 j	0.1150 ± 0.0012 j	0.1072 ± 0.0005 k
	B	0.1402 ± 0.0009 g	0.2325 ± 0.0018 b	0.1672 ± 0.0004 e	0.1678 ± 0.0008 e
3019	A	0.0212 ± 0.0005 s	0.0553 ± 0.0012 o	0.1267 ± 0.0020 h	0.0966 ± 0.0007 l
	B	0.1244 ± 0.0010 hi	0.1266 ± 0.0012 h	0.1829 ± 0.0012 d	0.1245 ± 0.0009 hi

[a]Means with the same letter are not significantly different. SE (Standard error). Tukey's test for multiple mean comparisons, $p \leq 0.05$. CM = Culture medium: (A) basal medium, (B) basal medium enriched with shrimp chitin.

Table 3. Enzymatic activity of trypsin-like protease Pr2 from *Metarhizium anisopliae* strains.

		Time (h)			
		24	48	72	96
Strain	CM	Mean ± SE[a]	Mean ± SE[a]	Mean ± SE[a]	Mean ± SE[a]
798	A	0.0827 ± 0.0004 qr	0.0888 ± 0.0007 p	0.1546 ± 0.0027 l	0.1664 ± 0.0011 jk
	B	0.1929 ± 0.0007 g	0.2875 ± 0.0013 b	0.2671 ± 0.0008 c	0.2833 ± 0.0008 b
6342	A	0.0326 ± 0.0012 w	0.0656 ± 0.0009 t	0.0638 ± 0.0015 t	0.0624 ± 0.0005 t
	B	0.3147 ± 0.0016 a	0.0854 ± 0.0012 pq	0.0957 ± 0.0010 o	0.0947 ± 0.0019 o
6345	A	0.0476 ± 0.0005 v	0.0486 ± 0.0003 v	0.0554 ± 0.0009 u	0.0718 ± 0.0010 s
	B	0.0442 ± 0.0015 v	0.2555 ± 0.0010 d	0.2454 ± 0.0009 e	0.2685 ± 0.0003 c
6347	A	0.0780 ± 0.0008 r	0.0781 ± 0.0014r	0.1339 ± 0.0012 m	0.1747 ± 0.0017 i
	B	0.1051 ± 0.0005 n	0.3193 ± 0.0005 a	0.2329 ± 0.0007 f	0.2453 ± 0.0005 e
3019	A	0.0461 ± 0.0003 v	0.0663 ± 0.0003 st	0.1615 ± 0.0006 k	0.1718 ± 0.0011 ij
	B	0.1835 ± 0.0009 h	0.2546 ± 0.0007 d	0.2360 ± 0.0011 f	0.2475 ± 0.0006 e

[a]Means with the same letter are not significantly different. SE (Standard error). Tukey's test for multiple mean comparisons, $p \leq 0.05$. CM = Culture medium: (A) basal medium, (B) basal medium enriched with shrimp chitin.

Table 4. Mortality of adults of *Prosapia* sp., and larvae of *Spodoptera exigua* caused by different *Metarhizium anisopliae* strains.

Strain[a]	Mortality (%) 48 h		Mortality (%) 7 days	
	Prosapia sp.	*Spodoptera exigua*	*Prosapia* sp.	*Spodoptera exigua*
M. anisopliae 798	30	60	30	67
M. anisopliae 6342	30	73	30	87
M. anisopliae 6345	20	67	20	87
M. anisopliae 6347	20	36	20	85
B. bassiana 3019	50	41	50	84
Control	5	4	5	4

[a]ARSEF.

only three genes [7]. It is expected, as mentioned by Freimoser *et al*. [7], that a larger number of subtilisins genes would imply different biological properties and function; however, although all of them are present in the *M. anisopliae* genome, we cannot assume that all of them are functional.

The total protease activity showed variations among the strains, but the 6345 strain showed the highest activity in both times recorded. Of course, this whole activity involves production of more and different enzymes, such as the proteolytic enzyme complex, which includes protease, aminopeptidase, and carboxypeptidase, whose production in culture begins before 24 h and increases rapidly after 48 h [14]. The activity of individual Pr1 and Pr2 proteases exhibited variation in time and in the presence or absence of inductor. These activities varied in a range of 0.0200 to 0.2465 U/ml for Pr1 and 0.0326 to 0.3147 U/ml for Pr2. A narrow range of enzymatic activity for Pr1 and Pr2 was observed. A possible comparison can be done with *Beauveria bassiana* isolates that exhibit higher values of enzymatic activity [5] than our strains; however, the enzymes are different. An important fact in this study is that the activity of Pr1 and Pr2 increased when strains were grown in minimal media supplemented with chitin. On the other hand, variation in mortality results at 48 h was observed for both insect species, and such variation remained the same for *Prosapia* and increased for *S. exigua* 7 days later. A low mortality was observed for *Prosapia* individuals, although all *M. anisopliae* strains tested in this study were recovered from the host *Aeneolamia varia*, which belongs to the same family and order as for *Prosapia*. These facts demonstrate that *S. exigua* is highly susceptible to these *Metarhizium* strains, even to *Beauveria bassiana*, which does not possess the same subtilisins or trypsin genes. The variation in mortality may be related to cuticle composition or susceptibility to protease action [3]. Strain 798 showed the highest enzymatic activity at 48 h, and the mortality caused at 48 and 72 h was lower than that of strain 6342. This strain showed its highest enzymatic activity until 96 h of growth in chitin and its biological activity was comparable to that of the 6345 strain, even though it is included among the highest enzymatic activity strains. Previous studies conducted for overexpression of genes coding for protease or even the production of fusion proteins have evidenced reduction in LT_{50} and LC_{50} [6, 17]. Thus, proteases as virulence factors should play an important role in the pathogenicity process. Although many studies indicate that protease genes are involved in the infection process, the presence and the number of subtilisin and trypsin genes and their proteases in the *M. anisopliae* strains in this work cannot be associated with virulence against the two insect pests. Probably, subtilisin and trypsin enzyme production is not a vital factor for pathogenicity, but its contribution is important. More studies on the particular participation of each gene are now being conducted in order to understand their expression during the pathogenic process.

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