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Pathogenicity of *Beauveria bassiana* and production of cuticle-degrading enzymes in the presence of *Diatraea saccharalis* cuticle

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The sugarcane borer, *Diatraea saccharalis,* is one of the worst pests in Brazilian sugarcane crop, causing high levels of financial losses every year. *Beauveria bassiana* is an entomopathogenic fungus widely used in the biological control of several agricultural pests. The aims of this study were to: (1) evaluate the pathogenicity of *B. bassiana* strains against *D. saccharalis* (2) investigate the production of proteases and chitinase by *B. bassiana* in the presence of the cuticle of sugarcane borer; and, (3) evaluate the relation between the production of enzymes and pathogenicity of the strains. All isolates tested were pathogenic to *D. saccharalis* and the mortality ranged from 36 to 88%. The production of enzymes was higher in the medium containing cuticle, showing that the process is stimulated by specific components found in the cuticle of the host. Pr1 activity was higher than Pr2 and both were produced at 24 h. The highest production of chitinase was obtained at 96 h of culture for all strains tested. Levels of specific cuticle-degrading enzymes such as proteases correlated positively with specific virulence parameters. *B. bassiana* URM2915 showed promising features to be used in a biological control program of *D. saccharalis*.

Key words: Biological control, sugarcane, subtilisin-like protease, trypsin-like protease, chitinase.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important source of sugar and alcohol in the Brazilian economy. However, part of the crop is lost due to the action of a number of insect pests, of which the borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) is one of the most important (Gallo et al., 2002; Oliveira et al., 2008). Due to its cryptic lifestyle, conventional control measures by deploying chemical insecticides targeted at the larvae are ineffective.

An alternative to chemical control is the use of entomopathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin. These fungi are widely used as biocontrol agents for a number of insect pests (Lacey, 2001; Hajek and Delalibera, 2010) and the efficiency of *B. bassiana* has already been proven against a number of Lepidoptera, such as *Castnia licus* Drury (Alves et al., 2002), *Ostrinia nubilalis* Hübner (Lewis et al., 2002), *Plutella xylostella* L. (Silva et al., 2003), *Spodoptera frugiperda* Smith (Vijayavani et al., 2009) and

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Table 1. Mortality (%) of *Diatraea saccharalis* larvae at seven days after application of different strains of *Beauveria bassiana*.

Access number	Insect host of origin	Site of origin	Mortality[a] (%) ± SD
URM2912	*Cyclonella sanguinea* (Coleoptera)	Paraná/BR	42 ± 3.42c
URM2915	*Nezara viridula* (Hemiptera)	Paraná/BR	88 ± 3.8a
URM2916	Coleoptera	Brasília/BR	76 ± 4.2ab
URM2920	*Anticarsia gemmatalis* (Lepidoptera)	Brasília/BR	68 ± 4.5b
URM2921	*Lebia concinna* (Coleoptera)	Paraná/BR	84 ± 5a
URM2923	*Deois flavopicta* (Homoptera)	Paraná/BR	76 ± 4.7ab
URM2924	*Deois flavopicta* (Homoptera)	Brasília/BR	36 ± 3.5d
URM2930	*Euschistus heros* (Hemiptera)	Paraná/BR	79 ± 5 ab
URM3447	*Castnia licus* (Lepidoptera)	Pernambuco/BR	86 ± 4.5 a
URM4548	*Diabrotica speciosa* (Coleoptera)	Buenos Aires/ARG	46 ± 5.1 c
Control			5.4±1e

[a]Mean followed by the same letter are not significantly different in the Tukey test at 5% probability. Original data, for statistical analysis were transformed into arcsine (X); SD: standard deviation.

Thaumetopoea pityocampa Den. & Schiff (Sevim et al., 2010). Entomopathogenic fungi have a number of determinants of pathogenicity, including production of cuticle-degrading enzymes, such as proteases, chitinases and lipases (Bidochka and Khachatourians, 1987). These enzymes are pointed out as important in the infection process, since they have already hydrolyzed polymer protein and chitin complexes, the major components of the insect's cuticle (St. Leger et al., 1986).

The best model to determining the level of pathogennicity in entomopathogenic fungi is based on a protease of the subtilisin-like, called Pr1, first studied in *M. anisopliae* by St. Leger et al. (1988). A trypsin-like enzyme (Pr2) belonging to the serine protease group also occurs during the early stages of cuticle colonization suggesting that it plays a hole in degrading extracellular proteins complementary to that of Pr1 (St. Leger et al., 1996).

Gupta et al. (1994) have shown a correlation between the production of high levels of chitinases and proteases and the virulence of *B. bassiana* against *Gallera mellonela* L. and *Trichoplusia ni* Hubner. In addition, Fang et al. (2005) proved that an overexpression of a chitinase gene (Bbchit1) enhanced the virulence of *B. bassiana* to aphids (*Myzus persicae* Sulzer), compared with a wild-type strain.

Only a few papers have focused on the production of these enzymes by *B. bassiana* strains on the presence of host's cuticle. Campos et al. (2005) have detected the presence of chitinases and proteases in the cuticle of *Boophilus microplus* Canestrini. In addition, Dias et al. (2008) and Montesinos-Matías et al. (2011) evaluated the production of Pr1 after growing the fungus in the presence of coffee-borer cuticle *Hypothenemus hampei* Ferrari and *Tenebrio molitor* L., respectively. These works suggest that the enzymes from *B. bassiana* were expressed differently according to the type of insect cuticle and there are not reports about the production of Pr1, Pr2 and chitinases in medium supplemented with *D. saccharalis* cuticle, important pest in sugarcane. The pathogenicity of *B. bassiana* strains were studied in order to verify if there is a correlation between the production of these enzymes and the virulence, contributing to the understanding of the parasite-host relation and to the selection of *B. bassiana* strains for biological control of *D. saccharalis*.

The objectives of this study were: (1) evaluate the pathogenicity of *B. bassiana* strains against *D. saccharalis* (2) investigate the production of proteases and chitinase by *B. bassiana* in the presence of the cuticle of sugarcane borer; and, (3) evaluate the relationship between the production of enzymes and pathogenicity of the strains.

MATERIAL AND METHODS

Diatraea saccharalis larvae

The second instar larvae of *D. saccharalis* were obtained from the Sugarcane Experimental Station of Carpina/Federal Rural University of Pernambuco/Brazil and were maintained with artificial diet, which basically consists in a solution of vitamins, sugar, soy meal, wheat germ, ascorbic acid and water, according to the protocol of Hensley and Hammond (1968). However, 24 h before the bioassays, each larva was confined individually in plastic containers (17 x 21 x 25 cm) with sugarcane stalks as the food source.

Reactivated inoculum

The *B. bassiana sensu latu* strains isolated originally from different hosts were supplied by Micoteca URM (University Recife Micology/UFPE) (Table 1). To reactivate the strains, they were inoculated in Petri dishes containing potato dextrose agar plus chloramphenicol (0.05% v/v), supplemented with 0.5% of yeast extract (PDAY) and incubated at 26°C for 12 days for conidiation. Following incubation, conidia harvest were prepared in 0.01% v/v Tween 80 in sterilized distilled water and were sprayed through the

use of micro-atomizer brand Paasche "VL" on ten *D. saccharalis* until insect death. Newly emerged conidia from the insect were subcultured not more than four times, at ten days intervals in PDAY and used to prepare the reactivated inoculum suspension containing 10^8 conidia/ml (Ito et al., 2007). To confirm viability, the conidia were spread on PDAY and incubated for 16 h at 26°C. Germination rates were scored at 400 × magnification by observing under microscope, at random, 100 conidia for the presence of germ tubes. Germination was at least 90% throughout the study.

Screening bioassay protocol

The basic measure of virulence generated in this study was the mortality recorded seven days post-inoculation. To assess infection, *D. saccharalis* larvae were placed in a sterile Petri dish (9 cm in diameter) and then sprayed with 1 ml of the reactivated inoculum suspension, as per as per reported in item Reactivated inoculum. The control group was sprayed, with sterile water containing 100 µl of Tween 80 (Tefera and Pringle, 2003). The design was completely randomized, with 11 treatments (10 strains of *B. bassiana* + Control).

For each treatment, five containers were used, each containing 10 larvae. The observations were taken daily for a period of seven days and larvae died were removed daily. To confirm the mortality by the fungus, the dead larvae were immediately surface sterilized with 70% alcohol for 10 s, followed in 1% sodium hypoclorite for 3 min and three rinses with sterile distilled water, placed on sterile wet filter paper in sterile Petri dishes. Mortality due the fungus was confirmed by microscopic examination of hyphae and spores on the surface of the cadaver.

Enzyme assays

B. bassiana strains that showed higher mortality against *D. saccharalis* (Table 1) and higher conidia production were selected for testing to enzyme production. A suspension containing $1x10^6$ conidia/ml was inoculated in 30 ml of the minimal liquid medium (MM) (Pontecorvo et al., 1953) as control and was inoculated in MM+ cuticle (MM + CUT): MM lacking a source of nitrogen and of glucose but with the addition of cuticle from larvae of *D. saccharalis* (0.5%) w/v. The larvae were dissected with a scalpel under an ocular for removal of the viscera and the cuticle was then oven-dried at 65°C for 1 h. After that, the cuticle was prepared using a solution of 0.2 M potassium tetra-borate and sterilized with water vapor without pressure. After, it was added to previously sterilized MM (121°C for 15 min) and again sterilized with water vapor without pressure for 5 min (Dias et al., 2008).

Cultures were incubated at 28°C and maintained under agitation (180 rpm) out to 24, 48, 72 and 96 h. After incubation, each culture was centrifugated at 8000 *g* for 15 min at 4°C to separate the mycelium and the supernatant obtained was stored at -20°C for enzyme assays. The design was completely randomized, in a factorial scheme 3 x 4 (3 strains x 4 incubation times), for a total of 12 treatments, with two repetitions. The subtilisin-like (Pr1) and trypsin-like (Pr2) activities were assayed using N-Suc-$(Ala)_2$-Pro-Phe-p-nitroanilide and N-benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma, St. Louis, MO) as substrates, respectively. Each assay consisted of 0.05 ml 1 mM substrate, 0.1 ml enzyme sample, and 0.85 ml 15 mM Tris-HCl, pH 8.0. The mixture was incubated for 30 min at 28°C and the reaction was stopped by adding 0.25 ml of 30% acetic acid and left to stand for 15 min in ice, after which samples were centrifuged at 1250 g for 5 min at 4°C. The supernatants were compared at 410 nm. One unit of enzyme activity (U) was defined as the amount of the enzyme able to release 1 µm of nitroanilide (NA) per milliliter per second at 28°C (Donatti et al., 2008).

Chitinase activity was assayed by the method of Nahar et al. (2004). The reaction mixture containing 1 ml of 1% colloidal chitin and 0.5ml of supernatant solution was incubated for 1 h at 50°C and the reaction was stopped by immersion in a bath of boiling water for 1 min. After centrifugation, 250 µL from the supernatant fluid was incubated with 50 µl of 0.8M sodium tetra-borate, pH 8.0 at 100°C for 3 min. After the mixture was cooled, 1.5 ml of p-dimethyl aminobenzaldehyde (DMAB) solution (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% v/v hydrochloric acid) was added and the mixture was incubated for 20 min at 37°C. Absorbance at 585 nm was measured against water as a blank. One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 µmol of *N*-acetylglucosamine per min under the conditions described above.

Statistical analysis

Percent mortality was corrected for control mortality (Schneider-Orelli, 1947) and normalized by arcsine (X) transformation before being subjected to analysis of variance (ANOVA). The Tukey test analysis was used to separate the means as a post-ANOVA procedure ($p<0.05$). Enzyme production data were subjected to the analysis of variance (ANOVA). The averages of the characteristics evaluated in response to the qualitative factor (strains) were subjected to Tukey test at 5% probability. These statistical analyses were performed using the ASSISTAT 7.5 beta software (Silva and Azevedo, 2002). Pearson's Correlation Coefficient was used to check the level of correlation between mortality caused by each strains and enzyme activity and it was calculated using enzyme's production at 96 h and mortality rate at 96 h post-inoculation. The analysis was performed using BioEstat software system, version 5.3.

RESULTS

Screening bioassay

Results of the screening assays are presented in Table 1. Mean mortality in the control was 5.4% seven days after the treatment. All the fungal isolates were pathogenic to larvae of *D. saccharalis* and there were significant differences in mortality between fungal isolates (F=75.26; df: 10; $p<0.01$). The mortality ranged from 36 to 88% (Table 1). Three strains caused mortality above 80% (URM2915, URM2921 and URM3447), while the URM2924, URM2912 and URM4548 caused below 50%. *B. bassiana* URM2915 is most indicated for the control of *D. saccharalis* because besides causing high mortality also showed good conidial production, fundamental characteristics to be used in programs for biological pest control.

Production of subtilisin-like and trypsin-like enzymes and chitinase in the presence of the cuticle of sugarcane borer

Based on the mortality rate (Table 1) and characteristics, like the production of conidia (data not shown), three strains were selected for production testing of Pr1, Pr2 and chitinase. The production of these enzymes was determined after the growth of the strains (URM2915,

Table 2. Factorial analysis of the strains, culture media and incubation times in the production of proteases (Pr1 and Pr2) and chitinases by *Beauveria bassiana* in the presence of *Diatraea saccharalis* cuticle.

Factor	Pr1			Pr2			Chitinase		
	df[a]	Mean square	*F*	*df*	Mean square	*F*	*df*	Mean square	*F*
Strains (S)	2	78.77	2.55*	2	0.357	0.45*	2	46.68	27.81*
Culture media (CM)	1	2020.5	654.82*	1	6441.5	8257.4*	1	2062.5	1228.7*
Time (T)	3	3881.5	125.82*	3	556.5	713.45*	3	318.55	189.77*
S × CM	2	238.8	7.74*	2	0.35	0.45*	2	52.27	31.13*
S × T	6	13.97	0.45	6	4.75	6.09*	6	2.89	1.72
CM× T	3	2490.9	80.74*	3	556.55	713.4*	3	287.60	171.3*
S × CM × T	6	35.27	1.14	6	4.75	6.09	6	2.29	1.36

*Significant to the 99% level of probability ($p < 0.01$); [a]*df*: degree of freedom.

Table 3. Subtilisin-like (Pr1) activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)[a]

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	1.53±0.04	0	46.83±0.08	0.83±0.02	68.83±0.19	2.92±0	70.79±0.19
URM2930	0	0.55±0.01	0	45.16±0.2	0	73.74±0.12	3.4±0.01	70.46±0.04
URM4548	0	1.04±0.02	15.26±0.24	40.70±0.57	15.89±0.17	68.70±0.40	17±0.26	66.70±0.25

[a]The Pr1 activities are means standard errors of the means based on three replicates.

Table 4. Trypsin-like (Pr2) activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)[a].

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	1.95±0.01	0	26.83±0.06	0	30.04±0.01	0	35.12±0.04
URM2930	0	1.46±0.04	0	25.71±0.08	0	30.59±0.07	0	34.71±0.04
URM4548	0	6.41±0.01	0	25.16±0.14	0	29.34±0.04	0	30.66±0.00

[a]The Pr2 activities are means standard errors of the means based on three replicates.

URM2930 and URM4548) in liquid medium in the presence and absence of cuticle of *D. saccharalis*. All strains of *B. bassiana* produced Pr1 and Pr2 in MM+CUT and there were significant differences between the strains (Table 2). For each of the three strains, Pr1 activity was detected at 24 h of growth. For URM2930 and URM4548, the peak of activity was observed at 72 h of growth. In the MM, Pr1 activity was detected at 48 h for URM4548, at 72 h for URM2915 and at 96 h of incubation only for the *B. bassiana* URM2930 (Table 3). Similarly, PR2 activity was detected at 24 h of incubation in the MM + CUT, and the peak of the activity was observed at 96 h. It was not possible to detect activity in the medium containing nitrate as the sole nitrogen source (MM) (Table 4). As shown in Tables 3 and 4, the strains studied showed higher levels of production of Pr1 than Pr2, highlighting URM2930 in production of Pr1 (73.74 U/ml) and URM2915 in production of PR2 (35.12 U/ml).

Chitinase activity was detected at 24 h of incubation on MM+CUT and at 72 h on MM for URM2915 and URM4548. The amount of secreted enzymes varied between the stains and the highest activity was observed for *B. bassiana* URM2915 strain (28.93U/ml) at 96 h. Enzyme activity was investigated for a period of 96 h and the highest chitinase activity was observed on the last time in the two culture media (Table 5). However, the chitinase activity was higher in MM + CUT at all incubation times compared to activities on MM.

Table 5. Chitinolytic activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)[a].

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	3.70±0.2	0	14.21±0.4	0.17±0	22.2±0.7	0.59±0.0	28.93±0.1
URM2930	0	0.17±0.0	0	10.01±0.4	0	16.73±0.1	0	22.2±0.2
URM4548	0	0.17±0.0	0	4.54±0.1	1.01±0.02	15.05±0.1	1.01±0.01	22.2±0.2

[a] The chitinase activities are means standard errors of the means based on three replicates.

There was significant interaction among the strains and culture media in the production of the enzymes and there were also interaction between culture media and incubation time in the production of proteases and chitinase (Table 2). These interactions were caused by the fact that enzyme activity was highest in medium containing cuticle and increased with the length of incubation. Correlation analysis between the percentage of mortality by three *B. bassiana* strains and Pr1(r =0.8207; P=0.0453) and Pr2 (r =0.8133; P=0.049) activity suggests a positive correlation of these variables in the medium containing cuticle. However there was no correlation with the chitinase activity (r=-0.0008; P=0.9987).

DISCUSSION

Although all the 10 fungal isolates tested were pathogenic to *D. saccharalis*, there were significant variations amongst the isolates. These variations have been reported in many arthropod pests (Bugeme et al., 2009; Godonou et al., 2009; Abood et al., 2010; Sevim et al., 2010) and emphasizes the need of screening for strain selection. The results were similar to those of Wraight et al. (2010). These authors evaluated virulence of *B. bassiana* against different species of Lepidoptera and observed that mortality was high and varied positively among the 43 isolates used, thus showing the potential of the fungus against different pests. Kaur and Padmaja (2008) evaluated the action of 23 *B. bassiana* isolates, obtained from different hosts and regions, against *Spodoptera litura* Fabricius and verified that pathogenicity varied among them, but there was no correlation between the variability and the host's geographic origin, as observed in this work.

The insect cuticle forms an effective barrier against organisms lacking an active cuticle penetration mechanism (virus, bacteria and protozoa). Only entomo-pathogenic fungus can penetrate their hosts through the cuticle, using physical and/or enzymatic mechanisms (St. Leger, 1995). In this paper was studied the production of the enzymes involved in the process of infection, in three strains of *B. bassiana* in the presence of *D. saccharalis* cuticle. We observed that when cuticle was added to the medium, there was an increase in production of Pr1 and Pr2, indicating that this was stimulated by cuticle components. By contrast, Tiago et al. (2002) demonstrated that *M. flavoviride* CG423 (syn. *M. anisopliae* var. *acridum*) has high levels of Pr2 only in minimal liquid medium without cuticle, with other negative effects being found in the presence of cuticle of *Schistocerca pallens* Thunberg.

In this study, production of Pr1 was higher as compared to Pr2, however the peaks of activity were the same (85 h), suggesting that there are different regulatory systems for these enzymes. Donatti et al. (2008) emphasized that the differences in the production of enzymes may reflect different functions in the infection process. In addition, both proteases were produced at 24 h of incubation in medium containing cuticle, suggesting their expression is not coordinated in this fungus. Similar data was observed in *B. bassiana* grown in *H. hampei* cuticle (Dias et al., 2008). Differently, Paterson et al. (1994) reported that Pr2 occurs before and would be involved in the activation or induction of Pr1 in *M. anisopliae* var. *anisopliae*. Differences between fungus species reveal that a complex mechanism is involved in the production of cuticle-degrading proteases.

Chitinase production by *B. bassiana* URM2915, URM2930 and URM4548 was higher during the last days of incubation in the medium containing cuticle as the sole source of carbon. The enhancing effect of cuticle on chitinase production suggests that this enzyme may be specifically induced by a cuticular component.

Studies have evaluated the effects of different sources of carbon in the production of this enzyme and observed that when the fungus was grown in medium with cuticle and glucose, production was lower than in medium containing only cuticle (Campos et al., 2005), and that there was repression of the enzyme when a easily available carbon source, glucose, was added to the medium (Dhar and Kaur, 2010). Chitinase production was lower than Pr1 and PR2 production, which can be explained by the composition of the insect's cuticle, a composite material consisting of arrangements of highly crystalline chitin nanofibers embedded in a matrix of protein (Vincent and Wegst, 2004). As reported by Fang et al. (2009), *B. bassiana* transformants secreting the fusion protein (protease and chitinase gene) penetrated the cuticle significantly faster than the wild type or transformants overexpressing either chitinase or protease

gene.

As reported in the literature, the production of cuticle-degrading enzymes is one of the pre-requisites for fungal infection (Mustafa and Khaur, 2010), and demonstrates the relation between production and virulence that has been targeted by several investigations. The current study has provided evidence for the relationship between enzyme production and virulence of *B. bassiana* against *D. saccharalis*. According to our results, Gupta et al. (1994) showed that levels of production of Pr1, Pr2 and NAGase (chitinase) have been related to virulence parameters in *B. bassiana*.

Moreover, Kim et al. (2010) showed that from the bioassay with the enzyme-inhibited supernatants processed by substrate inhibition, decreased aphicidal activities were observed for all three enzyme-inhibited treatments. This finding provides evidence that the enzymes (Pr1, Pr2 and most particularly the chitinase) in the supernatant of *B. bassiana* were strongly involved in the aphicidal activity.

Pelizza et al. (2011) screened 28 isolates of *B. bassiana* and nine isolates of *M. anisopliae* for chitinase production in solid medium and the results suggest a direct relationship between a high chitinolytic activity and an efficient virulence of the fungal strain against the tested insect pest (*Tropidacris collaris* Stoll). On the other hand, Silva et al. (2005) tested the larvicidal effect of *M. anisopliae* isolates against *Aedes aegypti* L. and the isolates showed a high variability of total protein production and NAGase activity after 48 and 72 h incubation in MM, but no relationship between enzyme levels and insecticidal activity could be detected, suggesting that other factors may be involved in the process.

The results presented in this study reveal that laboratory bioassays are a relevant stage in selecting the most efficient strains for controlling pests. The pathogenicity of *B. bassiana* can be influenced by strain used. These studies confirm that the production of cuticle-degrading enzymes by *B. bassiana* is influenced by specific components of the cuticle of *D. saccharalis*. This paper showed that there was a relationship between enzyme production and pathogenicity of *B. bassiana*, but many factors interfere directly in this process and more research is needed about this complex mechanism.

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