CHARACTERIZATION AND PHENETIC ANALYSIS OF GEOGRAPHICAL ISOLATES OF *BEAUVERIA* SPP.[1]

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ABSTRACT – Nine pathotypes fo *Beauveria bassiana* (Bals.) Vuill. and one pathotype of *B. brongniartii* (Sacc.) Petch were characterized according to extracellular enzyme production, growth rate on different culture media, conidial dimensions, conidiogenesis *in vitro*, isoenzyme variation, and virulence toward *Diatraea saccharalis*. Biological differences were found among isolates; only one isolate showed lipolytic activity. Two isolates had limited growth at three levels of temperature. LT_{50} values ranged from 5.9 days (virulent) to more than 8 days (non virulent). Electrophoretic analysis from α and β - esterases were performed to differentiate isolates. Pair-wise coefficients of similarity calculated from these parameters were used to construct phenograms in order to estimate the phenetic distance among the various fungal pathotypes. Isolates from the same regions had high coefficients of similarity, suggesting commonality within geographic populations.

Index terms: pathotype, microbial control, virulence, isozyme, entomopathogenic fungi.

CARACTERIZAÇÃO E ANÁLISE FENÉTICA DE ISOLADOS GEOGRÁFICOS DE *BEAUVERIA* SPP.

RESUMO – O objetivo deste trabalho foi caracterizar nove patótipos de *Beauveria bassiana* (Bals.) Vuill. e um patótipo de *B. brongniartii* (Sacc.) Petch, considerando produção de enzimas extracelulares (amilase, lipase e protease), taxa de crescimento em diferentes meios de cultura, dimensões dos conídios, conidiogênese *in vitro*, variação de isoenzimas e virulência para *Diatraea saccharalis*. Diferenças biológicas entre os isolados foram encontradas. Somente um isolado apresentou atividade lipolítica. Dois isolados apresentaram crescimento limitado em três níveis de temperatura. Os valores de Tl_{50} variaram de 5,9 dias para o isolado mais virulento, a 8 dias ou mais para os isolados menos virulentos. Outra característica utilizada para diferenciar isolados foram os padrões eletroforéticos dos sistemas de α e β esterases. Foram calculados coeficientes de similaridade, que permitiram a elaboração de fenogramas, com a finalidade de estimar a distância fenética entre os patótipos. Os isolados provenientes de uma mesma região tiveram altos coeficientes de similaridade, sugerindo variações entre as populações geográficas.

Termos para indexação: patótipos, controle microbiano, virulência, isoenzimas, fungos entomopatogênicos.

INTRODUCTION

The hyphomycete, *Beauveria bassiana* occurs frequently in many agroecosystems infecting a wide range of insects. In soybean grown in Argentina and Brazil, this fungus regulates populations of different coleopterous insects of the genera *Diabrotica, Colaspis,* and *Maecolaspis.* In Brazil, the fungus achieves high prevalence in populations of *Aracanthus*, an important pest of beans. Genetic diversity in this fungus has been reported by several authors (Tigano, 1985; Poprawski et al., 1988; Paccola-Meirelles, 1988; McCoy & Boucias, 1989). Therefore, it is important to identify the biochemical and physiological variability among strains, to select the best pathotypes with adaptive characteristics favouring its survival and effectiveness as a mycoinsecticide.

The purpose of this study was to determine variability within and among isolates of *Beauveria* spp., based on enzyme production,

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growth rate on different culture media, conidia dimensions, conidiogenesis *in vitro*, isoenzyme variation and virulence.

MATERIALS AND METHODS

Nine isolates of *B. bassiana* and one of *B. brongniartii* were compared biochemically and physiologically in the following ways: conidial size, extracellular enzyme production, growth rate and conidia production on different solid media at four temperatures

Isolates

The fungi were isolated from the respective host listed in Table 1. All isolates were subcultured no more than 3 times and then stored in skim milk adsorved on silica gel. The original inoculum was obtained after transferring the silica with fungi onto potato-dextrose-agar + yeast extract (PDA + Y) media.

Conidia size and radial colony growth rate

Conidia length and width were determined by preparing slides with spores mounted in glycerine and examining 50 conidia using an optical microscope (1000 x) with an Olympus micrometer with a barrel. To determine the radial growth rate of the fungus, six colonies of each isolate were inoculated, at three equidistant points per Petri dish, on PDA inverted plates at 22, 26, 30 and 34°C for 6 days. Spores obtained from the same colonies were removed by scrapping with a brush and agitation in water with 0.05% Tween 80, following determination of spore concentration with an hemocytometer.

Extracellular enzyme production

Extracellular production of amylase and lipase (Hankin & Anagnostakis, 1975) and protease (Gabriel, 1968) was assayed using agar plate techniques. Enzyme activity was expressed as the halo-diameter / colony-diameter ratio after incubation at 26°C for 6 days. Although the enzymatic index has been extensively used Rosato et al., 1981; Sosa-Gómez & Alves, 1983; Samuels et al. (1989), it does not correctly represent the enzymatic activity, because fungi with different growth rates may exhibit similar indices. Therefore, the halo diameter ratio was used (Rajamani & Hilda, 1987; Samad et al., 1989). Data were analyzed by ANOVA and means compared using the Tukey test ($P < 0.05$).

Enzyme preparations

Initially the isolates were grown on a complex cul-

TABLE 1. Accession number, date of collection, host, and geographic location of *Beauveria* spp. isolates.

Accession number	Collection date	Original host	Geographical origin	Species
ESALQ 353	3-15-85	*Pentatomidae*	Piracicaba, SP	B. bassiana
ESALQ 457	7-11-86	*Euschistus heros*	Goias	B. bassiana
ESALQ 458	7-11-86	*Piezodorus sp.*	Goias	B. bassiana
ESALQ 499*	3-28-86	*Diabrotica speciosa*	Blanco Pozo, Tucuman, Arg.	B. bassiana
ESALQ 500	6-86	*Nezara viridula*	Verba Bueno, Tucuman, Arg.	B. bassiana
ESALQ 501*	4-8-86	*Maecolaspis monrosi*	La Virginia, Tucuman, Arg.	B. bassiana
ESALQ 617 (ARSEF 737)	1-20-82	*Pentatomidae*	Highway Goiânia Nerópolis km 12, GO	B. bassiana
ESALQ 618 (ARSEF 933)	12-20-82	*Tibraca limbativentris*	Highway Goiânia Nerópolis km 12, GO	B. bassiana
ESALQ 619 (ARSEF 944)	3-16-83	*Pentatomidae*	Ponta Porã, MS	B. brongniarti
ESALQ 620 (ARSEF 1474)	4-2-84	*Nezara viridula*	Chapecó, SC	B. bassiana

* Collected during a period of high prevalence of the disease, probably in their epizootic phase.

ture medium to obtain a conidial suspension that was used to inoculate shaker flasks. After seven days of incubation in a rotary shaker with Sabouraud dextrose supplemented with 0.5% yeast extract (SDAY) medium at 25°C, mycelia were separated from residual broth by washing and centrifugation. Mycelial pellets were dried and weighed, following intracellular protein extraction in the presence of liquid N, by adding one ml of a 0.04 M Tris-HCl buffer (pH 6.8) to ca. 3 g of mycelia. Mycelial preparations were centrifuged at 20,000 g for 30 min at 4°C and the supernatants were placed in ampoules and frozen at -80°C.

Zymograms

Zymograms were obtained electrophoretically in 7.5% polyacrilamide gel slab prepared according to Hammes (1988). Following electrophoresis, the gel slab was incubated in α-naphthyl acetate, fast blue RR salt, 0.05 M tris-HCl, pH 7.1 buffer.

Bioassays

Bioassays were conducted using *Diatraea saccharalis* larvae obtained from the laboratory of Biological Control, IAA/PLANALSUCAR. Larvae were reared on a modified Hensley & Hammond diet (Mendes et al., 1980). All laboratory bioassay were conducted at 25 ± 1.5°C with a light: dark cycle of 14:10 hours. Fifty fourth instar larvae of *D. saccharalis* were exposed to each isolate by spraying with a suspension containing 2.7×10^8 conidia / ml. Larvae were examined daily for mortality. Dead larvae were placed on wet conditions to confirm the fungal infection and the data were subjected to probit analysis program based on Finney (1971) using the maximum likelihood estimation procedure. The data used in the analysis were obtained from two bioassays with similar results.

Phenetic analysis

The phenetic analysis was performed with a basic matrix constructed with 20 morphological and physiological characters codified in double stage for the following characteristics: length and width of conidia, growth rate on different culture media (PDA and media for detection enzymatic activities), lipolytic, proteolytic, and amylolitic activity, clearing zone, ratio of colony/clearing diameter, growth rate and sporulation at different temperatures, Lethal Time (LTs50), "b" value from mortality/time equation and zymograms. The eletrophoretic bands of α-esterase system were analysed to visually determine their relative similarity considering number, position and intensity for each isolate. Cluster analysis and principal components ordination were conducted according to Crisci & Armengol (1983) and Alves et al. (1986).

RESULTS AND DISCUSSION

Conidia length of *B. bassiana* isolates varied from 0.95 to 3.41 µm. The isolate 619 presented an average of 2.8 ± 0.2 µm, although some conidia reached 5.9 µm (Table 2). These dimensions agree with those cited by Mac Leod (1954) for *B. brongniartii*, even though Humber & Soper (1986) reported this isolate as *B. bassiana*, with original names CP-85 and ARSEF 944, respectively.

In general, radial growth on PDA culture medium increased at 26°C. At 22°C the growth was less affected than at 30°C. Some isolates were unaffected at 30°C such as 499 and 501 from Argentina, or at 22°C, as in the case of 619, an isolate of *B. brongniartii*(Table 3). This provides evidence of the great variability of strains regarding to thermic requirements, as reported by several authors (Sosa-Gómez & Alves, 1984; McCoy & Boucias, 1989; Samuels et al., 1989). Fungal development for all the isolates was limited at 34°C. The isolate 353 exhibited a high growth ratio at all other temperatures tested, while isolates 457 and 458 had limited growth in all conditions. These two isolates, 457 and 458, had the highest sporulation while 619 exhibited poor conidial production in the 3 temperatures (Table 4).

Extracellular amylase production varied between 0 and 1.8 (amylolitic index) on minimum and PDA medium (Table 5). The isolates with high activity were 457 and 458, but considering the halo diameter, the most active was isolate 619. Considering index and diameter of activity the less active was isolate 617. Isolate 619 showed the highest proteolytic activity.

The *Beauveria* spp. isolates were polymorphic for the α-esterase system producing 3 isozyme bands, *d*, *o* and *p*, which were virtually invariant electrophoretically. Sixteen sites of activity for the isolates were identified in the esterase zymograms (Fig. 1). Esterase patterns for isolates 457 and 458

TABLE 2. Conidia dimensions of *Beauveria* spp. isolates, produced on PDA medium.

	Length (µm)			Width (µm)		
Isolates	average	min.	max.	average	min.	max.
619	2.8 a	1.4	5.9	1.8	1.2	1.9
499	2.2 bc	1.5	3.2	1.8	1.5	1.9
458	2.2 bc	1.6	2.8	1.6	1.1	2.3
457	2.2 bc	1.7	3.4	1.5	1.0	2.2
501	2.1 bcd	1.2	2.9	1.6	1.0	2.4
618	2.0 cd	1.5	2.6	1.4	1.0	1.7
617	1.9 de	1.0	2.6	1.4	0.8	2.0
500	1.9 de	1.3	2.8	1.6	1.0	2.2
353	1.9 de	1.5	2.5	1.5	1.0	2.0
620	1.9 de	1.5	2.6	1.5	1.1	2.4

Means followed by the same letter are not significantly different at the 0.05 level (tukey test).

TABLE 3. Radial growth (mm) of *Beauveria* spp. isolates, colonies grown on PDA medium, after six days at three different temperatures [12 P:12 D].

Isol.	diam. 22°C	Isol.	diam. 26°C	Isol.	diam. 30°C	Isol.	diam. Geral
353	20.6 a	353	23.6 a	501	21.0 a	353	21.3 a
	B		A		B		
619	19.3 ab	500	23.6 ab	353	19.8 a	617	19.7 b
	A		A		B		
617	19.2 abc	617	22.5 abc	499	19.0 ab	500	19.4 b
	B		A		C		
500	18.2 bcd	501	20.7 c	617	17.3 bc	501	18.6 b
	B		A		C		
499	17.0 cde	620	19.6 c	619	16.6 c	499	18.4 bc
	B		A		A		
457	16.6 de	458	19.2 c	500	16.5 c	619	18.3 bc
	B		A		C		
618	15.8 ef	499	19.2 c	620	16.4 c	620	17.2 cd
	B		A		B		
620	15.8 ef	618	19.1 c	618	16.0 c	618	16.9 d
	B		A		B		
458	15.3 ef	619	19.1 c	457	12.6 d	457	16.0 de
	B		A		C		
501	14.2 f	457	18.8 c	458	11.8 d	458	15.5 e
	B		A		A		

Means followed by the same low case letter in a vertical line, or the same capital letter in a horizontal line are not significantly different at the 0.05 level (Tukey test)

were identical, although these isolates were obtained from different hosts. Isolates 500 and 501 produced esterase patterns similar to those from 353. Isolates 617, 618 and 619 shared many esterase bands. Poprawski et al. (1988) and McCoy & Boucias (1989) characterized biochemically

TABLE 4. Number of conidia (n x 10^6) of *Beauveria* isolates on PDA after 6 days

Isol.	22°C	Isol.	26°C	Isol.	30°C	Isol.	média Geral
457	425.4 a	457	367.2 a	617	100.3 a	457	278.8 a
458	374.7 a	458	277.8 ab	618	54.6 ab	458	228.3 ab
617	150.6 b	617	269.6 ab	457	43.8 ab	617	173.5 b
620	126.4 bc	501	218.8 bc	458	32.3 b	620	103.2 c
353	68.8 bcd	620	152.7 cd	620	30.4 b	501	102.5 c
501	67.7 cd	500	147.1 cd	353	30.1 b	353	61.3 cd
499	55.9 de	353	85.1 de	501	21.0 b	500	74.9 cd
500	57.8 de	499	78.8 de	619	19.6 b	499	51.9 de
619	10.8 e	618	39.4 e	499	21.0 b	618	34.2 de
618	8.5 e	619	37.3 e	500	19.7 b	619	22.6 e

Means followed by the same letter are not significantly different at the 0.05 level (Tukey test).

TABLE 5. Growth and enzyme production by *Beauveria* spp. isolates on solid medium, expressed in diameter (mm), after subculturing in minimal medium [26 ± 0.5°C and 12 h photophase].

	Diameter in mm[1]					
	protease		lipase		amylase	
Isolates	colony	clearing[1]	colony	clearing	colony	clearing
618	18.1 e	28.9 a	21.6 cd	0.0	6.0 d	6.6 b
619	25.4 a	29.2 a	14.3 f	28.0	15.5	16.0 a
499	23.6 ab	22.3 c	23.8 ab	0.0	13.8 ab	14.8 a
500	22.9 bc	24.4 b	25.2 a	0.0	14.1 ab	15.6 a
458	19.8 de	20.2 de	20.2 de	0.0	10.4 c	13.9 a
617	21.1 cd	20.8 d	22.5 bc	0.0	11.2 bc	0.0 c
620	20.8 d	20.9 cd	23.3 b	0.0	10.4 c	13.8 a
457	19.3 de	19.8 de	19.4 e	0.0	11.8 bc	13.7 a
353	20.7 d	20.6 d	23.2 b	0.0	15.2 a	15.2 a
501	20.2 d	19.1 e	22.4 bc	0.0	8.0 cd	8.2 b

[1] Average on 6th day

Means followed by the same letter are not significantly different at the 0.05 level (Tukey test).

isolates of *Beauveria* spp. to identify pathotypes in terms of isoenzyme variation. The use of zymograms of only one type of enzyme to differentiate isolates may lead to misidentification because of similarity in electrophoretic patterns. This risk may be diminished if several enzymatic systems are used.

LTs50 were obtained for all isolates (Table 6). Based on lack of overlap of the 95% fiducial limits of the LT50, there were significant differences among isolates, with isolates 619 and 618 being the most virulent.

The relationship among pathogenesis and enzymatic production is contradictory (Sosa-Gómez, 1990; Bidochka & Khachatourians 1990). Silva & Messias (1986) observed that mutants of *M. anisopliae*, with low lipolytic and amilolytic activity, were less virulent to the hemiptera *Rhodnius prolixus* when compared to the parental strains. However, the same tendency was not observed for

TABLE 6. Lethal time data obtained through bioassays with *Beauveria* spp. isolates on 4th instar *Diatraea saccharalis* larvae, treated with a suspension of 2.7 x 10^8 conidia / ml.

Isolates	LT50 (days)	Fid. lim. (95%)	equation[1]	total mortal. (%)
619	5.87 a	4.9 - 6.9	y = 1.5810 + 4.4475 .log x	76
618	5.95 a	5.3 - 6.7	y = -0.8347 + 7.5322 .log x	67
499	8.33 b	7.6 - 9.2	y = 2.3180 + 2.9127 .log x	67
457	9.28 b	8.1 - 10.7	y = 0.8563 + 4.2827 .log x	53
500	9.32 bc	8.0 - 10.8	y = 2.5261 + 2.5521 .log x	56
620	10.05 bcd	8.7 - 11.6	y = 2.5182 + 2.4762 .log x	56
617	10.27 cd	9.4 - 11.2	y = 1.9819 + 2.9835 .log x	56
458	11.21 cd	9.6 - 13.1	y = 2.1205 + 2.7432 .log x	50
501	12.30 d	11.1 - 13.6	y = 2.3508 + 2.4303 .log x	56
353[2]	—		————	24

(1) x = time in days and y = probit

(2) isolate with mortality below than 50%

The LT50 were compared by overlapping of the fiducial limits at 0.05 level.

proteolytic activity. The authors also found that, in the case of lipolytic and amylolytic enzymes, virulence could be restored in the revertants. St. Leger et al. (1988), on the other hand, observed that proteolytic enzymes were a key factor for the penetration process of *M. anisopliae* in the cuticle of *Manduca sexta*.

Considering that the relationship between lipolytic activity and virulence was observed for *B. brongniartii* isolates (Paris & Segretain, 1975 and Paris & Ferron, 1979), can be assumed that the virulence of isolate 619 may be due to its high level of lipolytic activity, but the isolate 618 of *B. bassiana*, which was ranked among the most virulent, did not produce extracellular lipase and had high levels of protease (Table 5).

In this study, no clear relationship between enzyme production and virulence of *Beauveria* isolates was observed. This is in agreement with Champlin et al. (1981) and Pekrul & Grula (1979), and indicates that the extracellular enzyme production is not the only factor involved in the virulence phenomena (Paris & Ferron, 1979) or in the sucess of the penetration process (Pekrull & Grula, 1979). Not all enzymes whith lipolytic, amylolytic and proteolytic activity may be involved in the pathogenesis; therefore, different methods utilized to detect this activity may lead to different conclusions.

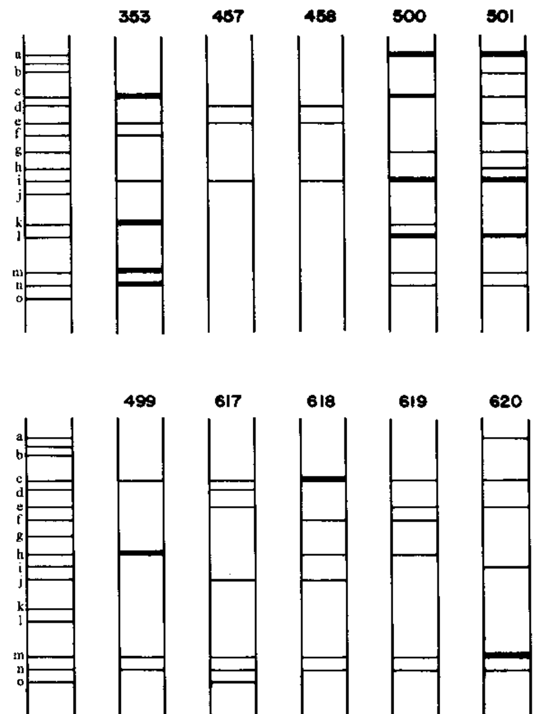

FIG. 1. α-Esterase banding patterns produced by nine *Beauveria bassiana* isolates (353, 457, 458, 499, 500, 501, 617, 618, 620) and a *B. brongniartii* isolate (619).

The phenogram obtained using the weighted pair group method with arithmetic averages (WPGMA) is shown in Fig. 2. The cophenetic correlation of the phenogram distance to the input distance matrix was 0.89. The principal component analysis for the first three factors accounted for 61.4% of the variance and supported the results of the WPGMA (Fig. 3). the lowest similarity was found with isolate 619 of *B. brongniartii* which was separated from all other isolates by a large phenetic distance.

The highest similarity was observed among strains from the same region, isolates 457 and 458 from Goiânia, Brazil, and 500, 501 and 499 from Tucuman, Argentina. This similarity suggests that variability among populations of close geographic regions is smaller than the variability among populations of distant regions. This same phenomenon was observed by Poprawski et al. (1988), although they considered only one character, isoenzyme patterns, in the analysis of the populations.

CONCLUSIONS

1. Numerical taxonomy is useful to differentiate groups of isolates based on morphological and physiological parameters.

2. Isolates can be agrupated according to geographical origin.

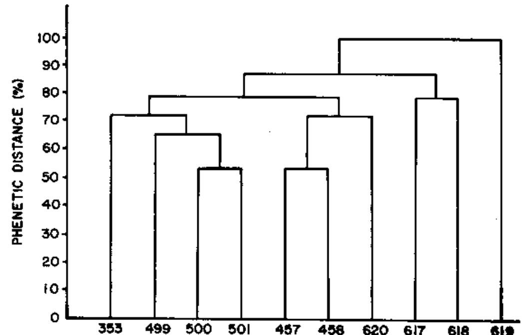

FIG. 2. Phenogram constructed by median euclidian distance. c = 0.50 and Cophenetic Correlation Coefficient. = 0.89.

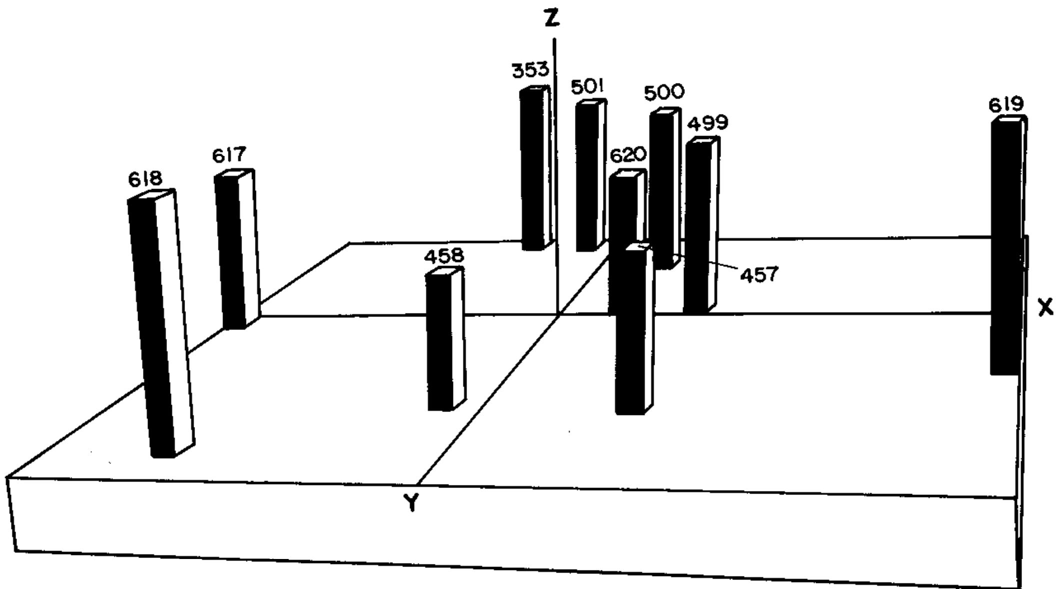

FIG. 3. Principal component analysis from *Beauveria* spp. Operational Taxonomic Units projection on the primary components, representating 61,4% of total variation.

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