Characterization and Pathogenicity of *Beauveria bassiana* Against *Diatraea saccharalis* (F.) (Lepidoptera: Pyralidae) in Argentina

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RESUMO - Foram analisados 21 isolados de *Beauveria bassiana* (Bals.) Vuill. quanto à patogenicidade em lagartas de *Diatraea saccharalis* (F.), importante praga da cultura do milho na Argentina. Os isolados obtidos originalmente de *D. saccharalis* foram patogênicos a essa praga, o que não ocorreu com isolados provenientes de outros hospedeiros. A mortalidade observada variou de 50 a 90%, com TL_{50} entre 2,1 a 8,4 dias. Os isolados apresentaram alta variabilidade quanto a germinação e produção de conídios em meio de cultura artificial. A análise de isoenzimas mostrou alta similaridade (>80%) entre os isolados obtidos originalmente de *D. saccharalis*, independente do local de origem (Argentina ou Brasil). As características analisadas não se relacionaram entre si, mas permitiram a seleção de isolados de *B. bassiana* com potencial para controle biológico de *D. saccharalis* na Argentina.

PALAVRAS-CHAVES: Insecta, broca da cana-de-açúcar, fungos entomopatogênicos, controle biológico, variabilidade genética.

ABSTRACT - The pathogenicity of 21 isolates of *Beauveria bassiana* (Bals.) Vuill. collected in different hosts and localities of Argentina and Brazil was evaluated on the sugar cane borer, *Diatraea saccharalis* (F.). Isolates originally obtained from this host were virulent against it in the laboratory, while isolates from other insect species showed no virulence towards *D. saccharalis* larvae. Mortality rates ranged from 50 to 90% with a TL_{50} of 2.1 to 8.4 days. High variability was observed among isolates in germination and conidia production on artificial culture medium. However, isozyme patterns of the isolates collected from sugar cane borer showed high similarity (> 80%) regardless of their origin (Argentina or Brazil). The analyzed characteristics did not show a clear relationship, but allowed the selection of *B. bassiana* isolates with potential as a biological control agent of *D. saccharalis* in Argentina.

KEY WORDS: Insecta, sugar cane borer, entomopathogenic fungus, biological control, genetic variability,

The sugar cane borer, *Diatraea saccharalis* (F.), is an important pest of maize, sorghum and sugar- cane crops in the Americas. In Argentina, this pest is responsible for 20% of reduction in maize production (E. Dagoberto & R.E. Lecuona, unpublished). Because the chemical control of sugar cane borer is not efficient, different strategies of control have been developed. In Brazil, *D. saccharalis* is mostly controled through the release of parasitoids (Botelho 1992). The entomopathogenic fungi *Metarhizium anisopliae* (Metsch.) Sorok. and *Beauveria bassiana* (Bals.) Vuill. have shown potential as biological control agents in sugar cane IPM in Brazil (Alves *et al.* 1984,1985, Lecuona & Alves 1988). In Argentina, *B. bassiana* was found infecting larvae of *D. saccharalis* (Dagoberto *et al.* 1981, R.E. Lecuona, unpublished). However, few studies were conducted to explore the potencial use of *B. bassiana* as a biopesticide against this pest. The utilization of isoenzymes to differentiate isolates and populations of *B. bassiana* was demonstrated (Poprawski *et al.* 1988, Mugnai *et al.* 1989, Tigano-Milani *et al.* 1990, St. Leger *et al.* 1992). The possibilities to use this entomopathogen as bioinsecticide depend on the availability of virulent strains with good capacity to produce conidia and high growth rate (Paccolla-Meirelles & Azevedo 1990). The aim of this work was to characterize Argentinean and Brazilian strains of *B. bassiana* to be used in the control of *D. saccharalis*. Isolates collected from different host species and localities were analyzed regarding biological parameters and isozyme patterns.

Material and Methods

Fungal Cultures. Twenty one strains of *B. bassiana* isolated from *D. saccharalis* and other insects in Argentina and Brazil were studied (Table 1). Monoconidial cultures were produced for each isolate and maintained on complete media agar plate (CMA): 0.4 g KH2PO4, 1.4 g NaHPO4, 0.6 g SO4Mg, 1 g KCl, 0.7 g NH4NO3, 10 g glucose, 15 g agar, 5 g yeast extract, 1 liter of distilled water. Conidia were obtained from cultural grown on CMA at 25°C for 15 days. Mycelia needed for isozyme studies were obtained by inoculating 50 ml of Sabouraud dextrose broth in 250-ml flasks with a final conidial suspension of 10^6 conidia/ml. Flasks were incubated on a rotary shaker (150 rpm, 26°C). Seven days after inoculation, the mycelia were separated from the supernatant by vacuum filtration.

Electrophoresis. The mycelial material obtained from liquid media was washed several times in sterile distilled water (SDW) and in a buffer (0.05 M Tris-HCL, pH 7.8) to remove residual broth. Mycelial were collected and concentrated by vacuum filtration, weighed, fragmented with liquid nitrogen, and centrifuged at 30,000 g for 30 min at 4°C. The supernatants were placed in 1.5 ml ampoules and frozen at -80°C. Hidrophilic proteins were separated via isoeletric focusing (IEF), as described by Riba *et al.* (1986). Zymograms were obtained using 10% polyacrylamide gel containing 6% ampholytes (Pharmacia, Sollentuna, Sweden). Twelve microliters of samples was layered on the gel, and IEF was done in a pH gradient from 3 to 10 at constant power of 8 W. After migration, the gels were incubated in the appropriate staining mixtures. Fifteen enzymes systems were tested, but only 6 were selected because they were polymorphic and presented well-resolved bands. All strains were then analyzed for peroxidase (PER), phosphoglucomutase (PGM), malato dehydrogenase (MDH), diaphorase (DIA), phosphogluconate dehydrogenase (PGD), and alcohol dehydrogenase (ADH) activity. Staining procedures were adapted from Shaw & Prasad (1970).

Bioassays. The isolates obtained from *D. saccharalis* were passed through this host before performing the bioassays. The pathogenicity of *B. bassiana* strains was determined by using third instar *D. saccharalis* larvae. Twenty larvae were

Table 1. Origin and phenetic classification of *Beauveria bassiana* isolates.

Isolate[1]	Host	Location	Year	Phenetic group[2]
Bb1 (CG276)	*Diatraea saccharalis*	Pergamino/BsAs/Argentina	1990	1
Bb2	*Diatraea saccharalis*	Pergamino/BsAs/Argentina	1990	2
Bb3(CG277)	*Diatraea saccharalis*	Colón/BsAs/Argentina	1990	1
Bb5(CG278)	*Diatraea saccharalis*	Lincoln/BsAs/Argentina	1990	2
Bb6(CG279)	*Diatraea saccharalis*	9 de Julio/BsAs/Argentina	1990	1
Bb7	*Diatraea saccharalis*	9 de Julio/BsAs/Argentina	1990	2
Bb8(CG280)	*Diatraea saccharalis*	25 de Mayo/BsAs/Argentina	1990	1
Bb9(CG281)	*Diatraea saccharalis*	Venado Tuerto/SFe/Argentina	1990	1
Bb10(CG282)	*Diatraea saccharalis*	Elortondo/SFe/Argentina	1990	1
Bb11(CG283)	*Diatraea saccharalis*	Rio Cuarto/Cba/Argentina	1990	1
Bb12(CG284)	*Diatraea saccharalis*	Huinca Renancó/Cba/Argentina	1990	1
Bb13	*Diatraea saccharalis*	Huinca Renancó/Cba/Argentina	1990	1
Bb32(CG16)	*Diatraea saccharalis*	Ipojuca/PE/Brazil	1983	1
Bb34(CG71)	*Diatraea saccharalis*	PE/Brazil	1984	2
Bb35(CG72)	*Diatraea saccharalis*	Araras/SP/Brazil	1983	1
Bb39(CG85)	*Diatraea saccharalis*	Iracemápolis/SP/Brazil	1985	2
Bb33(CG25)	*Anticarsia gemmatalis*	Brasília/DF/Brazil	1987	4
Bb36(CG74)	*Tibraca limbativentris*	Goiania/GO/Brazil	1982	4
Bb38(CG81)	*Tibraca limbativentris*	Chapecó/SC/Brazil	1984	3
Bb40(CG135)	*Euselasia* sp.	RS/Brazil	1988	1
Bb41(CG136)	*Deois flavopicta*	Piracicaba/SP/Brazil	1989	1
Bb37(CG78)	*Nezara viridula*	Yerba Buena/Tuc/Argentina	1986	3

[1]Bb, IMYZA-CICA-INTA Collection, Castelar, Argentina; CG, EMBRAPA/CENARGEN Collection, Brasília, Brazil.

[2]Group classification was based on clustering analysis of isozymes characters.

immersed in a conidial suspension (10^8 conidia/ml) for approximately 6 seconds. After treatment, the larvae were grown individually and incubated at 26ºC, 70% HR and photofase of 14 hs. Treatments were replicated four times. Control larvae were immersed in SDW. The mortality of larvae was observed daily to determine the median lethal time (LT_{50}).

Conidial germination. Conidia harvested from 15-days-old CMA spread plate cultures were used to prepare CMA spread plate, using 0.1 ml of a 10^7 conidia/ml suspension in SDW with 0.01% Tween 80 (Sigma, St. Louis, MO), incubated at 26ºC. The observation of the germination started 6 hs after the inoculation. Plate were assessed to determine the median germination time (GT_{50} = the time to germinate 50% of conidia).

Sporulation. Five-millimeter plugs from 15-days-old colonies were incubated on CMA plate at 26°C for 9 days. Then conidia produced were harvested following agitation in 10 ml of SDW containing 0.01% Tween 80. Conidia concentration was determined with a hemocytometer.

Statistical analysis. A dendrogram was constructed from the isozyme data using NTSYS-pc program (Rohlf, 1993). A similarity matrix was created using the Jaccard coefficient, and cluster analysis was done using the unweighted pair group arithmetic mean method (UPGMA) (Sneath & Sokal, 1973). The percentage of mortality and germination were transformed using Probit (Finney 1964) to calculate LT_{50} and GT_{50} respectively. For ANOVAs and mean comparisons, sporulation data were transformed to SQRT. Mean differences were compared using Tukey's test.

Results and Discussion

There were differences among the isolates regarding their capacity to infect *D. saccharalis* larvae, or their LT_{50} for the pathogenic ones (Table 2). The six strains originally isolated from other hosts than *D. saccharalis* were nonpathogenic to this insect (Bb 33, 36, 37, 38, 40 and 41). The isolates obtained from *D. saccharalis* in Argentina or Brazil were all pathogenic to their original host. Their virulence varied from 50% of larvae mortality for Bb 34 to 90% for Bb 12 (Table 2). In general the Brazilian isolates showed to be less virulents (50 - 70% mortality) than the Argentinean (60 - 90% mortality), possibly because they were obtained earlier than the Argentinean, and a large number of replications were made before being stored at CENARGEN collection. The LT_{50} ranged from 2.1 days to Bb 10 to 8.4 days to Bb 1, but this parameter was not related to the virulence. The ability of *B. bassiana* to infect *D. saccharalis* is already known from Brazilian populations of this pest in sugar cane (Lecuona & Alves 1988). However, in this study we had the first screening, in laboratory, of *B. bassiana* isolates against population of Argentinean sugar cane borer collected from maize crops. Lecuona & Alves (1988) showed that Bb 34 can infect 82% (LT_{50} of 7.8 days) of *D. saccharalis* larvae tested in Brazil. However, the same isolate caused 50% of mortality (LT_{50} of 7.4 days) on Argentinean *D. saccharalis*, which could be related to the difference of susceptibility in sugar cane borer populations (Argentinean and Brazilian). Other biological parameters analyzed, conidial germination and sporulation, also showed a wide variation among the isolates. The GT_{50} varied from 9.4 hours (Bb 38) to 20.0 (Bb 12). This characteristic was not correlated to pathogenicity as shown above with the extreme values presented, where Bb 38 was not pathogenic but showed the fastest germination in artificial media. In contrast, the most virulent strain, Bb 12, took longer time to germinate in similar conditions (Table 2). Similarly, in the sporulation analysis, not all of the most virulent isolates were good conidial producers in artificial medium. The sporulation rate ranged widely from 6×10^7 to 130×10^7 conidia/ml among the isolates analyzed.

Using isoelectric focusing (IEF), it was possible to analyze six enzymatic systems, more than in previous studies with *B. bassiana* using IEF or starch gel electrophoresis (Poprawski *et al.* 1988, Tigano *et al.* 1990, St Leger *et al.* 1992). The isozyme systems analyzed expressed a combination of bands in two or three patterns on the gels, depending on the isolate. The enzyme profiles for PGM, PER, MDH, DIA, PGD and ADH are shown in Fig. 1. In all enzymatic systems analyzed there was a common pattern, frequently found among the isolates. As shown in the dendrogram (Fig. 2), isolates were clustered in four main groups with > 70% similarity within them. In group 1 and 2 the isolates were identical. These groups clustered together with 87.3% of similarity. All the strains isolated from *D. saccharalis*, either in Argentina or Brazil fall in these groups. However, there was not a clear correlation between host and phenetic group. Groups 1 and 2 also contained an isolate, Bb 41, obtained from another host. The two other groups observed were less homogenous than the first ones. The two isolates Bb 37 and Bb 38 from group 3, clustered together at 79.4% of similarity, while Bb 36 and Bb 33 from group 4, presented 72.7% of similarity. The minimum of similarity (54.8%) was observed between group 4 and the rest of the isolates analyzed,

Table 2. Pathogenicity to *Diatraea saccharalis*, conidia germination and sporulation of *Beauveria bassiana* isolates.

Isolate	Pathogenicity to *D. Saccharalis*[1] Mortality(%)[2, 3]	LT_{50} (days)[4] and slope	GT_{50}(hours)[4, 5] and slope	Sporulation (n x 10^7 conidia/ml)[2, 6]	
Bb 12	90 *a*	7.8 (7.1245)	20.0 (8.8388)	36.7	(6.035 *c*)
Bb 10	89 *a*	2.1(4.7092)	13.4 (11.0467)	9.1	(2.940 *ef*)
Bb 5	85 *ab*	5.1 (4.7675)	14.2 (14.7655)	26.7	(5.159 *cd*)
Bb 3	81 *ab*	7.3 (8.1877)	16.3 (9.7453)	71.5	(8.453 *b*)
Bb 6	80 *abc*	7.7 (5.9426)	14.1(12.5939)	15.5	(3.877 *ed*)
Bb 11	75 *abcd*	6.9 (6.2307)	18.0 (12.2520)	38.0	(6.131 *c*)
Bb 13	75 *abcd*	7.2 (7.8382)	12.5 (17.6725)	69.2	(8.311 *b*)
Bb 7	70 *bcde*	7.5 (7.2809)	14.2 (10.4773)	28.0	(5.252 *cd*)
Bb 9	70 *bcde*	7.3 (6.0837)	15.1 (12.3979)	29.5	(5.398 *c*)
Bb 32	70 *bcde*	6.9 (4.9642)	12.6 (16.5622)	33.2	(5.727 *c*)
Bb 1	64 *cdef*	8.4 (4.2018)	17.3 (8.6046)	105.0	(10.241 *a*)
Bb 8	60 *def*	7.4 (2.7830)	13.4 (11.1092)	113.0	(10.626 *a*)
Bb 2	60 *def*	7.4 (3.1186)	14.3 (11.4876)	130.0	(11.398 *a*)
Bb 39	60 *def*	7.7 (4.1762)	15.2 (10.8117)	6.6	(2.519 *efg*)
Bb 35	55 *ef*	7.7 (2.6983)	13.3 (11.8998)	33.0	(5.709 *c*)
Bb 34	50 *f*	-	15.1 (13.0491)	22.5	(4.689 *cd*)
Bb 33	0	-	13.5 (15.2189)	0.6	(0.760 *h*)
Bb 36	0	-	12.4 (18.3338)	3.8	(1.927 *fgh*)
Bb 38	0	-	09.4 (11.6915)	6.4	(2.481 *efg*)
Bb 40	0	-	13.6 (11.5641)	0.9	(0.903 *h*)
Bb 41	0	-	13.4 (19.3347)	3.5	(1.813 *fgh*)
Bb 37	0	-	13.5 (12.3728)	2.1	(1.411 *gh*)

[1]Values are means of four replicates with 20 larva/replicate.

[2]Means followed by the same letter were not significantly different at $\alpha = 0.05$ using Tukey test.

[3]The percentage was calculated over infecting larvae. MSE= 44.9218, DF= 48, F= 13.09, CV= 9.46

[4]Median lethal time and germination time calculated by Probit.

[5]Calculated on 400 conidia in CMA.

[6]Data were transformed to SQRT. MSE= 0.3277, DF= 66, F= 123.07, CV= 11.27.

showing less diversity among the isolates of this study than observed in another study with several *B. bassiana* strains isolated from different geographic regions and hosts (St. Leger *et al.* 1992). Even using a smaller sample of isolates, the results obtained here agree with previous observations made for *B. bassiana* and other entomopathogenic fungi, when it was difficult to correlate molecular markers to any other characteristic of the isolates (St. Leger *et al.* 1992, Sosa-Gómez *et al.* 1994, Tigano-Milani *et al.* 1995b).

The strain pairs Bb 1, Bb 2, Bb 6, Bb 7, Bb 12, and Bb 13, although isolated at the

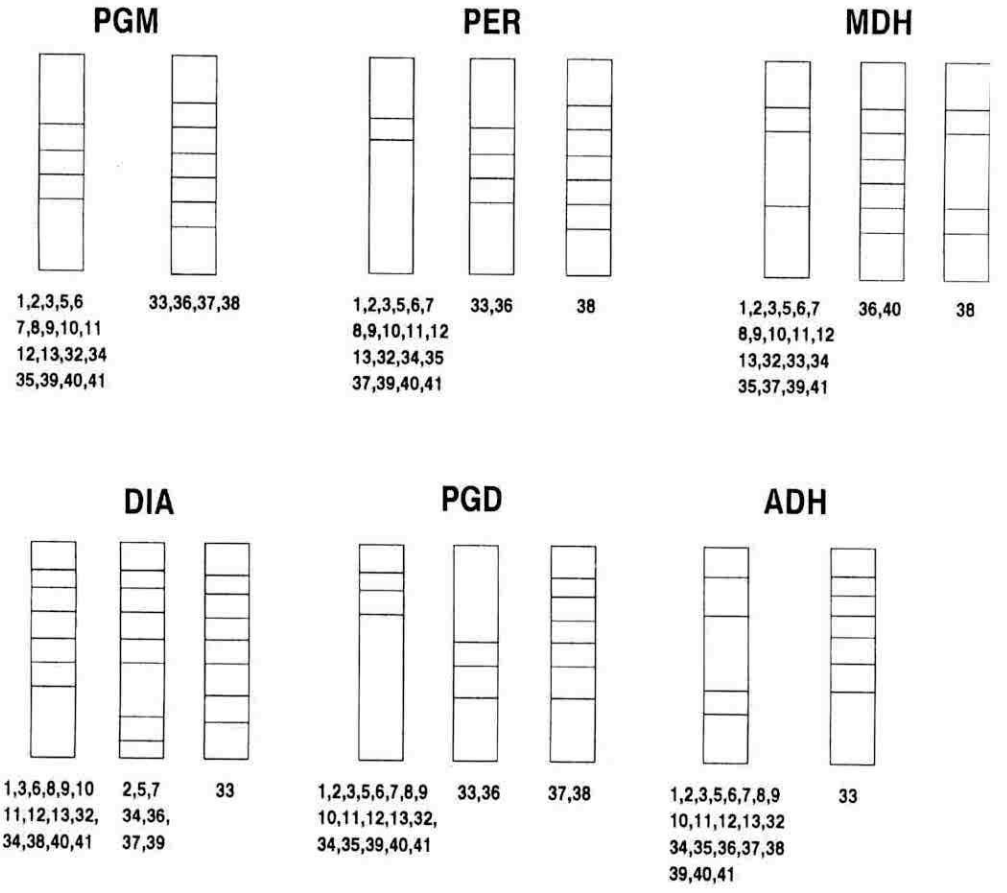

Figure 1. Electrophoretic polymorphism of peroxidase (PER), phosphoglucomutase (PGM), malato dehydrogenase (MDH), diaphorase (DIA), phosphogluconate dehydrogenase (PGD), and alcohol dehydrogenase (ADH) systems in *Beauveria bassiana* isolates. The numbers represent the isolate (Table 1).

same place and time, but from different *D. saccharalis* larvae (Table 1), showed different biological characteristics (Table 2). These differences were confirmed for the 1st strain pair at isozymatic level (phenetic group 1 and 2, Table 2). This fact was also observed by Tigano-Milani (1995a, b) for *Nomuraea rileyi* (Farlow) Samson for biological characters and for *Paecilomyces fumosoroseus* (Wize) Brown et Smith at the DNA level.

The strain Bb 37, although native from Argentina, was placed in a different phenetic group (group 3), as compared to all others

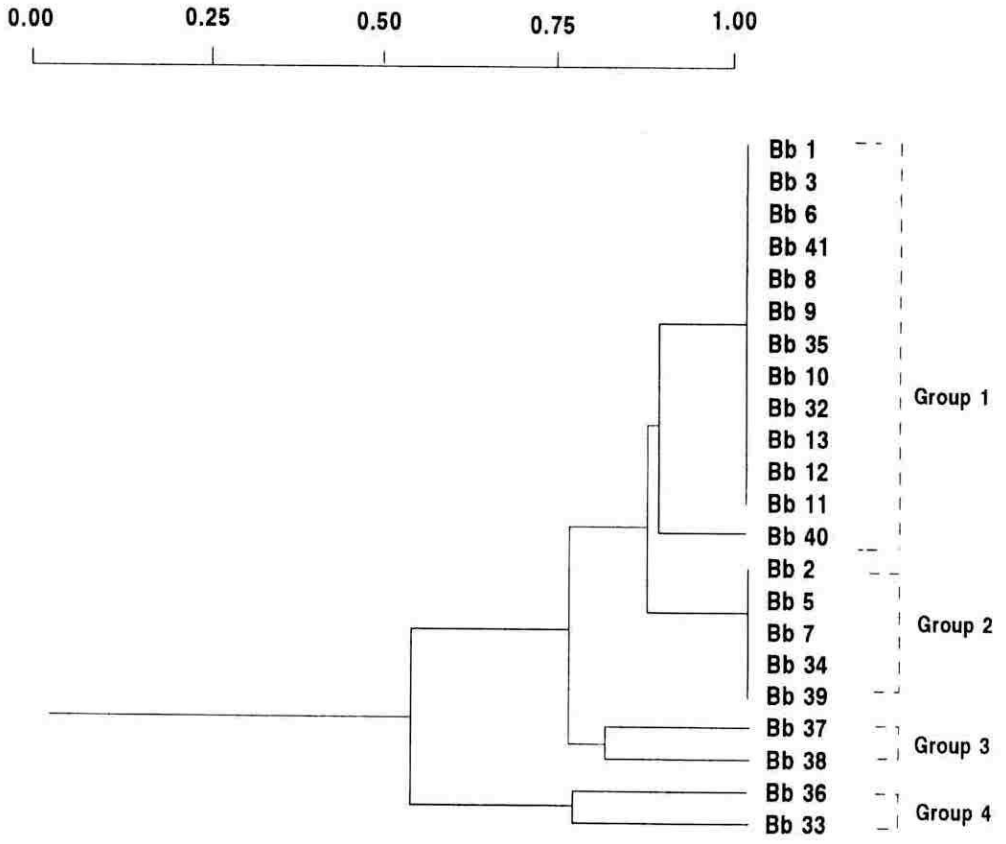

Figure 2. Dendrogram constructed from isozymes data, indicating the relationships among *Beauveria bassiana* isolates. A similarity matrix was calculated based on Jaccard coefficient, and the tree was generated from this matrix by the unweighted pair group method arithmetic mean (UPGMA).

Argentinean strains, which are in the groups 1 and 2. However, this strain was the only Argentinean one obtained from other host than *D. saccharalis* (Table 1).

The different parameters used to characterize the isolates allow the selection of the ones with higher performance toward developing a biopesticide to control *D. saccharalis* in Argentina. In fact, four isolates, Bb 10, 5, 3, and 6, were selected for further analysis concerning their capacity to infect and kill sugar cane borer, in a short period of time, as well as for their capacity to be produced efficiently in artificial media.

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