MOLECULAR CHARACTERISATION AND APPLICATION OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN IN THE BIOLOGICAL CONTROL AGAINST *DIATRAEA SACCHARALIS* (FABRICIUS)

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Abstract

MASSIVE application of the entomopathogen *Beauveria bassiana* in sugarcane fields is important both from an agronomic and ecological perspective. This has attracted interest for the development of reliable tools for the identification of the various species in use. The present work had as objectives the molecular characterisation of *B. bassiana* by inter-microsatellite (ISSRs) markers and the evaluation of the application of the hyphomycete in the biological control of *Diatraea saccharalis* (Fabricius). Genomic DNA was extracted from lyophilised mycelia. The amplified products were analysed with 100 ISSR primers. A high level of polymorphism (near 80%) was found among eleven isolates using fourteen ISSR primers selected. Seven different isolates showed exclusive bands and ISSR primer 873 distinguished all of them. The application of *B. bassiana* in the field was able to diminish live larvae of *D. saccharalis* by 43.7%, therefore demonstrating their potential as a biological control agent for sugarcane borer.

Introduction

Diatraea saccharalis (Fabricius) is the main pest of sugarcane in Cuba and it causes serious losses and damage to plantations. In order to reduce the population levels of the borer, the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. is used.

Natural occurrence of the fungus in Cuba was reported in larvae and pupae of *D. saccharalis* by Estrada *et al.* (2004). The pathogenicity of different isolates of the fungus was also determined in larvae of this Lepidopteran by Estrada *et al.* (1995).

There are several reports on the molecular characterisation of *B. bassiana* with the objective to study their genetic structure (McGuirre *et al.* 2005) and on the monitoring of the application of the fungus in the field (Wang *et al.* 2003).

The objective of the present work was to conduct a molecular characterisation of *B. bassiana* by intermicrosatellites, Inter Simple Sequence Repeats (ISSRs) and to determine the efficacy of application of the fungus in the biological control against *D. saccharalis*. The variability and the phylogenetic relationships between the eleven isolates of *B. bassiana* were analysed using ISSR markers as described by Estrada *et al.* 2007.

Materials and methods

Molecular characterisation of isolates of *B. bassiana* by ISSRs

Fungal isolates

Eleven isolates of *B. bassiana* from different geographic and entomological origins were used (Table 1). The isolates were grown in static liquid medium as described by Adamek (1965) and incubated at 25°C for four days. The fresh mycelia were harvested and lyophilised for 24 h.

Table 1—*Beauveria bassiana* isolates used in this study.

Isolates	Strains	Entomological origin	Geographic origin
1	Quivicán	*D. saccharalis*	Cuba
2	252	*Artiples floridus*	USA
3	MG1	*D. saccharalis*	Cuba
4	18	*D. saccharalis*	Cuba
5	India	*Insecta*	India
6	Bórer	*D. saccharalis*	Cuba
7	PCC	*D. saccharalis*	Cuba
8	93	*Insecta*	Guadalupe
9	60	*Insecta*	Bulgaria
10	CC	*D. saccharalis*	Cuba
11	156	*D. saccharalis*	Cuba

Genomic DNA extraction

The lyophilised mycelia were used for genomic DNA extraction. The extraction was carried out using a small-scale DNA isolation method using Dneasy Plant Mini Kit, from Qiagen.

Primers used in the PCRs test

One hundred primers based on dinucleotide, tetradinucleotide or pentanucleotide repeats were used. These oligonucleotides were obtained from the University of British Columbia (UBC primer set 100/9). A total of fourteen oligonucleotides were selected based on the number and consistency of the amplified fragments.

PCR and analysis of the amplified profiles

Each amplification reaction (final volume of 20 µL) consisted of 1 µL of total genomic DNA (10 ng/µL), 1 µL of the corresponding primer (5 µM), 10 µL of Taq-PCR master mix (Qiagen) and 8 µL of ultra pure distilled water. DNA amplifications were performed in a PTC-100 Thermocycler with an initial step of 5 min at 94^{o}C, followed by 45 cycles of 30 s at 94^{o}C, 45s at 52^{o}C and 2 min at 72^{o}C with a final 6 min extension at 72^{o}C.

The amplification reactions were stored at 4^{o}C until resolved by electrophoresis. Samples of 20 µL PCR products were analysed on 1.5% agarose gels after running at 90 V for 2 h and staining with ethidium bromide.

ISSR markers behave as dominant markers. They were scored as presence (1) or absence (0) of homologous bands (bands with the same size) for all the isolates. Dendrograms were constructed by UPGMA (unweighted pair-group method, arithmetic average).

The cluster analysis was performed using different coefficients –the simple matching coefficient (SM), the DICE coefficient and the Jaccard coefficient (J) and using the NTSYS-pc version 1.6 package.

Evaluation of the application of *B. bassiana* in field trials

A randomised block design was used which consisted of eight plots of 96 m^2 each with 120 stools of a three-months old first ratoon crop of variety CB4452.

The plots were in parallel and separated by a distance of 200 m. To assure a homogeneous population of the pest, all the stools of the plots were infested artificially with egg batches of *D. saccharalis* 'in black head' stage (non developed embryos).

The infestation was carried out with 960 egg batches and the average number of eggs/batch was 26.71. Before the application of *B. bassiana*, the percentage of larvae hatching was calculated and it was 76.78%. *B. bassiana*-isolate 3 (MG1) was applied at a rate of 10^{12} conidial/ha in the form of an aqueous suspension at a concentration of 6×10^9 conidial/g.

It was applied in four experimental plots by means of a hand-operated knapsack sprayer. The control plots were treated with water.

Samplings were carried out at day 7, 15, 30, 45, 60 and 101 after the application of the fungus.

n each sampling, four stools/plot were selected at random which were cut and analysed in the laboratory.

The number of affected stalks and internodes, the presence of live larvae of *D. saccharalis*, and presence of other natural enemies of the pest were recorded.

Considering the development cycle of the pest, at 30 days of sampling, the percentage of efficacy of the application of *B. bassiana* was determined by means of the Abbott formula (CIBA-GEIGY, 1981), which is used when the infestation of the pest is homogeneous before the application of the treatments:

$$\% \text{ efficacy} = \frac{(Cd - Td)}{Cd} \cdot 100$$

where:

Cd: number of live individuals in the control plots after the treatment.

Td: number of live individuals in the treated plots after the treatment.

A simple classification variance analysis was carried out and the means were compared by means of Newman Keuls's test ($p < 0.05$) (STATITCF, 1988).

Results and discussion

Molecular characterisation of isolates of *B. bassiana* using ISSRs

ISSR amplification

The sequences of these 14 primers used seem to indicate that the microsatellites that are more frequent in *B. bassiana* contain the repeated dinucleotides (AG)n, (AC)n and (GT)n (Table 2).

Table 2—ISSR primer set # 100/9 UBC (University British Columbia) and the respective oligonucleotide sequence. Note B = C, G, T; D = A, G, T; H = A, C, T; R = A, G; V = A, C, G; Y = C, T.

Primer	Sequence	Primer	Sequence
808	$(AG)_8C$	846	$(CA)_8TR$
809	$(AG)_8G$	849	$(GT)_8YA$
810	$(GA)_8T$	850	$(GT)_8YC$
818	$(CA)_8G$	873	$(GACA)_4$
821	$(GT)_8A$	885	$BHB(GA)_7$
828	$(TG)_8A$	889	$DBD\ (AC)_7$
842	$(GA)_8YG$	891	$HVH(GT)_7$

The 14 ISSR primers used for the PCR amplifications of DNA from the 11 isolates of *B. bassiana* were selected from the primers from the set 100/9 UBC, and gave reproducible amplification products (Figure. 1).

The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes (NG), resolving power (Rp) (Prevost and Wilkinson, 1999) and number of exclusive bands (NEB) obtained with each primer are shown in Table 3.

The total number of amplified products was 172 with an average of 12.28 bands/primer, ranging from 300 to 3000 bp, with 135 (78.49%) polymorphic DNA fragments.

The maximum number of amplified products was 18 (primers 809 and 810) and the minimum was 6 (primer 818) (Table 3).

Seven different isolates (five from Cuba, one from Bulgaria and one from USA), showed a total of 11 exclusive bands that could be transformed in Sequence Tagged Site (STS) markers.

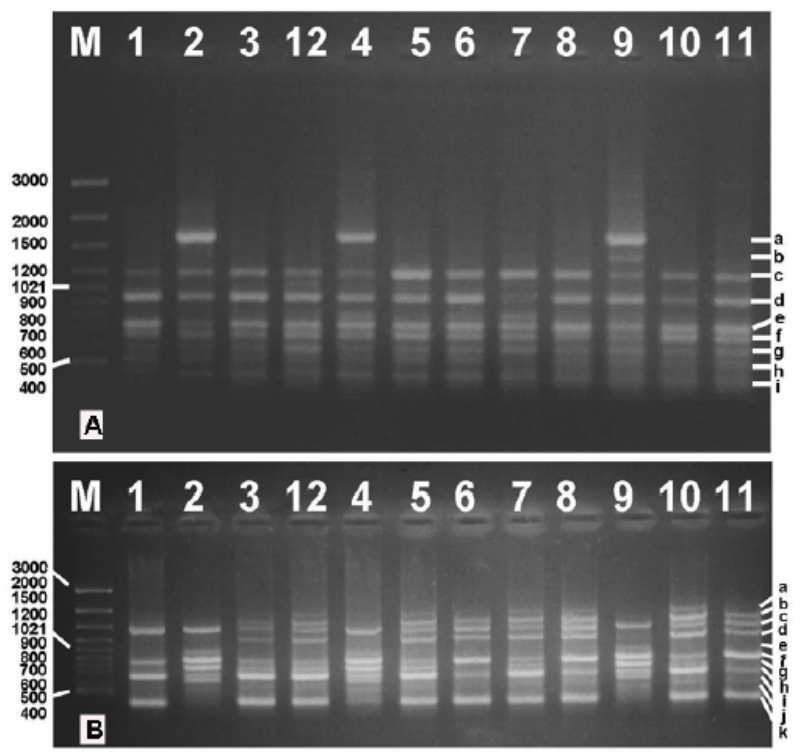

Fig.1—ISSR amplification products obtained from eleven isolates studied with ISSR primer 846 (A) and ISSR primer 889 (B).

Lanes/strain

1- strain MG_1
2- strain 252
3- strain 18
12- repetition of strain 18
4- strain India
5- strain Bórer
6- strain Quivicán
7- strain PCC
8- strain 93
9- strain 60
10- strain CC
11- strain 156
M- molecular wt marker

Table 3—The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes or isolates identified (NG), resolving power (Rp) and number of exclusive bands (NEB) obtained each ISSR primer.

Primer	TNB	NPB	P%	NG	Rp	NEB
808	15	12	80	8	6.3	1
809	18	15	83.4	8	7.9	0
810	18	16	88.9	7	6.6	3
818	6	4	66.7	4	2.0	0
821	11	9	81.8	7	4.4	1
828	11	8	72.7	6	4.2	1
842	14	8	67.2	10	3.4	0
846	10	4	40.0	6	1.4	1
849	14	13	92.8	10	5.6	2
873	11	11	100	11	6.2	1
885	10	10	100	6	6.6	0
888	16	16	100	7	8.9	1
889	11	7	63.6	4	4.7	0
891	7	2	28.6	3	1.1	0

Total	172	135	78.5	97	69.5	11

ISSR is easy to employ and highly reproducible. In this study, nearly 80% of the bands generated, using ISSR, were polymorphic. This shows the high level of genetic variation that exists between the different isolates.

These fungi display parasexual reproduction, which normally confers high genetic variability. In the same way, the ISSR markers proved to be an efficient marker system because of their capacity to reveal several informative bands in a single amplification (a mean of 9.6 bands/primer). Furthermore, it was possible to identify all the isolates with a single primer (873) (Figure 2).

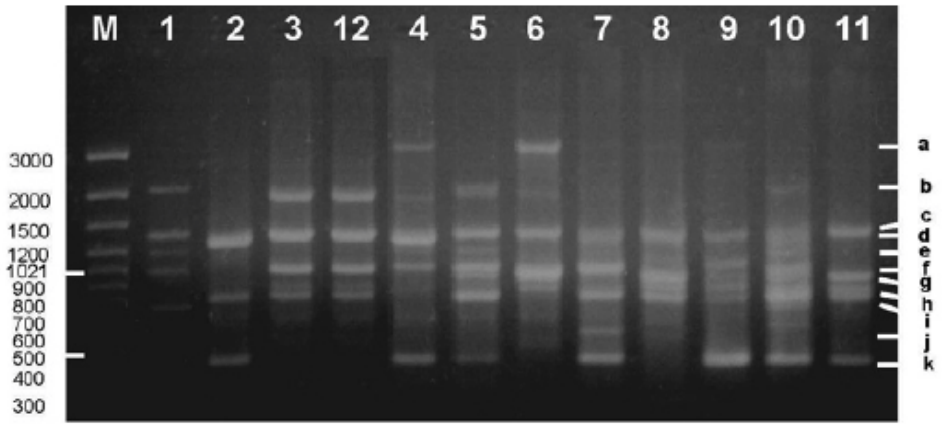

Fig. 2—ISSR amplification products obtained from eleven isolates studied with primer 873. Lanes labelled as per Figure 1.

The dendrograms obtained using the SM, DICE and J coefficients showed similar structure with the same clusters. The dendrograms generated have two distinct clusters (Figure 3). Cluster A contained the isolates from the Caribbean region and B had the isolates from other geographical locations.

The bootstrap values for the Cluster A and B were very high, 97% and 100%, respectively. The different isolates of cluster A exhibited nearly 75% similarity. Sub-cluster A1 contained just one isolate, MG1 from Cuba. This isolate is clearly different from the rest of the Caribbean isolates.

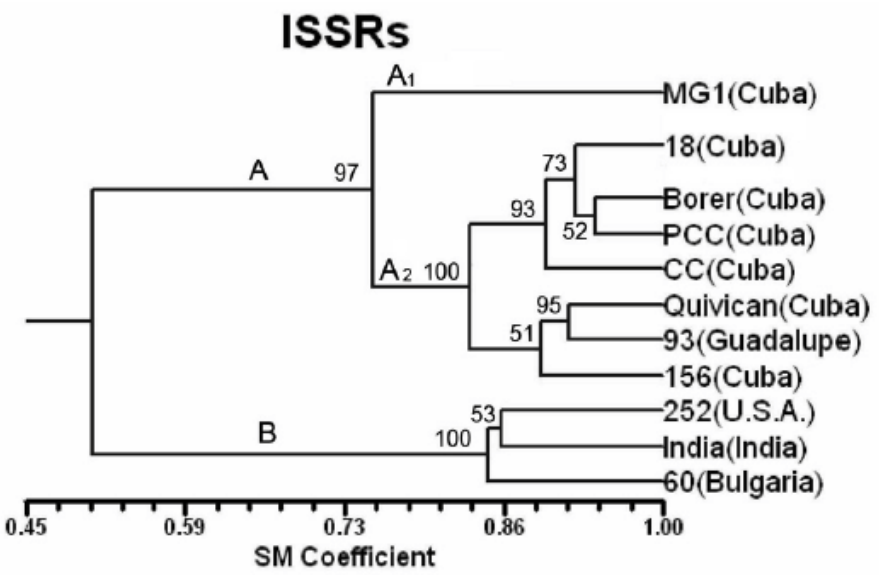

Fig. 3—UPGMA dendrograms showing the genetic relationships between the eleven isolates based on the ISSR markers (SM coefficient). Node numbers at the forks refer to bootstrap values. These numbers show the percentage of time the

group consisting of isolates, which are to the right of the fork occurred.

This information is valuable because this isolate shows higher virulence than any of the other isolates used in Cuba. Also, it has been used in the sugarcane plantations in Cuba for biological control of the larval populations of *D. saccharalis*.

A specific diagnostic band for this isolate was obtained, and in future it could be possible to obtain a specific molecular marker for this isolate. The other Caribbean isolates exhibited a similarity greater than 82%, and are grouped in sub-cluster A2. It also had a high bootstrap value (100%).

The isolates from regions other than the Caribbean are in cluster B, and displayed more than 80% similarity. Between the two clusters, the similarity exhibited is less than 50%. This is a low value of homology, and showed the high genetic variability present between the isolates from the Caribbean region and the rest of the isolates.

The worldwide distribution of the *B. bassiana* isolates analysed using AFLPs was found to be very dissimilar (Uma *et al.*, 2006). In another study involving 50 *B. bassiana* isolates of worldwide distribution, a very close similarity (80%) in AFLP fingerprints was reported by De Muro *et al.* (2003). The differences in the two studies could be explained by the fact that the different markers used detected polymorphisms in different regions of the genome.

Application of *B. bassiana* isolate 3 in field trials

Table 4 shows the results of the larval populations of *D. saccharalis*, the damage originated in the treated and untreated plots with the isolate 3 of *B. bassiana.* A significant difference among the evaluated treatments is observed in all the samplings carried out. The number of live larvae in the plots where the hyphomycete was applied was significantly inferior to the number of live larvae in the untreated plots.

Similarly the number of bored internodes was significantly lower in the treated plots. There was also a correlation between the presence of live larvae and the number of bored internodes showing that the fungus reduces the number of *D. saccharalis* larvae and consequently reduces damages caused to sugarcane fields.

Table 4—Larval population of *D. saccharalis* and damage in sugarcane treated plots with *B. bassiana* and control plots.

Samplings	Number of live larvae (mean)		Number of bored internodes (mean)	
Days	Plots treated	Plots control	Plots treated	Plots control
7	1.75 (a)	2.50 (b)	0.75 (a)	3.75 (b)
15	2.00 (a)	2.25 (b)	2.75 (a)	5.50 (b)
30	2.50 (a)	3.50 (b)	6.00 (a)	9.00 (b)
45	4.75 (a)	6.75 (b)	11.50 (a)	15.75 (b)
60	3.25 (a)	5.75 (b)	9.50 (a)	16.25 (b)
101	0.56 (a)	1.70 (b)	4.25 (a)	11.50 (b)
Percentage of efficacy (%): 43.7				

( ) Newman Keuls's test ($p < 0.05$). Difference significant between treated and untreated plots.

The percentage of efficacy of the application of *B. bassiana* was 43.7% (Table 4). This value is very important by considering that, at 30 days after the application of the hyphomycete, it is possible to reduce the 43.7% of the live larvae of *D. saccharalis* that are able to bore into the sugarcane stalk and therefore to cause damage to the crop.

Alves *et al*. (1985) obtained similar results in Brazil when applying suspensions of conidia of *B. bassiana* at different concentrations in artificially infested plots with *D. saccharalis* larvae.

These authors found 47% and 56% of efficacy and they demonstrated that, in the treated plants, the hyphomycete not only diminished the number of live larvae but also reduced the level of

damage caused by the pest.

Conclusions

ISSR fingerprints provide a useful tool for establishing a rapid and rational approach for differentiating between isolates of entomopathogenic fungi.

In the National Programme of Biological Control against *D. saccharalis,* it is very important to apply *B. bassiana* in the field because this imperfect filamentous fungus reduces larval population and the damage caused by this pest.

The results indicate that intermicro-satellites could be used to evaluate the efficacy of different isolates of *B. bassiana* and their persistence in sugarcane fields. Using these markers, a genetic profile that identifies the most important isolates currently used in the biocontrol of the sugarcane borer in Cuba was obtained.

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CARACTÉRISATION MOLÉCULAIRE DU *BEAUVERIA BASSIANA* BALSAMO) VUILLEMIN ET L'APPLICATION DANS LE CONTRÔLE BIOLOGIQUE DU *DIATRAEA SACCHARALIS* (FABRICIUS)

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MOTS CLÉS: *Diatraea saccharalis*, Lutte Biologique, Caractérisation Moléculaire, Inter-Microsatellites, Canne à Sucre.

Résumé

L'APPLICATION massive de l'entomopathogène *Beauveria bassiana* dans les champs de canne est importante dans une perspective agronomique et écologique. Ceci a suscité de l'intérêt pour le développement des outils pour identifier les espèces utilisées. Cette étude avait pour objectifs de caractériser *B. bassiana* par marqueur moléculaire en utilisant les inter-microsatellites (ISSRs) et d'évaluer l'application du hyphomycète dans la lutte biologique du *Diatraea saccharalis* (Fabricius). L'ADN génomique a été extrait à partir des mycéliums lyophilisés. Les produits amplifiés ont été analysés avec 100 amorces ISSRs. Un fort pourcentage de polymorphisme (presque 80%) a été observé parmi onze isolats caractérisés avec les quatorze amorces ISSRs sélectées. Sept isolats ont produit des bandes uniques avec l'amorce 873 et cela a permis de les différencier. L'application de *B. bassiana* au champ a réduit le nombre de larves vivant de *D. saccharalis* par 43.7%, démontrant ainsi le potentiel de l'entomopathogène dans la lutte biologique du foreur de la canne à sucre.

CARACTERIZACIÓN MOLECULAR Y LA APLICACIÓN DE BEAUVERIA BASSIANA (BALSAMO) VUILLEMIN EN EL CONTROL BIOLÓGICO CONTRA DIATRAEA SACCHARALIS (FABRICIUS)

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Resumen

EL INTERÉS agronómico y ecológico de las aplicaciones masivas de *B. bassiana* precisan de métodos confiables que permitan su identificación en el agrosistema cañero. El presente trabajo tuvo como objetivos la caracterización molecular de *B. bassiana* por intermicrosatélites (ISSRs) y la evaluación de la aplicación del hifomiceto en la lucha biológica contra *Diatraea saccharalis* (Fabricius). Para ello el ADN genómico fue extraído del micelio liofilizado. Se analizaron los productos de amplificación con 100 cebadores de ISSRs. El mayor polimorfismo (80%) fue encontrado en 14 de los cebadores ISSR seleccionados. Siete aislamientos diferentes mostraron bandas exclusivas y el cebador ISSR 873 distinguió una banda entre todas. La aplicación de *B. bassiana* en el campo logró disminuir el 43.7% de las larvas vivas del barrenador lo que demostró su potencialidad como agente para el control biológico de esta plaga.