Identification and characterization of a *Beauveria bassiana* (Bals.) Vuill. isolate having a high potential for the control of the *Diatraea* sp. sugarcane stem borer

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RESEARCH

ABSTRACT

A native isolate of the *Beauveria* sp. fungus obtained from the *Diatraea* sp. larva was studied and identified as *B. bassiana* by analyses based on ribosomal internal transcribed spacer (ITS) sequences. The code given to the isolate was Bv062 and its insecticidal activity was evaluated on the second instar larvae of *D. saccharalis*, *D. indigenella*, *D. tabernella* and *D. busckella*, under laboratory conditions, producing mortality rates of 73.3, 83.3, 67.6 and 24.1 %, respectively. Subsequently, the activity of the fungus was evaluated on *D. saccharalis* eggs. Egg mortality was of 82.2 % when spraying a conidial suspension adjusted to a concentration of 1×10^8 conidia/mL on the eggs. The mean lethal concentration (LC_{50}) was found to be 9.04×10^5 conidia/mL and the lethal concentration ninety (LC_{90}) was of 2.07×10^7 conidia/mL on the second instar larvae of *D. saccharalis*. *In-plant* bioassays under semi-controlled conditions showed that fungus efficacy was above 50 % with the sporulation of dead larvae of 80 %, using concentrations between 1.0×10^7 and 5.0×10^7 conidia/mL. Results demonstrated the high potential of *B. bassiana* Bv062 as a biocontrol agent for the development of a bio-insecticide to be used in the management of different species of the sugarcane stem borer complex.

Keywords: entomopathogenic fungi, biological control, *Diatraea saccharalis*

RESUMEN

Identificación y caracterización de un aislamiento de *Beauveria bassiana* (Bals.) Vuill. con alto potencial para el control del barrenador del tallo de la caña de azúcar *Diatraea* spp. Se estudió un aislamiento nativo del hongo *Beauveria* sp. obtenido de una larva de *Diatraea* sp., el cual fue identificado molecularmente como *B. bassiana* mediante el análisis basado en las secuencias de la región espaciadora interna transcrita del ADN ribosomal (ITS). El aislamiento de *B. bassiana* fue codificado como Bv062 y se evaluó su actividad insecticida sobre larvas de segundo ínstar de *D. saccharalis*, *D. indigenella*, *D. tabernella* y *D. busckella* en condiciones de laboratorio, donde causó mortalidades del 73.3, 83.3, 67.6 y 24.1 % respectivamente. Posteriormente se evaluó la actividad del hongo sobre huevos de *D. saccharalis* y se encontró una mortalidad de huevos del 82.2 % al asperjarlos con una suspensión de conidios ajustada a una concentración de 1×10^8 conidios/mL. Se determinó una concentración letal media (CL_{50}) de 9.04×10^5 conidios/mL y una concentración letal noventa (CL_{90}) de 2.07×10^7 conidios/mL sobre larvas de segundo ínstar de *D. saccharalis*. Ensayos de actividad *in planta* en condiciones semicontroladas evidenciaron una eficacia del hongo superior al 50% con una esporulación de las larvas muertas superior al 80 %, al utilizar concentraciones entre 1.0×10^7 y 5.0×10^7 conidios/mL. Los resultados demostraron el alto potencial de *B. bassiana* Bv062 como agente de biocontrol para el futuro desarrollo de un bioinsecticida para el manejo de diferentes especies del complejo del barrenador del tallo de la caña de azúcar en este y otros cultivos hospederos.

Palabras clave: hongos entomopatógenos, control biológico, *Diatraea saccharalis*

Introduction

Diatraea saccharalis (Fabricius) is known to be one of the most economically important pests in sugarcane (*Saccharum officinarum*) production, where significant losses in yield are found in many parts of the United States and South America [1, 2]. This insect causes direct damage to the sugarcane by penetrating and opening galleries in the stem. The damaged tissue affects the weight of sugar production and produces the deterioration of the stems used as seed, as well as indirect damages occurring by allowing the entrance to opportunistic fungi that digest sugar and contaminate the juice, thereby decreasing its quality for sugar production [1, 3].

In Colombia there is a complex of borer species that affects sugarcane plantations in different areas of the country, with the presence of up to six species in the same region [1]. The losses produced by the insect are estimated as a reduction of 145 kg of

1. Vargas G, Gómez LA, Michaud JP. Sugarcane stem borers of the Colombian Cauca River Valley: Current pest status, biology, and control. Florida Entomol. 2015;98:728-35.

2. Guevara LA, Wiendl FM. Aspectos da biologia, en condições de campo, da broca da Cana-de-Açucar (*Diatraea saccharalis* (Frab,1794)). Agric Técnica. 1980;1:32-9.

sugar per hectare, per each infestation percentage unit, where the estimated decrease in tons of harvested sugarcane is around 0.826 % [4]. Agrochemicals have been used to control this pest, most of them have been inefficient due to the insect's cryptic habits, since its larval cycle develops within sugarcane stems [5]. Furthermore, the high toxicity and adverse effects of agrochemicals on the environment and human health have led to the search of other environmentally friendly alternatives. Biological control through natural enemies such as parasitoids and predators has been mostly used for pest control [6]; nonetheless, different factors have affected the efficacy of this strategy for the control of the borer complex. One of them is the presence of different species of *Diatraea* spp., for which there is a differential response to the parasitoids, indicating the need for several approaches in the pest management of these new species [7].

At the same time, some controller species cannot be produced massively, or their extensive use is limited, and they must be preserved in the agroecological system, which is, however, hampered by the indiscriminate use of agrochemicals and the disappearance of wild plants [1]. For this reason the use of entomopathogenic fungi as a complement to biological control with parasitoids is an interesting tool that should be included in integrated pest management programs; this is due to their high pathogenicity against different species, the fact that they may be produced and formulated at an industrial scale and that they can generate natural epizootic diseases in the field, as well as their natural dissemination in the environment [8, 9]. Several fungi (Hypocreales: Clavicipitaceae) including *Beauveria bassiana*, *B. brongniartii*, *M. anisopliae*, *I. fumosorosea*, *Hirsutella* sp., *Cylindrocarpon* sp., and *M. rileyi* have been isolated from *Diatraea* spp. and have become important agents for their microbial control in America [6]. In Colombia, in spite of its high potential, this strategy has been poorly used, and currently there is no biological pesticide registered for the control of any of the species of the sugarcane borer [10]. This underlines the importance of exploring the use of entomopathogenic fungi that may be developed into biopesticides for the control of *Diatraea* spp.

Among the previously mentioned fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Hyphomycetes: Moniliales) is one of the best known, widely distributed and used entomopathogenic fungi species for the control of many crop pests worldwide. It is a ubiquitous filamentous fungus with a high potential for the control of insects of different orders, such as Hepteroptera, Lepidoptera, Coleoptera, Hymenoptera and Diptera [8]. Its action mechanism is through the contact with the cuticle of the host, where it penetrates the exoskeleton reaching the hemocoel, through enzymatic mechanisms; later it invades the hemolymph where it consumes the necessary nutrients for its growth, thus colonizing all tissues and producing the death of the insect [11]. In this paper, an isolate of *Beauveria* sp. that had been recovered from a *Diatraea* spp. larva that was naturally infected in the field and obtained in a sugarcane plantation for panela (a piece of unrefined whole cane sugar) production in the Department of Boyacá

(Colombia), was identified and characterized, determining its potential as a biocontrol agent of the stem borer complex.

Materials and methods

Microorganism and culture medium

The microorganism used in this research was the isolate of *Beauveria bassiana* obtained from one larva of *Diatraea* sp. coming from a sugarcane plantation and collected in the Department of Boyacá; it was supplied by the Germplasm Bank for Microorganisms of Interest in Biological Control of the Colombian Corporation for Agricultural Research (AGROSAVIA), and coded as Bv062. The *B. bassiana* strain was grown in potato-dextrose agar (PDA), supplemented with 0.1 % chloramphenicol and incubated for 7 days at 25 °C.

Insects

The experiments were carried out with *D. saccharalis* larvae from the colony located at the Insect Rearing Unit of AGROSAVIA at the Research Center "Tibaitatá" (Mosquera-Colombia), which was established with larvae donated by Cenicaña (Colombian sugarcane Research Center). The larvae of *D. tabernella*, *D. indigenella* and *D. busckella* were also donated by Cenicaña from its rearing unit located at the Experimental Center in the Cauca Valley (Cali-Florida). The larvae were kept at 25 ± 5 °C with a relative humidity of 60 ± 10 % and a photoperiod of 16 h of light: 8 h of darkness, using an artificial diet described by Lastra and Gómez [12].

Ribosomal DNA amplification and sequencing

Fungal spores and mycelia were recovered with a loop from massive sporulated cultures grown on Petri dishes with PDA. The conidia (20 mg) were suspended in 200 μL of sterile water. The genomic DNA extraction was carried out with the ZR Fungal/Bacterial DNA MicroPrep™ Kit (Zymo Research) and the DNA was stored at –20 °C until it was used. For molecular identification, amplification by PCR was made at a ribosomal DNA (rDNA) region with universal primers, a DNA ribosomal region was amplified with primers that flanked ITS. This region is widely used for genera and specie classification and to calculate genetic distance among entomopathogenic fungi [13]. PCR amplification was performed in a total volume of 25 μL, which include 50 ng genomic DNA.

The PCR product was separated in 1 % agarose gel and visualized using SYBR® Safe (Thermo Fisher Scientific) in an UV transluminator. The PCR product was purified with the Wizard® SV Gel Kit and the PCR Clean-Up System (Promega, USA) and cloned in the pGEM-T (Promega, USA) plasmid. The fragment was finally sequenced in both directions with universal T7 and M13 primers. The sequences obtained were edited and aligned to obtain a consensus sequence that was compared to the representative sequences of different fungi species from the database of the National Center for Biotechnology Information (NCBI). Phylogenetic reconstruction was made with the Neighbor-Joining method, using the nucleotide substitution method of Kimura 2 parameters, with the MEGA program version 6 [13]

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including 25 sequences of the genera *Beauveria*, *Isaria*, *Metarhizium* and *Nomuraea* (Table 1).

Evaluation of the insecticidal activity of *B. bassiana* Bv062 against larvae of different *Diatraea* species

A seven days-old culture of the isolate Bv062 was used to prepare a suspension that was adjusted to 1×10^7 conidia/mL, which was topically inoculated by placing 2 µL on the dorsum of second instar larvae of *D. saccharalis*, *D. indigenella*, *D. tabernella* and *D. busckella*. The inoculated larvae were individually placed in plastic half-ounce containers with a grain of tender corn as the feeding substrate. The control treatment consisted of larvae that did not undergo any treatment. The completely randomized experimental design had three replicates per treatment with 10 larvae per replicate. Larval mortality was assessed every two days until all individuals died or formed the pupa. Mortality results obtained with treatments were corrected with the mortality in the control to calculate the efficacy using the formula of Schneider-Orelli [14].

Evaluation of the insecticidal activity of *B. bassiana* Bv062 against *D. saccharalis* eggs

A bioassay was carried out using the nebulization technique described by Carrera *et al.* [15] consisting of the use of the PulmoMate Sunrise® nebulizer connected to a PVC tube that was the pulverization chamber of the fungal suspension. Five suspensions of conidia adjusted to five concentrations of: 5×10^6, 1×10^7, 5×10^7, 1×10^8, 5×10^8 conidia/mL in a 0.1 % Tween 80 solution, were prepared. A volume of 5 mL was taken for each treatment and sprayed on three egg-masses of *D. saccharalis* collected 24 hours after laying, with a known number of eggs estimated by counting through a stereoscope. The spraying lasted two minutes and the inoculated eggs were left to dry in the room. The control treatment consisted of *D. saccharalis* eggs sprayed with a 0.1 % solution of Tween 80. The eggs were placed individually in half-ounce plastic containers with a grain of tender corn and a piece of dampened cotton. After 7 days, the infected eggs and the neonate larvae were counted using a stereoscope. The larvae were placed individually in half-ounce plastic containers with a grain of corn. Larval mortality was assessed every two days until all individuals died or formed the pupa. To confirm fungal infection, the dead larvae were collected and placed in a humid chamber at 25 °C for approximately 7 days.

Determination of the lethal concentrations of Bv062 on *D. saccharalis* larvae

A bioassay was carried out following the above described methodology, for which suspensions of conidia were prepared; these were adjusted to six concentrations of 5×10^5, 1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8 conidia/mL with which the *D. saccharalis* larvae were inoculated. Each larva was placed in a plastic container containing a grain of corn; they were allotted in groups of 10 units by triplicate, in Selloplus® boxes of 64 ounces, and incubated at 26 ± 2 °C, with 70 % RH, and mortality and sporulation were

Table 1. Fungus isolates used in the analysis of phylogenetic relationships

No.	Species	Isolate	Accession number
1	*Beauveria bassiana*	IMI 386696	AJ560672.1
2	*Beauveria bassiana*	IMI 386693	AJ560673.1
3	*Beauveria bassiana*	PL 6111	AJ345089.1
4	*Beauveria bassiana*	ARSEF 8259	HQ880801
5	*Beauveria bassiana*	ARSEF 2964	HQ880802
6	*Beauveria bassiana*	ARSEF 8257	HQ880800
7	*Beauveria pseudobassiana*	F-917	AB831685.1
8	*Beauveria pseudobassiana*	F-1200	AB831696.1
9	*Beauveria pseudobassiana*	F-1772	AB831710.1
10	*Beauveria australis*	ARSEF 4598	HQ880789
11	*Beauveria australis*	ARSEF 4580	HQ880788
12	*Beauveria brongniartii*	ARSEF 7058	HQ880773
13	*Beauveria sungii*	ARSEF 7281	HQ880815
14	*Beauveria malawiensis*	BCC 17613	HQ880824
15	*Beauveria malawiensis*	ARSEF 4755	HQ880823
16	*Isaria coleopterora*	CBS 102.73	AY624176
17	*Isaria japonica*	BCC 2808	AY624199
18	*Isaria fumosorosea*	CBS375.70	AY624183
19	*Isaria fumosorosea*	CBS107.10	AY624184
20	*Isaria ghanensis*	CBS105.73	AY624185
21	*Isaria fumosorosea*	CBS244.31	AY624182
22	*Isaria farinosa*	CBS 262.58	AY624179
23	*Isaria farinosa*	CBS 541.81	AY624180
24	*Metarhizium robertsii*	ARSEF 23	HM055440.1
25	*Nomuraea rileyi*	CG 129	EU553337.1

evaluated every two days. The control treatment consisted in non-inoculated larvae. The results of mortality were subjected to a Probit analysis with the POLO Plus program to determine lethal concentrations. To confirm fungal infection, the dead larvae were collected and placed in humid chambers at 25 °C for approximately 7 days.

Determination of insecticidal activity of *B. bassiana* Bv062 against *D. saccharalis* larvae on sugarcane plants

The bioassay was developed in the municipality of Barbosa, Santander (Colombia), having an average temperature of 22 ± 5 °C and relative humidity (HR) of 63.5 ± 10 %. Sugarcane plants of the RD7511 variety of three months of age were planted individually in pots and sprayed with 2 mL per plant with the treatments using a manual nebulizer. The treatments consisted in conidial suspensions of *B. bassiana* Bv062 adjusted to three concentrations (5×10^6, 1×10^7, 5×10^7 conidia/mL) and a control treatment where plants were sprayed with water. After applications, each plant was infested with five *D. saccharalis* larvae of five eight-day-old (second instar), and plants were placed outdoor and protected with a poly-shade net. Thirty plants per treatment were distributed in three experimental units of 10 plants each. The experimental design was completely randomized. After eight days, a destructive sampling was carried out, in which the plant was cut longitudinally, and all larvae found were removed (alive and dead). The live larvae were placed individually in plastic half-ounce containers with a grain of tender corn and taken to the laboratory to evaluate mortality, according to the above described methodology. The efficacy of the treatments was estimated with the formula of Abbott [16].

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Statistical analysis

The homogeneity of variances and the normality of results were determined through the Bartlett and Shapiro-Wilk tests with a 95 % confidence, respectively. After demonstrating normality, differences between treatments were determined by variance analysis (ANOVA) and Tukey test (95 %) through the statistical program Statistix (8.0).

*R*esults and discussion

Molecular identification of the selected isolate

For the molecular identification of the Bv062 isolate based on an ITS fragment, an amplimer of 595 bp was obtained (Figure 1).

The inferred phylogenetic reconstruction with the Neighbor-Joining method using 26 isolates from the GenBank, showed that the Bv062 isolate is grouped in the same clade as other *B. bassiana* isolates, with a high bootstrap value (1000 replicates) (Figure 2). In the tree there is a clear separation per group of isolates of the *Beauveria* genus, in relation to other genera such as *Isaria* or *Metarhizium*. The genetic distance calculated with the Kimura 2-parameter method showed that the distance between the Bv062 isolate compared to the three *B. bassiana* isolates of different geographical origin (United Kingdom, United States and Hungary) is less than 0.007, while the distance from other *Beauveria* species ranges between 0.01 (*B. varroae*) and 0.05 (*B. malawiensis*) (data not shown).

Previous studies have used the regions between ITS ribosomal genes for the identification of different genera of entomopathogenic fungi, demonstrating the usefulness of this region to discriminate isolates of the *Beauveria* sp, *Metarhizium*, and *Isaria* [17, 18] genera. Considering its morphological characteristics and its molecular identification, the Bv062 isolate was classified as *B. bassiana*.

Evaluation of the insecticidal activity of *B. bassiana* against larvae of different *Diatraea* species

The isolate of *B. bassiana* Bv062 was pathogenic for the second instar larvae of the four borer species evaluated in this study under laboratory conditions. This was observed through the mortality of the individuals, followed by mycelial growth, and finally abundant white sporulation, which produced the mummification of the bodies. These symptoms correspond to typical signs of infection by the *B. bassiana* isolate [19]. The mortality of the larvae ranged from 24 to 83 % (Figure 3); these values were not significantly different between the first three species, but they were different from *D. busckella* (F = 18.8, df = 11, p = 0.0006), indicating a lower degree of pathogenicity of the fungus in this species compared to the others.

The *B. bassiana* isolate evaluated in this study was obtained from a naturally infected larva from the field, which was collected in the sugarcane plantation of the department of Boyacá (Colombia). Here the *D. saccharalis*, *D. rosa* and *D. busckella* species have been identified within the borer complex that affects the crop, where the latter has a higher incidence compared to the other species (unpublished data). Possibly, the coexistence of the fungus with this species
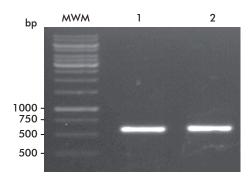

Figure 1. Agarose gel with amplimers obtained from an ITS region of *B. bassiana* Bv062. Molecular weight (MW) marker of DNA Ampigene® (DNA Ladder 250-10 000 bp). Lane 1: Bv062, Lane 2: Positive control.
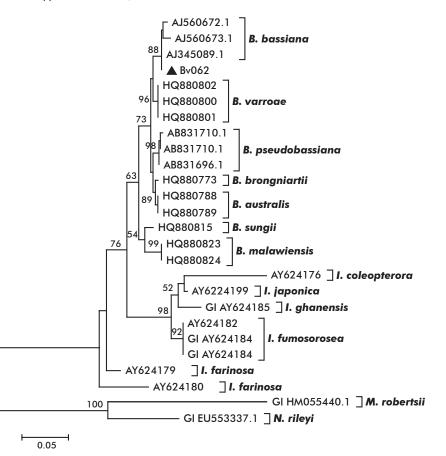

Figure 2. Phylogenetic relationships of the Bv062 isolate of *B. bassiana* (comparison with 25 isolates of the GenBank of the National Center for Biotechnology Information NCBI) based on the ITS region. Construction with the Neighbor-Joining method (bootstrap 1000 replicates) and distance by Kimura 2-P. The percentage of the replicate is shown above each branch.

in the same geographical region has generated the establishment of the *B. bassiana* in this host population without producing a drastic mortality because of the adaptation processes or their coevolution, resulting in a lower pathogenicity of the fungus or a greater resistance of the host [20]. Another hypothesis suggests that when there are different host populations within the same ecosystem, variations can be produced in

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the characteristics of the pathogen, such as its transmission, infection rate and fitness [21].

It should be noted that most of the work related to the entomological pathogenicity of the sugarcane borer reported in the literature has been carried out with the *D. saccharalis* species, and information is scarce on the activity of the *B. bassiana* on other species of the complex.

Evaluation of the insecticidal activity of *B. bassiana* Bv062 on eggs of *D. saccharalis*

The infection of the D. saccharalis eggs produced by the *B. bassiana* Bv062 isolate, ranged from 2 to 25 % (Table 2), where the highest concentration, was significantly higher compared to the others (F = 49.5; df = 14; p = 0.00). Mycelial growth was observed on the surface of the eggs, and there was an evident reddish color, which is a characteristic symptom of entomopathogenic fungus infection on insect eggs, as reported by other authors [22].

In relation to the recovery of the larvae hatching from the eggs, significantly lower values were found when the eggs were treated with the fungus at the three highest concentrations, compared to the control treatment, in which hatching was of 95.32 % (F = 19.8; df = 17; p = 0.00) (Table 2). Finally, the mortality of the hatched larvae was evaluated, ranging between 65 and 91 % according with the different evaluated concentrations. Larvae mortality was significantly higher when eggs were inoculated with the highest concentrations of 5×10^7, 1×10^8 and 5×10^8 conidia/mL (F = 30.6; df = 14; p = 0.00). The mortality of hatched larvae could be the result of fungal infection, due to the penetration of the fungus inside the egg, or the indirect acquisition of the inoculum from the contaminated chorion, during hatching [22].

These results demonstrate the ability of *B. bassiana* to produce the insecticidal effect, not only on the larvae of the insect, but also on the eggs, thereby extending the action spectrum of the fungus, since it can affect the pest insect during two stages of its development, *i.e.* eggs and larvae.

Determination of lethal concentrations of Bv062 on *D. saccharalis* larvae

The mortality of *D. saccharalis* larvae was directly dependent on fungal dose, which increased as the concentration of the inoculated suspension increased. During the initial days of infection, a pinkish coloring and the hardening of the body of the larva, were observed. Subsequently, a superficial mycelial growth was found on the dermis of the dead larva, until it was completely covered with the white mycelia and powdery white sporulation (Figure 4), all of which are typical symptoms confirming that the cause of death was the infection by *B. bassiana*.

Lethal concentrations LC_{50} and LC_{90}, the confidence limits and the slope, were calculated from the average of three replicates, and the results are presented in Table 3. Lethal concentrations were lower than those found for other isolates of *B. bassiana* evaluated on *D. saccharalis* larvae. For example, Zappelini [23] determined the LC_{90} of 1×10^8 conidia/mL, corresponding to five times that estimated
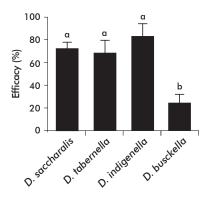
Figure 3. Mortality of larvae of different species of *Diatraea* sp. inoculated with a conidial suspension adjusted to a concentration of 1×10^7 conidia/mL of *B. bassiana* Bv062. Treatments with the same letter do not differ significantly according to the Tukey test with 95 % confidence.

Table 2. Effect of the inoculation of *D. saccharalis* eggs with different concentrations of *B. bassiana* Bv062

Concentrations (conidia/mL)	Egg infection (%)*	Larvae hatched (%)*	Corrected mortality of the larvae (%)*	Accumulated corrected mortality (%)*
Witness	–	95.32 a	–	–
5×10^6	2.28 c	94.40 ab	64.86 d	65.0 d
1×10^7	5.33 c	91.94 abc	72.89 cd	75.02 c
5×10^7	6.12 c	85.65 bc	81.72 ab	81.95 b
1×10^8	17.96 b	83.67 c	78.81 bc	82.34 b
5×10^8	25.51 a	73.41 d	87.21 a	90.89 a

* Treatments with the same letter show do not differ significantly according to the Tukey test with 95 % confidence.
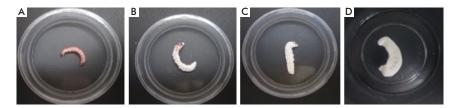
Figure 4. Progression of the postmortem infection of a *D. saccharalis* larva infected by conidia of *B. bassiana* Bv062. A) Completely rigid larva of a pink color (1-2 days postmortem); B) Notable growth of the white mycelia of Bv062 on the body of the larva (3 to 4 days postmortem); C) Body of the larva (5-6 days postmortem) completely coated with mycelia and conidia of Bv062; D) Mummified cadaver of a *D. saccharalis* larva with abundant and powdery sporulation of Bv062(7-8 days postmortem).

Table 3. Lethal concentrations of *B. bassiana* Bv062 on the 2nd instar larvae of *D. saccharalis*

LC_{50} (conidia/mL)	Limits of the CI		LC_{90} (conidia/mL)	Limits of the CI		Heterogeneity	
	Lower	Upper		Lower	Upper	χ^2	Slope
9.04×10^5	4.4×10^5	1.6×10^6	2.07×10^7	1.5×10^7	7.2×10^7	0.649	0.876

in this study for Bv062. Wenzel *et al*. [24] also estimated the LC_{50} of 1.58×10^7 conidia/mL for the Brazilian isolate IBCB 66. In another study, Alves *et al*. [25] obtained the LC50 of 4.8×10^6 conidia/mL for the Brazilian isolate *B. bassiana* 447 on the third stage larvae of *D. saccharalis*. However, it is not possible to compare the results obtained because of the methodological differences.

In relation to the mean lethal time (LT_{50}), this was of six days, and lethal time ninety (LT_{90}) was of approximately 12 days (Table 4). These values contrast with other studies such as those of Zappelini [11] who

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reported LT_{50} of 5.4 days, Wenzel *et al.* [24] with LT of 6 days at concentration of 1.58×10^7 conidia/mL and Díaz and Lecuona [26] with LT ranging from 2.9 to 7.78 days.

Determination of insecticidal activity of *B. bassiana* Bv062 against *D. saccharalis* larvae on sugarcane plants

After eight days of the evaluation, a high number of dead larvae were found inside plants and on their surface. Many of them were mummified, and showed evident sporulation. These results demonstrate that the foliar application of the entomopathogenic fungus enables the infection of larvae that crawl on the foliar surface, which is a normal behavior in borer larvae during the initial instars. The corrected mortality, using the mortality of the control treatment (7.9 %) was expressed as efficacy, and it is shown in Figure 5. Efficacy showed values of 46, 75 and 69 % with concentrations of 5×10^6, 1×10^7 and 5×10^7 conidia/mL respectively.

The efficacy obtained with the highest concentration (T1) was not significantly different from that obtained with the medium concentration (T2), but it was significantly higher than that of the lowest concentration (T3) ($F = 12.1$; $n = 2$; $p = 0.0078$). Sporulation of dead larvae was over 80 % in the three treatments, thus confirming the cause of death as a fungal infection. Considering that there are no significant differences in efficacy with T1 or T2, but there is a numerical difference between them, the concentration of 1×10^7 conidia/mL was selected as the minimum effective dose for further evaluations of this entomopathogenic agent in the field.

Considering that the volume of insecticides applied under field conditions ranges from 200 to 400 L/ha, the dose based on the selected concentration would range from 2×10^{12} to 4×10^{12} conidia/ha. This concentration is similar to that used by Estrada *et al.* [27], who applied 1×10^{12} conidia/ha in a homogeneous population of this pest in a sugarcane plantation in Cuba, where results showed a significant reduction in the population levels of *D. saccharalis* and the damage produced by it, with an important increase in yield. In another study, a commercial myco-insecticide (Mycotrol®) was evaluated under field conditions in a sugarcane plantation in Texas (USA) using a dose of 5×10^{13} conidia/ha. Although results showed a reduction in the incidence and the damage done by *D. saccharalis*, this reduction was not related to greater yields or the quality of the sugarcane juice; the authors considered that there was an insufficient coverage of the biological agent on the foliar surface [28]. Furthermore, the fungus used as the active ingredient in this biological agent was not isolated from *Diatraea* spp., for which reason its activity on this insect and its behavior, may be inferior to other strains.

Another important factor in the possible future use of this fungus in sugarcane is the effect on non-target organisms, considering that especially for *Diatraea* spp. natural enemies are important in the regulation of this pest. Although this fungus has a broad spectrum of action, current evidence suggests that the impact on other, non-target organisms, is minimum because

Table 4. Lethal times LT_{50} and LT_{90} of *B. bassiana* Bv062 on 2nd instar of *D. saccharalis* larvae

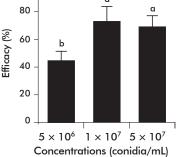| LT_{50} (days)	Confidence Limits		LT_{90} (days)	Confidence Limits		Heterogeneity	
	Lower	Upper		Lower	Upper	χ^2	Slope
6.07	3.77	8.09	12.25	9.067	23.08	28.77	4.14

Figure 5. Efficacy of Bv062 against *D. saccharalis* larvae in sugarcane plants under semicontrolled conditions. Error bars represent the standard deviation of three replicates. Results with the same letter do not differ significantly according to the LSD test at 95 % confidence.

of its specificity [8]. In previous studies, the use of entomopathogenic agents such as *B. bassiana* and *M. anisopliae* showed no effect against *Cotesia flavipes* (Hymenoptera: Braconidae) and on *Trichogramma* sp. (Hymenoptera: Trichogrammatidae) [29, 30], which are the main parasitoids of *D. saccharalis*. This suggests that these two control tools can be used simultaneously within an integrated pest management program for sugarcane plantations, although specific studies should be carried out with the *B. bassiana* Bv062 isolate.

Conclusions

The *B. bassiana* Bv062 isolate is a potential agent for the control of eggs and larvae of *D. saccharalis* and some species of the borer complex affecting sugarcane in Colombia. Under controlled conditions, *B. bassiana* Bv062 is able to reduce the population of insect pests, which makes it a viable alternative for the development of a biopesticide. Studies should be continued on the formulation of the fungus, its evaluation under field conditions by spraying the crop, the determination of persistency, studies on non-targeted species, among others, to define the viability of its use in sugarcane plantations.

Acknowledgements

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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