

Efficacy of entomopathogenic fungi against *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) under laboratory conditions

Eficiencia de hongos entomopatógenos contra *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) bajo condiciones de laboratorio

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ABSTRACT

Rhyzopertha dominica (Fabricius) (Coleoptera: Bostrichidae) is a primary insect pest of stored grains. The aim of this study was to evaluate the efficacy of 10 different isolates of entomopathogenic fungi as biocontrol agents against *R. dominica*. The chosen isolates were *Beauveria bassiana* CEP 545, CEP 560 and CEP 567, *Metarhizium robertsii* CEP 381 and CEP 401, *M. anisopliae sensu lato* CEP 615, CEP 616 and CEP 617 and *Cordyceps (=Isaria) fumosorosea* CEP 303 and CEP 309. Insects were sprayed with a conidial suspension of each fungus and incubated for 15 days under laboratory conditions. Insect mortality (expressed as percentage) and median survival times (MST) were estimated. *Beauveria bassiana* caused the highest mortality (47-65%), whereas the other fungal isolates caused a maximum mortality of 21%. The *B. bassiana* CEP 545 and CEP 567 isolates led to an MST of 8 and 9 days, respectively. Among all the isolates tested, *B. bassiana* CEP 545 was the most efficient as a biocontrol agent. The median lethal concentration (LC₅₀) of this isolate was calculated with five concentrations on day 15 after the treatment. The insect mortality increased with the highest isolate concentrations and the LC₅₀ was estimated in 9.54x10⁹ conidia mL⁻¹. This study confirmed that some *B. bassiana* isolates, especially CEP 545, could be used as biocontrol agents against *R. dominica*.

Keywords

biological control • *Beauveria bassiana* • stored grains • *Rhyzopertha dominica* • alternative control

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RESUMEN

Rhyzopertha dominica (Fabricius) (Coleoptera: Bostrichidae) es una plaga de insectos primaria en granos almacenados. El objetivo de este estudio fue evaluar la eficiencia de 10 diferentes aislamientos de hongos entomopatógenos como agentes de biocontrol contra *R. dominica*. Los aislamientos elegidos fueron *Beauveria bassiana* CEP 545, CEP 560 y CEP 567, *Metarhizium robertsii* CEP 381 y CEP 401, *M. anisopliae sensu lato* CEP 615, CEP 616 y CEP 617 y *Cordyceps (=Isaria) fumosorosea* CEP 303 y CEP 309. Los insectos se rociaron con una suspensión de conidios de cada hongo e incubaron por 15 días bajo condiciones de laboratorio. Se estimó la mortalidad de los insectos (expresada como porcentaje) y el tiempo de supervivencia media (TSM). *Beauveria bassiana* causó la mayor mortalidad (47-65%), mientras que los otros aislamientos fúngicos causaron una mortalidad máxima de 21%. Los aislamientos de *B. bassiana* CEP 545 y CEP 567 condujeron un TSM de 8 y 9 días, respectivamente. Entre todos los aislamientos evaluados, *B. bassiana* CEP 545 fue el más eficiente como agente de biocontrol. La concentración letal media (CL₅₀) de este aislamiento se calculó con cinco concentraciones el día 15 después del tratamiento. La mortalidad de los insectos incrementó con las concentraciones más altas y la CL₅₀ se estimó en 9.54x10⁹ conidios mL⁻¹. Este estudio confirmó que algunos aislamientos de *B. bassiana*, especialmente CEP 545, podrían usarse como agentes de biocontrol contra *R. dominica*.

Palabras clave

Control biológico • *Beauveria bassiana* • granos almacenados • *Rhyzopertha dominica* • control alternativo

INTRODUCTION

Insect pests are a major cause of post-harvest losses in stored grains, which are between 10 and 25% per year. The presence of insects affects the weight, nutrients, and percentage of germination of grains, thus decreasing the value of their commercial quality (1). Among these insects, the lesser grain borer *Rhyzopertha dominica* (Coleoptera: Bostrichidae), which is found in the post-harvest handling of grains in several regions of the world (7), is the most destructive insect pest and the most difficult to control because it can develop inside the grains (18). In Argentina, this becomes essential because marketing standards prohibit the sale of merchandise infected with live insect pests (6).

Conventional control of insect pests in the post-harvest handling of cereal crops has been traditionally based on chemical insecticides. However, their excessive use has caused insecticide resistance, chemical residuality, elimination of beneficial insects, environmental pollution, and human toxicity (3). Thus, several researchers explored the possibility of using biological control and found that some insect pests can be controlled by entomopathogenic fungi as *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Sordariomycetes) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Sordariomycetes) under laboratory conditions and field assays (4, 10, 12, 14, 15, 16, 19). These fungi are of low cost production, are easy to formulate, exist naturally, and exhibit variable efficiency against different insect species (5, 8). In addition, they are safe biocontrol agents, of low environmental impact, without residual activities or mammalian toxicity, and less vulnerable to resistance development (2).

In this sense, we hypothesized that entomopathogenic fungi can be used as control agents against insect pests of stored grains. Thus, the aim of the present study was to evaluate the efficacy of 10 isolates of four different species native to Argentina as biocontrol agents against *R. dominica*, under laboratory conditions.

MATERIALS AND METHODS

Adults of *R. dominica* were reared at our laboratory, located in La Plata (34°54'39" S, 57°55'46" W), Province of Buenos Aires, Argentina. These insects were maintained in glass jars (150 mL) with pearl barley whole grains. The jars were covered with a muslin cloth and

incubated at 25 ± 2 °C and 60 ± 5 % relative humidity (RH). All the individuals used in the experiments were 3-week-old mixed-sex adults.

The fungal isolates used came from different geographical areas of Argentina and had been isolated from different sources (soil or insects). The isolates were deposited and preserved at the Entomopathogenic Fungal Culture Collection at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE) in La Plata (Buenos Aires, Argentina), and the ARSEF Culture Collection (USDA-ARS) in Ithaca (N.Y., USA). The virulent capacity of the fungal isolates was stimulated by using them to infect adults of *R. dominica*, from which they were later re-isolated in pure cultures before using them to analyze their biological activity through bioassays (table 1).

Table 1. Source, host, geographical origin, and germination percentage of the fungal isolates used.

Tabla 1. Fuente, huésped, origen geográfico y porcentaje de germinación de los aislados de hongos utilizados.

Fungal isolates	Collection number	Source of isolates	Original host (Order: Family)	Geographical origin	Germination of conidia (%) \pm SE
<i>B. bassiana</i>	CEP 545	Insect	<i>Acromyrmex lobicornis</i> (Hymenoptera: Formicidae)	Neuquén	98.92 \pm 4.23
<i>B. bassiana</i>	CEP 560	Insect	<i>Edessa meditabunda</i> (Hemiptera: Pentatomidae)	Santa Fe	93.67 \pm 9.94
<i>B. bassiana</i>	CEP 567	Insect	<i>Acromyrmex lundii</i> (Hymenoptera: Formicidae)	Buenos Aires	98.42 \pm 5.10
<i>M. robertsii</i>	CEP 381 - ARSEF 727	Soil	<i>Tenebrio molitor</i> (Coleoptera: Tenebrionidae)	San Juan	98.00 \pm 5.72
<i>M. robertsii</i>	CEP 401 - ARSEF 2575	Soil	<i>Tenebrio molitor</i> (Coleoptera: Tenebrionidae)	San Juan	97.42 \pm 6.48
<i>M. anisopliae sensu lato</i>	CEP 615	Soil	<i>Tenebrio molitor</i> (Coleoptera: Tenebrionidae)	Buenos Aires	97.67 \pm 6.16
<i>M. anisopliae sensu lato</i>	CEP 616	Soil	<i>Tenebrio molitor</i> (Coleoptera: Tenebrionidae)	Buenos Aires	97.25 \pm 6.68
<i>M. anisopliae sensu lato</i>	CEP 617	Soil	<i>Tenebrio molitor</i> (Coleoptera: Tenebrionidae)	Buenos Aires	97.17 \pm 6.77
<i>C. fumosorosea</i>	CEP 303	Insect	<i>Trialeurodes vaporariorum</i> (Hemiptera: Aleyrodidae)	Buenos Aires	98.33 \pm 5.23
<i>C. fumosorosea</i>	CEP 309	Insect	<i>Trialeurodes vaporariorum</i> (Hemiptera: Aleyrodidae)	Buenos Aires	97.42 \pm 6.48

Three isolates of *B. bassiana* (CEP 545, CEP 560 and CEP 567), two of *Metarhizium robertsii* (CEP 381 and CEP 401), three of *M. anisopliae sensu lato* (CEP 615, CEP 616 and CEP 617) and two of *Cordyceps fumosorosea* (CEP 303 and CEP 309) were cultured either on Potato Dextrose Agar (CEP 545, CEP 560, CEP 567, and CEP 401) or Sabouraud Dextrose Agar + yeast (CEP 615, CEP 616, CEP 617, CEP 303, CEP 309, and CEP 381) in sterile Petri dishes (90 mm in diameter), and incubated at 25 ± 1 °C for 15 days in darkness. After that time, conidia were harvested by scraping them with a sterile spatula and suspended in 0.01% (v/v) Tween 80 (sodium polysorbate) in sterile distilled water. The conidial suspension was vortexed for 2 minutes to homogenize it. The concentration of conidial suspension was adjusted to 2×10^8 conidia·mL⁻¹ according to preliminary tests and quantified by using a hemocytometer (Neubauer chamber). The conidial viability of each fungal isolate was calculated through germination, according to Lane *et al.* (1988). The conidial germination percentage of all fungal isolates used was > 90%.

A total of 20 adults of *R. dominica* per replicate were used. Controls without fungal application were performed for each fungal isolate, and two replicates of each treatment were conducted. The experiment was repeated twice at different times. Before the bioassay, the adult insects were maintained under starvation for 24 h. The conidial suspensions were applied by spraying, using an airbrush (FENGDA, BD-180, China). Insects were sprayed with 100 μ L of each conidial suspension (2×10^8 conidia·mL⁻¹ in 0.01% (v/v) Tween 80), in sterile distilled water (final concentration: 1×10^7 conidia·mL⁻¹). Control insects were sprayed with only 100 μ L of 0.01% Tween 80 in sterile distilled water. After inoculation, the insects were

introduced into a glass jar (150 mL) with three grams of pearl barley grains. The glass jars were covered with a muslin cloth and incubated for 15 days at 25 ± 2 °C, 60 ± 5 % RH and a photoperiod of 12:12 h (light:dark). Dead insects were removed daily, surface-sterilized with 70% alcohol and sterile distilled water, and then placed in plastic Petri dishes (60 mm in diameter) with water agar (1%). Cultures were then incubated at 25 ± 2 °C for additional seven days to allow the development of fungi on the dead insects. The mortality caused by the entomopathogenic fungi was confirmed by observing sporulation on the dead insects, and by the fact that it was possible to subculture them in pure cultures. For that purpose, the dead insects were inoculated in sterile media and fungal growth on each insect was confirmed (figure 1). Fungal infection was also confirmed by dissecting the insects to observe their tissues under an optical microscope. Insect mortality showing fungal outgrowth (%) and the median survival time (MST) were calculated.

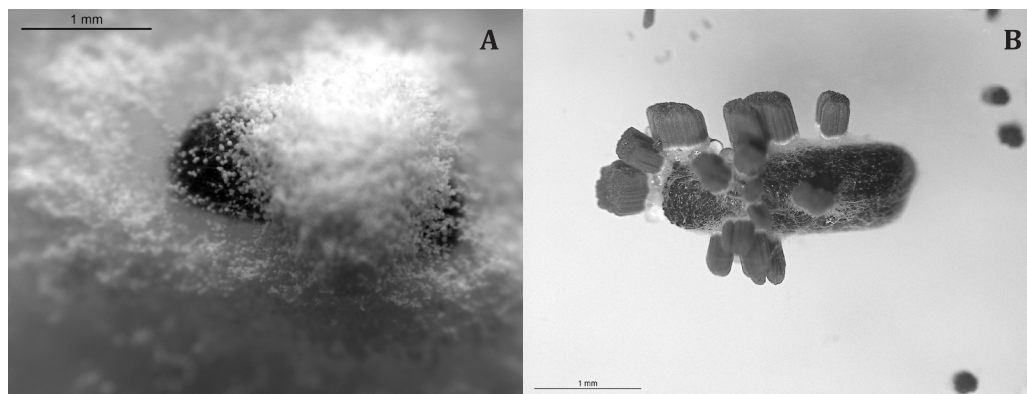


Figure 1. *R. dominica* adults infected by (a) *B. bassiana* and (b) *M. robertsii* (1 mm).

Figura 1. Adultos de *R. dominica* infectados por (a) *B. bassiana* y (b) *M. robertsii* (1 mm).

The results of this first bioassay showed that, among all the isolates tested, *B. bassiana* CEP 545 was the most efficient as a biocontrol agent. Thus, its median lethal concentration (LC_{50}) was next evaluated with five concentrations of conidial suspension: 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , and 1×10^{10} conidia·mL⁻¹. This second bioassay was carried out under the same conditions and procedure as those described above. A total of 20 adults of *R. dominica* per replicate were used. Controls without fungal application were performed for each fungal isolate, and two replicates of each treatment were conducted. The experiment was repeated twice at different times.

Statistical differences among the percentages of mortality of each isolate were analyzed by a chi-square test. The MST of *R. dominica* was computed with the Kaplan-Meier (K-M) method, and statistical differences among them were analyzed by a log-rank test. When a significant difference was found ($p < 0.05$), the comparisons of pairs were adjusted according to the Sidak method (11). The LC_{50} was calculated using Probit analysis.

Analyses were performed using software R version 3.5.1, and the LC_{50} was analyzed with the 'lc' function described by Savi *et al.* (2017).

RESULTS AND DISCUSSION

The viability of all the fungal isolates, evaluated through the average germination percentage of conidia, ranged from 93.67 to 98.92% (table 1, page 319). This is in agreement with the results of Mahdneshin *et al.* (2009), who also indicated that the germination of all *B. bassiana* and *M. anisopliae* isolates tested ranged from 80 to 94%.

Several studies have shown the high potential of entomopathogenic fungi as an effective and economically feasible alternative for the control of insect pests in stored products (1, 14, 16, 19). In his review on this topic, Edde (2012) explained that the mortality achieved depends on the insect species which is intended to control, with *R. dominica* being the insect pest of stored products most difficult to control with insecticide grain protectants in many countries.

The percentage of mortality of *R. dominica* found in the present study for each fungal isolate tested is presented in table 2. The results indicated that three out of the 10 fungal isolates tested were effective against *R. dominica* at conidial concentrations of 2×10^8 conidia·mL⁻¹, with *B. bassiana* isolates being more pathogenic than those of *M. robertsii*, *M. anisopliae sensu lato*, and *C. fumosorosea*. The *B. bassiana* isolates CEP 545 and CEP 567 caused a mortality rate higher than 50% (between 47.50 and 65%).

In particular, the *B. bassiana* isolate CEP 545 caused a mortality rate of 65% (n=80), in contrast to the *M. anisopliae sensu lato* isolate CEP 381, which caused a mortality rate of 8.75% (n=80). These results are in agreement with those of Padín *et al.* (1995), who reported that the *B. bassiana* isolates studied by them showed a high percentage of mortality against *R. dominica* at 6 and 15 days after starting the treatment, whereas those of *M. anisopliae*, *Metarhizium (=Nomuraea) rileyi* and *Lecanicillium lecanii* showed a percentage of mortality lower than 30% for the dusting and spraying techniques, although the dusting technique was more effective than the spraying technique.

Similarly, Mahdeshin *et al.* (2009) indicated that the cumulative mortality of *R. dominica* after 7 days varied from 14.78 with the lowest concentrations of *M. anisopliae* (1.5×10^4 conidia·mL⁻¹) to 89.35% (1.1×10^{10} conidia·mL⁻¹) with the highest concentration of *B. bassiana* by using the immersion technique. In contrast, Abdel-Raheem *et al.* (2015) showed that *M. anisopliae* isolates were more effective against insect pests than *B. bassiana* isolates, where the LC₅₀ values were 1.2×10^5 and 2.7×10^5 conidia·g⁻¹, respectively, and the mortality percentages were 79.3% conidia g⁻¹ and 50.3% conidia g⁻¹ respectively. These authors attributed these differences to the pathogenicity effect of the different isolates (13).

Other reports, such as that by Moino *et al.* (1998), showed that the effectiveness of different isolates of *B. bassiana* and *M. anisopliae*, applied by direct inoculation with sporulating fungal colonies, caused high mortality against *R. dominica*. Lord (2001) also reported high mortalities of *R. dominica* when *B. bassiana* was applied in combination with diatomaceous earth (95.8%) as compared with that without diatomaceous earth (84.2%), as a result of the synergism between the cuticle of the insect and diatomaceous earth. Barra *et al.* (2013) evaluated 20 isolates of *Purpureocillium lilacinum* and reported that the percentages of mortality of *R. dominica* varied from 15 to 65% and that the median lethal time (LT₅₀) varied from 9.3 to 48.38 days.

Table 2. Mortality (%) (mean ± SE) and median survival time (MST± SE) of *R. dominica* adults treated with fungal isolates 15 days after inoculation.

Tabla 2. Mortalidad (%) (media ± ES) y Tiempo de Supervivencia Media (TSM± ES) de adultos de *R. dominica* tratados con aislamientos de hongos después de 15 días de inoculación.

Collection number	Fungal isolates	Mortality (%) ± SE	MST (days) ± SE
CEP 545	<i>Beauveria bassiana</i>	65 ± 5.33 a	8 ± 0.52
CEP 560	<i>Beauveria bassiana</i>	47.50 ± 5.58 a	15 ± 0.52
CEP 567	<i>Beauveria bassiana</i>	60 ± 5.48 a	9 ± 0.51
CEP 381	<i>Metarhizium robertsii</i>	8.75 ± 3.16 b	15 ± 0.27
CEP 401	<i>Metarhizium robertsii</i>	10 ± 3.35 b	15 ± 0.39
CEP 615	<i>Metarhizium anisopliae sensu lato</i>	10 ± 3.35 b	15 ± 0.24
CEP 616	<i>Metarhizium anisopliae sensu lato</i>	21.25 ± 4.57 b	15 ± 0.38
CEP 617	<i>Metarhizium anisopliae sensu lato</i>	17.50 ± 4.25 b	15 ± 0.39
CEP 303	<i>Cordyceps fumosorosea</i>	12.50 ± 3.70 b	15 ± 0.36
CEP 309	<i>Cordyceps fumosorosea</i>	21.25 ± 4.57 b	15 ± 0.40

*Mean of the percentage of mortality with the same letter does not show differences ($p < 0.05$).

*Media del porcentaje de mortalidad con la misma letra no muestra diferencias ($p < 0,05$).

In the present study, all the fungal isolates evaluated decreased the survival rate of *R. dominica* (figure 2, page 322). In particular, all *B. bassiana* isolates, with the exception of *B. bassiana* CEP 560, caused a higher mortality rate and shorter MST than the *M. robertsii*, *M. anisopliae sensu lato*, and *C. fumosorosea* isolates. The *B. bassiana* CEP 545 and CEP 567 isolates led to an MST of 8 and 9 days, respectively, whereas the other fungal isolates led to an MST of 15 days (table 2). The survival time of *R. dominica* adults treated with the fungal

isolates was significantly different from that of the untreated controls. However, there were no significant differences between the MST caused by the same isolates. The results of Mahdneshtin *et al.* (2009) showed that *B. bassiana* and *M. anisopliae* isolates were pathogenic and caused mortality of *R. dominica*, but reported a different LT_{50} with an average of 7.77 days for *B. bassiana* and an average of 7.86 days for *M. anisopliae*. Similar results were obtained by Mohamed *et al.* (2016), who recorded LT_{50} of 7.80, 7.19, 6.48, and 5.48 days for concentrations of 5×10^6 , 5×10^7 , 5×10^8 , and 5×10^9 conidia·mL⁻¹ of *B. bassiana* respectively. Furthermore, Akmal *et al.* (2017) showed an LT_{50} of 6.78 days at a concentration of 2×10^8 conidia·mL⁻¹ of *B. bassiana* and found that the time required to cause 50% mortality decreased as the concentration of entomopathogenic fungi increased. Batta (2005) reported high mortalities of adult *R. dominica* 7 days after treatment with *M. anisopliae*, which ranged from 86.7% to 93.3% depending on the application methods used. They pointed out that the treatment with the dust formulation led to high levels of adult mortality (86.7–88.0%) and only very low infestation rates by the insect (1.0%). Rice and Cogburn (1999) recorded mortality rates of 42%, 69% and 88% after 7, 14 and 15 days, respectively with *B. bassiana* when *R. dominica* was reared on red flour beetle medium containing 2×10^8 conidia g⁻¹. These results show that the effectiveness of entomopathogenic fungi depends on both the fungal concentration and the time elapsed after the treatment.

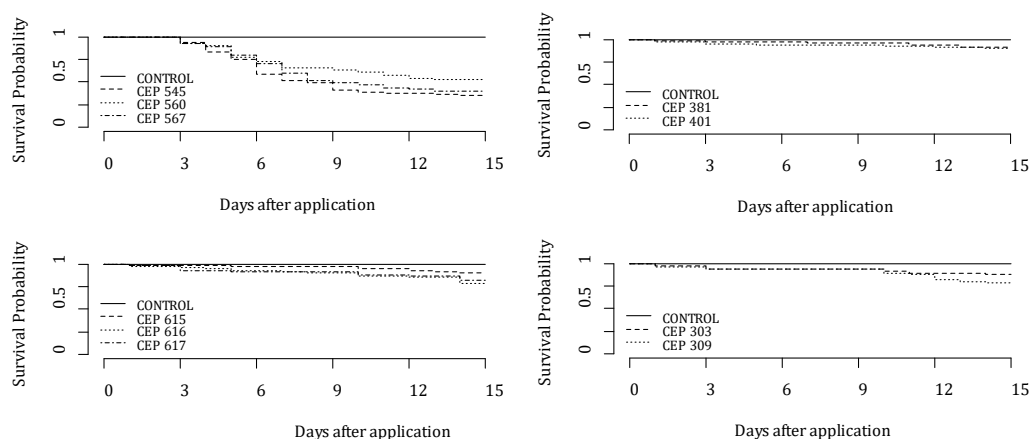


Figure 2. Survival of *R. dominica* adults after treatment with *B. bassiana* isolates CEP 545, CEP 560, and CEP 567, *M. robertsii* isolates CEP 381 and CEP 401, *M. anisopliae sensu lato* isolates CEP 615, CEP 616, and CEP 617, and *C. fumosorosea* isolates CEP 303 and CEP 309.

Figura 2. Supervivencia de adultos de *R. dominica* después del tratamiento con aislamientos de *B. bassiana*: CEP 545, CEP 560 y CEP 567, aislamientos de *M. robertsii* CEP 381 y CEP 401, aislamientos de *M. anisopliae sensu lato* CEP 615, CEP 616 y CEP 617 y aislamientos de *C. fumosorosea*: CEP 303 y CEP 309.

In the present study, the percentage of mortality with *B. bassiana* CEP 545 increased as the concentration increased. The highest percentage of mortality reached was 78.75% at a concentration of 1×10^{10} conidia·mL⁻¹. The LC_{50} was estimated in 9.54×10^9 conidia·mL⁻¹, 15 days after fungal application (table 3, page 323). This is in agreement with that previously reported by Mahdneshtin *et al.* (2009), who showed that isolates of *B. bassiana* and *M. anisopliae* were more effective against adult *R. dominica* with higher conidial concentrations and LC_{50} values of 9.6×10^5 to 1.9×10^7 conidia·mL⁻¹. The results from Akmal *et al.* (2017) when testing *B. bassiana* and *Isaria fumosorosea* (*C. fumosorosea*) against *R. dominica* showed that the mortality of adults and immature stages was dose-dependent and increased as concentrations of entomopathogenic fungi increased. Similarly, Wakil and Ghazanfar (2010) demonstrated that an increase in the concentration and exposure interval led to an increase in mortality of *R. dominica* when treated with *M. anisopliae* (8×10^9 conidia·kg⁻¹) and, correspondingly, to decreases in the production of the progeny of adults. Abdel-Raheem *et al.* (2015) recorded the maximum cumulative mortality percentage of *R. dominica* (50.3%)

11 days after treatment with the highest concentrations of *B. bassiana* (0.42×10^6 conidia g^{-1}) and the minimum mortality (4%) 3 days after treatment with the lowest concentrations (0.12×10^6 conidia g^{-1}). Also, in agreement with these results, Mohamed *et al.* (2016) determined that the percentage of mortality for the adult of *R. dominica* increased (77%) with the concentration of *B. bassiana* (5×10^9 conidia mL^{-1}) after 7 days, and that the LC_{50} value was 5.48×10^7 conidia mL^{-1} . Therefore, the results of our tests regarding the increase in the percentage of mortality at the highest doses are in accordance with previous reports.

Table 3. Mortality (%) (mean \pm SE) of *R. dominica* adults treated with conidia *B. bassiana* CEP 545 at different concentrations.

Tabla 3. Mortalidad (%) (media \pm ES) de adultos de *R. dominica* tratados con conidios de *B. bassiana* CEP 545 a diferentes concentraciones.

Concentration (conidia mL^{-1})	Mortality (%) \pm SE
Control	5 \pm 3.45 a
1×10^6	12.50 \pm 3.70 a
1×10^7	30 \pm 5.12 ab
1×10^8	46.25 \pm 5.57 b
1×10^9	52.50 \pm 5.58 b
1×10^{10}	78.75 \pm 4.57 c

*Mean of the percentage of mortality with the same letter does not show differences ($p < 0.05$).

*Media del porcentaje de mortalidad con la misma letra no muestra diferencias ($p < 0,05$).

CONCLUSION

The *B. bassiana* native strains used in this research, especially CEP 545, showed high mortality rates and low LT_{50} . Thus, these isolates could be potentially used as biocontrol agents of *R. dominica* adults in stored grains. Although further research needs to be carried out, our preliminary results showed that some *B. bassiana* native isolates could be studied more deeply to develop the best formulation methods to be used against *R. dominica* in field trials.

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