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Potential of Two *Metarhizium anisopliae* (Clavicipitaceae) Isolates for Biological Control of *Diatraea saccharalis* (Lepidoptera: Crambidae) Eggs

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Abstract

Chemical pesticides tend to accumulate in soil, resulting in human and environmental health risks. Hence, alternative methodologies involving chemical pesticides are beneficial for the control of agricultural pests. *Metarhizium anisopliae* is an entomopathogenic fungus that acts on different developmental stages of pest insects such as *Diatraea saccharalis*, a holometabolic lepidopteran with high potential for infestation in sugarcane crops. The present study evaluated the biocontrol effect of *M. anisopliae* isolates MT and E9 on *D. saccharalis* eggs at different ages by investigating the external and internal morphological alterations in treated eggs. Conidial suspensions of *M. anisopliae* isolated from MT and E9 at concentrations of 10^7 conidia/mL were applied to eggs of *D. saccharalis* aged 0, 24, 48, 72, 96 and 120 h. The eggs were observed every 24 h during development (0 h to 144 h). Samples were collected for observational, histological, and ultrastructural analyses. We found that the MT isolate caused 100% inviability of eggs aged 0 - 72 h, 144 h after the bioassays, while the effect of the E9 isolate varied between 49.40% and 93.75%. Melanization was observed on the periphery of the eggs 24 h after the bioassays. Fungal hyphae developed 48 h after bioassays, crossed the egg chorion, and dispersed through the yolk region, inhibiting embryonic development. After 72 h, hyphae and conidiophores were observed on the eggs, which persisted for 144 h. In sum, *M. anisopliae* MT isolate can be used as a biological controller for *D. saccharalis* eggs.

Keywords

Entomopathogen, Fungi, Management, Sugarcane, Sustainability

1. Introduction

Chemical pesticides (e.g., fungicides, herbicides, nematicides, and insecticides) [1] play an important role in the development of different agricultural cultures [2]. However, several active molecules accumulate in the soil [3] [4] [5] and pose a risk to human health and the environment [6] [7] [8] [9].

Biological control is an alternative method of managing agricultural pests, reducing chemical pesticide consumption [10]. *Metarhizium anisopliae* (Sorokin 1883) (Clavicipitaceae) is an entomopathogenic fungus present in soil [11], that demonstrates resistance to UV radiation [12], and acts on different stages of insect development [13] [14] [15]. *M. anisopliae* synthetizes and secretes enzymes (lipase, protease, and chitinase) that facilitate the penetration of fungal hyphae through insect barriers [16] [17]. Moreover, fungal hyphae disperse and colonize insects, resulting in inanition or septicemia [18].

The biocontrol potential of *M. anisopliae* includes its action on coleopterans [19], dipterans [20] and lepidopterans [21]. *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) is a holometabolous lepidopteran that causes damage to sugarcane during the larval phase [22] [23]. There are several studies on the effects of entomopathogenic fungal isolates on the larvae and pupae of insects; however, there is a lack of research on the biological control of eggs [15] [24] [25] [26] [27].

The utilization of bioinsecticides is safe for environmental and agricultural use [28] [29] [30] and the application of different *M. anisopliae* isolates could reduce the viability of *D. saccharalis* eggs by interfering or inhibiting the embryonic development. Consequently, the present study evaluated the biocontrol effect of *M. anisopliae* isolates MT and E9 on *D. saccharalis* eggs at different ages by observing the external and internal morphologic alterations. This study provides data on the entomopathogenic action of *M. anisopliae* isolates. This information may be used for developing new methodologies in integrated pest management (IPM) and models for future research on the target organisms.

2. Material and Methods

2.1. Insects

D. saccharalis eggs at ages 0, 24, 48, 72, 96 and 120 h were provided by the Laboratory of Biological Control, Morphology and Cytogenetics of Insects, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá (UEM), Maringá, Paraná, Brazil. The eggs were washed in sterilized distilled water (pH 7, and temperature of 25˚C) and kept in Petri dishes (90 cm

[diameter] × 1.5 cm [height]) at 25˚C ± 2˚C, at a relative humidity (RH) of 70% ± 10%, and 12:12 light and dark cycles [31] [32].

2.2. Fungal Isolates

Two isolates of *M. anisopliae* (MT and E9) were provided by the Laboratory of Microbial Biotechnology, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá (UEM), Maringá, Paraná, Brazil. The isolates were cultivated in Petri dishes (90 cm [diameter] × 1.5 cm [height]) with Potato-Dextrose Ágar® (PDA) (Heywood, United Kingdom) for 240 h.

2.3. Bioassays

Conidial suspensions of MT and E9 at a concentration of 10^7 conidia/mL were diluted in Tween® 80 0.02% (LabSynth, Diadema, Brazil). The Tween® 80 was diluted in sterilized distilled water at pH 7 and 25˚C (v/v). The conidial concentration was standardized in a Neubauer chamber. *D. saccharalis* eggs into different ages (0, 24, 48, 72, 96 and 120 h) were separated into groups (n = 50 for age), and 100 μL of MT and E9 was applied on their surfaces. Control eggs were treated with a 0.02% solution of Tween® 80. We performed three repetitions for each treatment.

2.4. Observational Analysis

D. saccharalis eggs were observed at intervals of 24 h, from 0 to 144 h after the treatments, using a Zeiss stereomicroscope (Carl Zeiss, Gottingen, Germany). Samples were stained with toluidine blue and observed using an Omicron medical microscope Axioskop 40 (Carl Zeiss, Gottingen, Germany) and acquired using AxioCam MRc (Carl Zeiss, Oberkochen, Germany).

2.5. Light Microscopy

Both control and (0 h) *M. anisopliae* MT treated *D. saccharalis* eggs, were collected 48-h after the treatments (n = 10) and fixed in Bouin solution (picric acid, formaldehyde, and acetic acid in 7.5:2:0.5 (v/v) ratio) for 24 h at room temperature (25˚C ± 2˚C). Subsequently, the eggs were dehydrated in a graded alcohol series (70%, 80%, 90%, and 100%; v/v), diaphanized in xylol, embedded in Paraplast® (Leica Biosystems, Wetzlar, Germany), and sectioned to 7 μm using a Leica RM 2250 microtome (Leica Biosystems, Wetzlar, Germany). The samples were then stained with periodic acid Schiff (PAS). Images were analyzed using an Omicron Medical microscope (Axioskop 40; Carl Zeiss, Gottingen, Germany) and captured with an Axiocam Mrc (Carl Zeiss, Oberkochen, Germany).

2.6. Scanning Electron Microscopy

For SEM, control *D. saccharalis* eggs at 0 h of age and eggs treated with *M. anisopliae* isolate MT were collected 24, 48, 72, and 96 h after the beginning of the bioassays (n = 4 per period). The eggs were fixed in alcoholic Bouin solution

(picric acid, formaldehyde, and acetic acid in 7.5:2:0.5 (v/v) ratio for 24 h at room temperature (25˚C). The samples were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%, v/v). After dehydration, the samples were subjected to critical point drying (Leica EM CPD030, Leica Biosystems, Wetzlar, Germany). All the samples were then coated with a layer of gold in an IC-50 metalizer (Shimadzu, Kyoto, Japan) and analyzed using a Quanta 250 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Microscopy Center of the Complex of Research Support Centers of the State University of Maringá, Paraná, Brazil.

2.7. Statistical Analyses

The viability of *D. saccharalis* eggs into different ages was determined using Prisma 2.1 software version 5.0. The data were compared applying the analysis of variance (ANOVA). The results that pointed differences between the values were submitted to the test of multiple comparison of Tukey ($p < 0.05$) [33] [34]. The isolate with the greatest biocontroller potential was selected for further analysis (observational, histological, and ultrastructural analyses).

3. Results

3.1. Inviability of *D. saccharalis* Eggs

Both isolates of *M. anisopliae* (MT and E9) tested in our study had negative effects on the viability of *D. saccharalis* eggs. The MT isolate reduced the viability of 100% of the eggs aged 72 h or less, while the E9 isolate reduced the viability of 93.75% of the eggs at the same age. In contrast, eggs aged more than 96 h demonstrated strong resistance, and the percentage of inviability was lowered by 60% for both isolates (**Table 1**).

3.2. Observational Analyses

Control eggs demonstrated complete embryonic development after 144 h post-treatment (**Figure 1(A)**). Eggs treated with *M. anisopliae* MT isolate (10^7 conidia/mL) showed melanization in the peripheral regions after 24 h (**Figure 1(B)**). The process persisted for 48 h, and we observed the development of fungal hyphae in the eggs (**Figure 1(C)**). After 72 h, the surface of the eggs was covered by conidia, which persisted for 144 h (**Figure 1(D)**).

3.3. Light Microscopy

The control eggs showed translucid chorion and a dark region (yolk), indicating embryo development (**Figure 2(A)**). The eggs treated with *M. anisopliae* isolate MT were similar to the control; however, there was no embryo development in the yolk region (**Figure 2(B)**). Fungal hyphae can be observed on the chorionic surface (**Figure 2(B)**). Conidiophores (reproductive structures) appeared on the chorion after 96 h of treatment (**Figure 2(C)**) and persisted until 144 h (**Figure 2(D)**).

3.4. Histochemical Analyses

The control eggs had a clear chorion and developing embryo in the yolk region (Figure 3(A)). In eggs treated with the MT isolate, the yolk region was reduced; the hyphae crossed the chorion and dispersed throughout the yolk region, inhibiting embryonic development (Figure 3(B)). Among the hyphae in the yolk region, energids were observed (Figures 3(C)-(D)).

Table 1. Inviability (mean ± standard deviation) of *Diatraea saccharalis* eggs at different ages (0, 24, 48, 72, 96, and 120 h) treated with *Metarhizium anisopliae* isolate, MT and E9, solution at concentration of 10^7 conidia/mL, 144 h post-treatment.

Old (h)	Control	MT	E9	*p* value
0	10.50 ± 1.79^a	100.00 ± 0.00^b	93.75 ± 1.52^c	1037***
24	14.50 ± 2.05^a	100.00 ± 0.95^b	83.40 ± 1.75^c	967***
48	21.00 ± 2.50^a	100.00 ± 0.67^b	55.15 ± 1.87^c	105***
72	21.00 ± 1.32^a	100.00 ± 0.00^b	49.40 ± 6.27^c	884***
96	22.00 ± 3.92^a	59.05 ± 4.28^b	18.45 ± 4.69^a	21.4***
120	12.00 ± 1.50^a	43.95 ± 3.78^b	16.80 ± 3.62^a	22.8***

***$p < 0.001$. Different letters indicate differences between treatments.

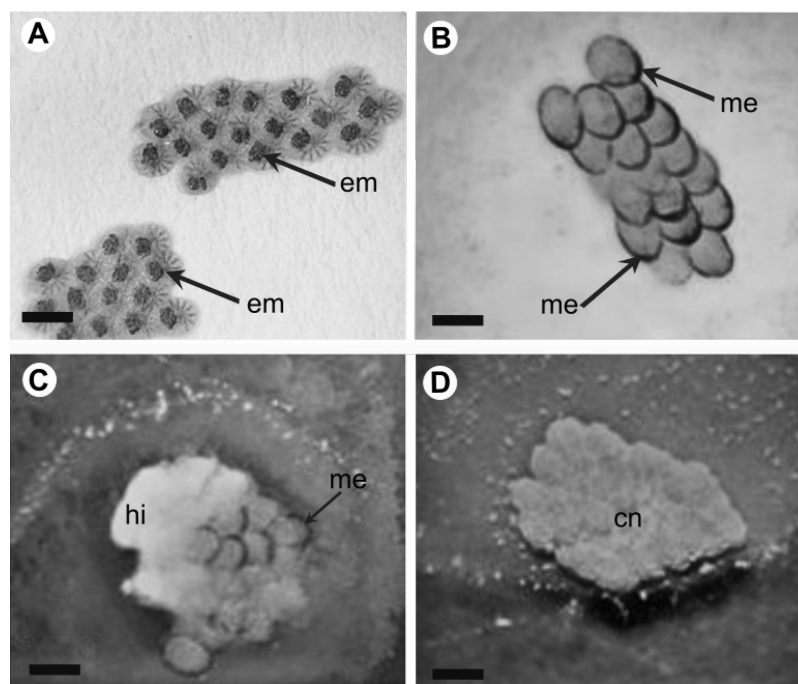

Figure 1. Whole mounting of *Metarhizium anisopliae* development on *Diatraea saccharalis* eggs at 0 h of age: control and eggs treated with *M. anisopliae* MT solution (10^7 conidium/mL). (A) Control eggs (144 h): embryo (em) demonstrated full development. (B-D) Different ages of eggs treated with *M. anisopliae* MT solution (10^7 conidium/mL). (B) 24 h after the treatments: melanization (me) is visible in egg extremity. (C) 48 h after the treatments: melanization persists and hyphae (hi) development is observed. (D) 96 h after bioassays: eggs are fully covered by conidiophores (cn), which persist to 144 h. Scale Bar A-D = 1 mm.

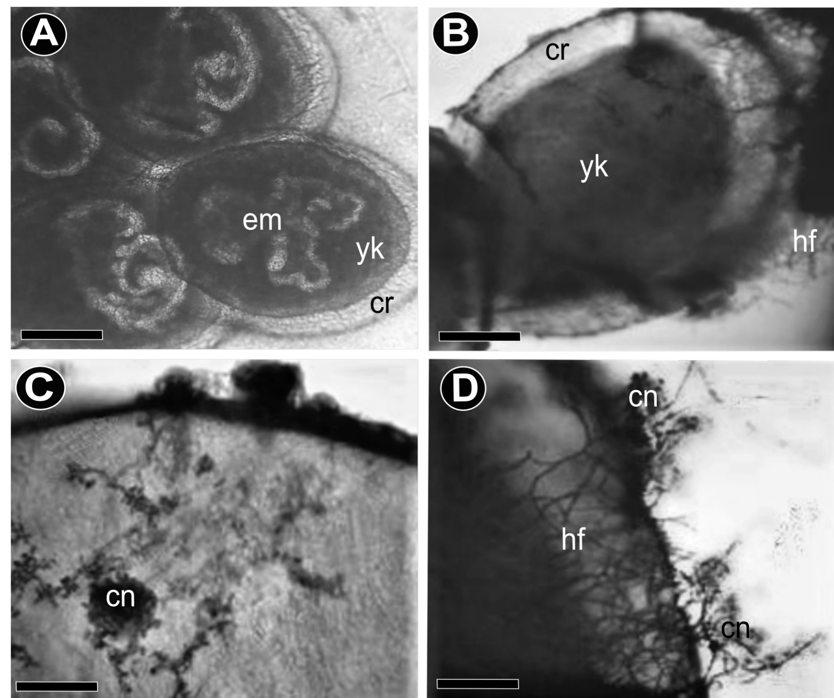

Figure 2. Whole mounting (light microscope) of *Metarhizium anisopliae* development on *Diatraea saccharalis* eggs at 0 h old. (A) Control eggs at 48 h old: chorion (cr) delimits the yolk (yk) where embryo (em) development occurs. (B-D) Eggs at 48 and 96 h old, treated with *M. anisopliae* MT solution (10^7 conidium/mL) and stained with Toluidine Blue. (B) 48 h after treatment: there is no embryo development of *D. saccharalis* into the yolk: hyphae (hf) cover the chorion. (C-D) 96 h after treatment: eggs are covered with conidiophores (cn) and hyphae. Scale bar in A = 500 µm; B-D = 100 µm.

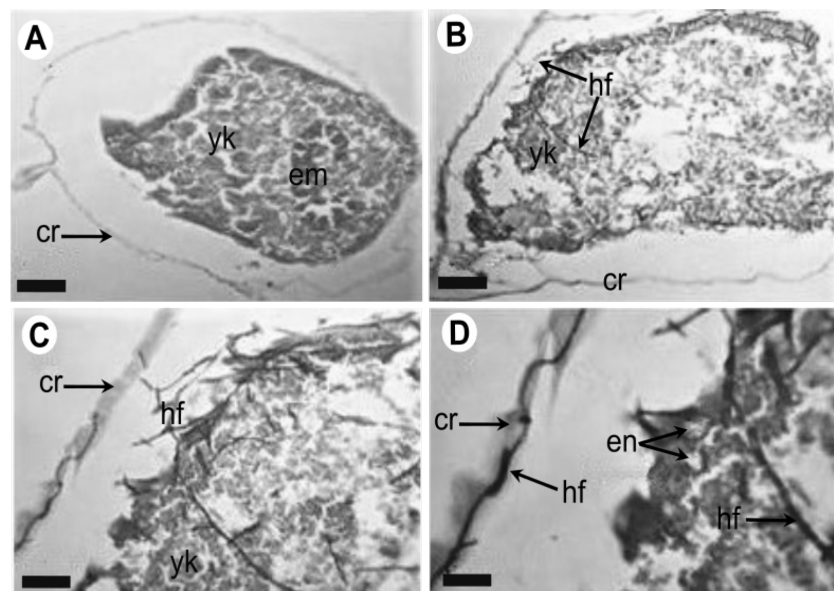

Figure 3. Light microscopy of *Diatraea saccharalis* eggs at 0 h old, control eggs and eggs treated with *M. anisopliae* MT solution (10^7 conidium/mL), observed after 48 h of treatment (sections of 7 µm stained with PAS). (A) Control eggs: chorion (cr), yolk (yk), and embryo development (em). (B-D) Eggs treated with *M. anisopliae* MT solution: energids in the yolk; hyphae (hf) penetrate chorion and disperse to yolk. Scale bar A-D = 50 µm.

3.5. SEM

D. saccharalis eggs showed an imbricated disposition (**Figure 4(A)**). The chorion showed hexagonal and heptagonal structures (**Figure 4(B)**) with disposed circular aeropyles (**Figure 4(B)**). SEM showed hyphae measuring 5 μm on the surface of eggs 48 h after treatment. The hyphae were close to the chorion, which showed circular aeropyles with a diameter of 3 μm (**Figures 4 (C)-(E)**). Conidia (**Figure 4(D)**) and conidiophores (**Figure 4(F)**) appeared on the egg surface after 96 h. We observed times of *M. anisopliae* (MT) development on *D. saccharalis* eggs, considering adhesion, germination, penetration, and extrusion (**Table 2**).

4. Discussion

There is a lack of studies on the effects of entomopathogens on pest insect eggs [25] [32]. Here, we demonstrated that *M. anisopliae* isolates (MT and E9) have entomopathogenic action on *D. saccharalis* eggs of different ages. Moreover, the MT isolate had more potential than the E9 isolate to control the pest insect eggs. Similar results were observed with different isolates of *M. anisopliae* in the control of arachnids [35] [36] [37], nematodes [38], and lepidopterans [33].

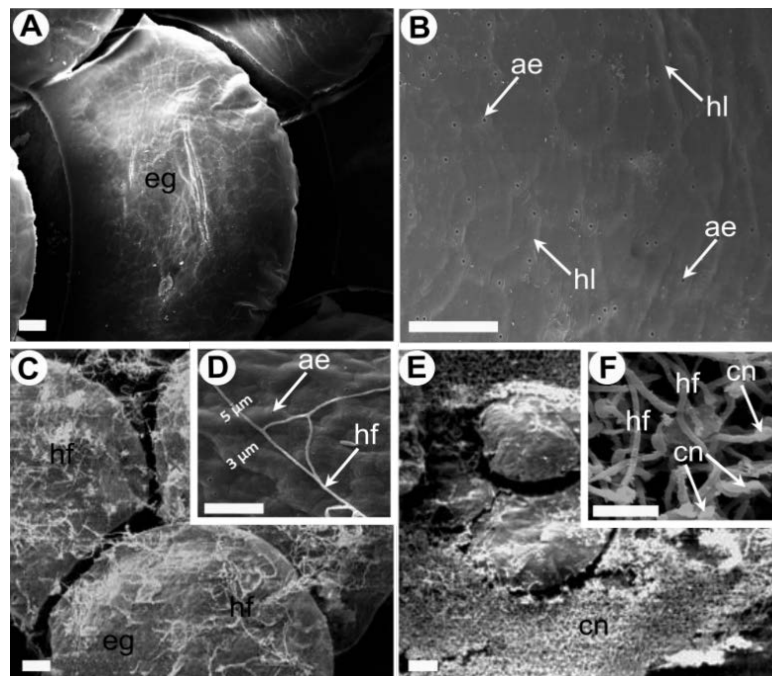

Figure 4. Scanning electron micrograph of *Diatraea saccharalis* eggs including the control and eggs treated with *M. anisopliae* MT solution (10^7 conidium/mL). (A-B) Control eggs. (A) Imbricate disposition of eggs (eg). (B) Egg surface with chorion sculptures (hl) in hexagonal and heptagonal format and aeropyles (ae) in extremity. (C-F) Eggs treated with *M. anisopliae* MT solution (10^7 conidium/mL). (C and E) 24-h old and (D and F) 96-h after the treatments; (C) Imbricate disposition of eggs covered by hyphae (hf). (D) Hyphae and aeropyles with diameter of 5 and 3 μm respectively. (E) Conidiophores (cn) (reproductive structures) cover most eggs' surfaces. (F) Amplification of a conidiophore region; note the hyphae and conidiophores. Scale bar A-F = 500 μm.

Table 2. Periods of the infection process of *Diatraea saccharalis* eggs treated with *Metarhizium anisopliae* MT isolate solution at a concentration of 10^7 conidia/mL.

Period (h)	Adhesion	Germination	Penetration	Extrusion
0	+	−	−	−
24	+	+	+−	−
48	−	+	+	−
72	−	−	−	+−
96	−	−	−	+
120	−	−	−	+

(−) Absence or low frequency of the process; (+−) start of preview; (+) bulk preview of the process.

Infection by *M. anisopliae* starts passively and is related to hydrophobic interactions [39]. *D. saccharalis* eggs treated with *M. anisopliae* isolate MT matched the sequential description of Moraes *et al.* [13], who analyzed different stages of development in insects infected by entomopathogenic fungi.

We found that 100% of the *D. saccharalis* eggs (0 - 72-h old) infected by the MT isolate (10^7 conidia/mL) were inviable. This result is superior to the inviability potential of *Blissus Antilles* (Hemiptera: Lygaeidae) eggs (24-h old) infected by the ESALQ818 isolate (10^4 conidia/mL), at 96.7% [40]. Other studies have evaluated the mortality of *D. saccharalis* eggs treated with different solutions: Daquila *et al.* [32] observed 34.98% inviability in eggs (0 - 24-h old) infected by *Bacillus thuringiensis* isolate Aizawai GC-91 (Bacillales: Bacillaceae) and Canazart *et al.* [31] observed that the alternative control with garlic essential oil (0.5%) made more than 60% of the eggs unfeasible.

Eggs over 96-h old showed resistance to the fungal isolates used, which may be related to their morphology and defense process. Insect eggs have specialized structures, for example, the chorion and extraembryonic membranes [41] [42] [43] [44], which increase their resistance to chemical and biological controls [41] [44] [45].

The chorion of *D. saccharalis* eggs has a thin and translucid structure [31], formed by two structures, the exochorion and endochorion [32]. These structures are synthesized by ovarian follicular cells [46]. On the surface of the exochorion, irregularly shaped structures are present, with disposed aeropyles at the extremities. The aeropyles allow the exchange of gases between the external and internal environment [47]. Extraembryonic membranes are present below the chorion, which act as barriers that limit the passage of macromolecules and microorganisms to the inside [42] [43].

Proteins, lipids, and glycogen are the main components of the egg cytoplasm [48]. The glycogen (yolk) is crucial for embryonic development. Therefore, toxins in the yolk may interrupt or inhibit the embryonic development of *D. saccharalis* eggs [32]. The presence of energids indicates the beginning of embryonic development; however, this process is interrupted by the toxins released by

fungal hyphae. After fungal penetration, a dimorphic transition takes place, resulting in the formation of hyphae that have dispersal potential and secrete toxins and enzymes that inhibit the metabolic process of the insects, leading to death [13] [22]. Consequently, the toxins and enzymes released by *M. anisopliae* isolate MT may activate cell death in insects. Eggs of *D. saccharalis* express different esterases at different ages [31] [49], which activate metabolic processes crucial to embryonic development and survival [50].

The melanization process observed in this study is similar to those described by other authors who treated *D. saccharalis* eggs treated with different formulations (essential oil and entomopathogens) [31] [32] [51]. Protease controls the melanization process [43] [52] and activates the serine protease cascade. Serine protease activates prophenoloxidases, which control melanogenesis, and phenoloxidases, which oxidizes tyrosine in dihydroxyphenylalanine. This produces dihydroxyphenylalanine and dopamine, which are melanin precursors [53] [54]. The presence of these enzymes in lepidopteran eggs was confirmed by Canazart *et al.* [31], Kanost and Clem [52], and Maki and Yamashita [55].

Studies on the control of pest insect eggs are crucial for the development of methodologies for IPM. We demonstrated the biocontrol potential of *M. anisopliae* isolate MT on different ages of *D. saccharalis* eggs and encourage its utilization in sugarcane crops.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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