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Diatomaceous earth and *Beauveria bassiana* in polyvinyl alcohol matrices, a combined effect for *Pieris brassicae* control

Tierra de diatomea y *Bauveria bassiana* en matrices de alcohol polivinilico, un efecto combinado para el control de *Pieris brassicae*

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ABSTRACT
Cabbage larvae (*Pieris brassicae*, Lepidoptera: Pieridae) are an important pest in organic farming; therefore, biological pest control alternatives are being sought. The effect of *Beauveria bassiana* (Bb) combined with diatomaceous earth (DE) in a polyvinyl alcohol flask (BbI), as well as the combination between them (Bb+DE, BbI+DE) on *P. brassicae* and *Galleria mellonella* larvae inoculated by aspersion or immersion was evaluated. Prior to the experiment, the viability of *B. bassiana* was assessed and one of three commercial brands of diatomaceous earth was selected based on the shape and damage caused to *G. mellonella*. Radial shapes with and without edges were more common in the diatomaceous earth types, but these did not define efficacy on cuticle damage. The result showed that the Bb+DE treatment had greater damage, followed by the BbI+DE treatment applied by aspersion. The highest mortality in *G.mellonella* was 70.1 % with Bb at 120 h after sprinkled, while in *P. brassicae*, mortality reached 42.1 % with BbI+DE; thus, it is important to highlight the importance of the inoculation method and larvae types used when effectiveness bio-trials are performed.
Keywords: Polyvinyl alcohol, Aspersion, *B. bassiana*, Mortality, Plague.

RESUMEN
La larva de la col (*Pieris brassicae*, Lepidóptera: Pieridae), es una plaga importante en la agricultura orgánica, por lo que se buscan alternativas para su control. En este estudio, se evaluó el efecto de *Beauveria bassiana* sola (Bb) y combinada con tierra de diatomea (TD) en matrices de alcohol polivinílico (BbI), así como la combinación entre estos (Bb+TD, BbI+TD) en larvas de *P. brassicae* y *Galleria mellonella* inoculadas por aspersión y por inmersión. Previo al experimento, se evaluó la viabilidad de *B. bassiana*, y seleccionó una de tres marcas comerciales de tierra de acuerdo con su forma y daño provocado en *G. mellonella*, donde las formas radiales con y sin borde fueron mas frecuentes en los tipos de tierra, sin embargo, estas no definieron la eficacia en daño de cutícula. El resultado mostró que el tratamiento Bb+TD tuvo mayor daño, seguido del tratamiento BbI+TD aplicados por aspersión. La mortalidad más alta en *G. mellonella* fue 70.1 % con Bb a las 120 h después de aperjado, mientras que en *P. brassicae* la mortalidad alcanzó 42.1 % con BbI+TD, por lo que hay que destacar la importancia del método de inoculación y el tipo de larva utilizada al realizar bioensayos de efectividad.
Palabras clave: Alcohol polivinílico 1, Aspersión, *B. bassiana* 2, mortalidad 3, plaga.

INTRODUCTION
Cabbage is one of the most relevant crops in the Central America and Caribbean zones, also with great economic importance since it is one of the cruciferous vegetables of greatest consumption (Mujica *et al*., 2009); there is an annual production of 204 thousand tons only in Mexico (SIAP, 2019). Although it is susceptible to damage by Lepidoptera species, *P. brassicae* stands out, this plague is capable of devouring a great number of leaves, leading to important production losses (Abbas *et al*., 2021).

The last decade has given special importance to ecological soil handling and nutrition, recognizing the organic production in Latin America, Asia, and European countries, due to its contribution to sustainable development (Schwentesius and Gómez, 2015; Willer, 2021) and, with the chemical products elimination, keeping the soil quality and fertility. On the other hand, the excessive use of pesticides harms the environment, indirectly contributing to the elimination of the natural enemies of plagues (Del Puerto *et al*., 2014; Castro *et al*., 2022). Recently, special attention has been given to the use of entomopathogenic microorganisms, which cause lethal infection of pests and have been shown to have significant broad-spectrum effects on pest species that damage crops (Bacca and Lagos, 2014; Pacheco *et al*., 2019). *B. bassiana* species are entomopathogenic fungi with great potential in diverse insect plague management, due to the biosynthesis of diverse cyclopeptide compounds, that act against fungal virulence (Wang, 2017). However, when applied in field conditions, entomopathogen microorganisms are affected by several factors, such as temperature, humidity, and solar radiation. Therefore, they need to be formulated in a method that enhances their efficacy, and to achieve this, the fungi are retained in immobilization matrices that protect the spores from environmental threats, thereby extending their shelf life when applied in the field (Azamar-Jiménez, 2018; Litwin *et al*., 2020).

On the other hand, the use of additives in biological control products is of great importance as it increases the efficacy of the formulations; one of these products is diatomaceous earth, which consists of crystalized algae microfos-

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sils that cause injuries in the insect body (Losic and Korunic, 2018; Bachrouch *et al*., 2022). However, little is known about the combined effect of diatomaceous earth with *B. bassiana* matrices for plague control. Therefore, the aim of this research was to evaluate the effect of diatomaceous earth in combination with the entomopathogenic fungi *B. bassiana* immobilized in polyvinyl alcohol matrices, as a biological agent against *P. brassicae* in cabbage crops.

MATERIALS AND METHODS

Obtaining *B. bassiana* strains, *P. brassicae* larvae and diatomaceous earth

B. bassiana OM839761

The strain was obtained from the collection of the Facultad de Ciencias Agrícolas y Pecuarias of the Benemérita Universidad Autónoma de Puebla. Sample was defrosted and 100 µL were inoculated in solid Agar Dextrose Sabouraud (ADS) medium, and then incubated at 32 °C± 2 for 5 d, and the grown mycelium was collected with the aid of a spatula to create the treatments.

Larvae used in the experiment

Ninety *G. mellonella* larvae were used as reference insects, obtained from the Escuela Tecnológica Superior of Zacapoaxtla; also, 90 *P. brassicae* larvae were obtained from a cabbage crop naturally infested, located at the Facultad de Ciencias Agrícolas y Pecuarias.

Diatomaceous earth used

Food grade quality, micronized, and organic Diatomaceous earth was acquired from GOSA©, Echinocactus©, and Lombriz MX© brands, and purchased from different commercial stores.

Selection of diatomaceous earth by the shape and percentage damage to larval cuticle prior to experiment

Present shapes in terms of diatomaceous earth brand

To select the diatomaceous earth, the efficacy of three brands was evaluated and three 600 mL solutions were prepared at concentrations of 0.01 g/mL, 0.002 g/mL, and 0.001 g/mL. With a Neubauer camera in a Leyca DM750 compound microscope the percentage of algal shell shapes present in each sample was quantified; then, 10 *G. mellonella* larvae in the last instar were added and manually shaken for 10 seconds at each concentration according to Vargas-Flores (2003). Finally, were placed over pieces of sterile moist paper towels contained in 140 x 20 mm capacity Petri dishes.

Diatomaceous earth damage on the cuticle of *G. mellonella*

After 48 h of inoculation, a 5 mm^2 incision was made on the dorsal part of the larval skin. The incision was then cleaned of fat residues and observed under the microscope to determine the percentage of spots (damage) over the cuticle. This data was subsequently correlated with the percentage of shell shapes.

Treatment preparation and inoculation of *G. mellonella* and *P. brassicae* with *B. bassiana* larvae

For the prepration of the treatment one (Bb) which consisted of *B. bassiana* 1 x 10^6 conidia/mL, conidia were collected from a Petri dish containing 5-day-old *B. bassiana* and added to 600 mL sterile distilled water to a concentration of 1 x 10^6 conidia per mL. The preparation of treatment two (DE) consisted of diatomaceous earth 10 mg/mL in 600 mL sterile distilled water, 6 g of GOSA diatomaceous earth were added to obtain a concentration of 0.01 g/mL, and the mixture was vortexed with a Hermann® Vortex until a homogenized mixture was obtained. While for treatment three (BbI), which was Immobilized *B. bassiana* 2% and 12 g of GOLDEN BELL® polyvinyl alcohol, was weighed and added to 600 mL of sterile distilled water contained in a 1 L flask, the solution was heated until the alcohol was dissolved and sterilized in an autoclave at 121 °C for 15 min; once the solution was cold, *B. bassiana* conidia from a Petri dish were added and incubated for 5 days until a concentration of 1x10^6 conidia per mL was obtained, according to the methodology described by Barranco-Florido *et al*. (2009). For treatment four (Bb+DE), which consisted of *B. bassiana* 1 x 10^6 conidia/mL + diatomaceous earth 10 mg/mL, the combination of treatment one and treatment two was made, it was formulated as a 600 mL solution 1:1 contained in a 1L flask. Treatment five (BbI+DE) consisted of immobilized *B. bassiana* 2 % + diatomaceous earth 10 mg/mL, a combination of treatment two and treatment three was formulated and a 600 mL solution 1:1 was contained in a 1L flask. Finally for the treatment six (Witness) 600 mL of sterile distilled water were used. The viability was determined in SDA following the methodology by Berlanga-Padilla and Hernández-Velázquez (2002) for all treatments that included conidia. In 5 different spots of a Petri dish (100 x 15 mm), a circle was marked with SDA agar, over which 2 µL of a 1 x 10^7 conidia/mL suspension was added, then coverslips were placed and after 24 h, the total number of conidia germinated in each spot was observed and registered using a Leyca DM750 microscope.

The *G. mellonella* and *P. brassicae* with *B. bassiana* larvae inoculation process consisted of two events: first, 60 last instar *G. mellonella* larvae were simultaneously placed on moist sterile paper towels and 140 x 20 mm Petri dishes and inoculated by aspersion with 3 L capacity TRUPER® sprinkler at 38 PSI for each treatment, plus 30 larvae with the same characteristics inoculated by immersion. For the second event, 90 last instar *P. brassicae* larvae were collected from a naturally infested cabbage crop and treatments were applied similarly to the *G. mellonella* larvae without immersion applications.

Damage in larvae cuticle

Damage percentage was evaluated at 24, 48, 72 and 120 h after inoculation, for this, the damage on the larvae cuticle was observed in a Leyca brand stereoscope. No damage in *P. brasicae* and *G. mellonella*, was observed, but cuticle melanization appeared.

Mortality of *G. mellonella and P. brassicae*

Mortality was evaluated at 24, 48, 72 and 120 hours after inoculation, values calculated and adjusted using the corrected mortality percentage formula (Abbott, 1925) to ensure normality and homogeneity of variances, then the data were subjected to analysis of variance (ANOVA).

Statistical Analysis

SAS version 9.0 for Windows 2008 was used with prior confirmation of normal data distribution. In the evaluation of the three diatomaceous earth brands, a factorial design was used to know the effect, with three simultaneous concentrations and a Tukey's multiple comparison test with $\alpha = 0.05$ to select the best one. In *G. mellonella*, an evaluation of the variability among treatments in cuticle damage and larval mortality was determined by a Tukey's multiple comparison test with $\alpha = 0.05$, and the relationship was determined by a Pearson's correlation coefficient between cuticle damage and the percentage of shapes found in each treatment. For the second evaluation with *P. brassicae*, it was determined if there was a significant variance in the percentage of dead larvae, and the best treatment was determined with a Tukey's mean comparison with $\alpha = 0.05$.

RESULTS

Selection of diatomaceous earth by the shape and percentage damage to larval cuticle prior to experiment

Present shapes in terms of diatomaceous earth brand

There were four shapes of diatomaceous earth from the Gosa brand: Radial with rim (RB), radial shape without rim (RSB), meanwhile Echinocactus brand only one in rod shape (V), and other in ellipse shape (E) and the shape of Lombriz MX showed a lot of incomplete particles. The statistical analysis of shell shapes found showed significant differences (P ≤ 0.05), radial with rim shape (RB), was found more frequently followed by radial shape without rim (RSB), this last one being different in its ellipse shape (E) at the same time, its mean was lower (Figure 1).

In addition, data analysis did not show significant differences between brands and diatomaceous earth-shell concentrations. Nevertheless, the Tukey test ($P \leq 0.05$) showed a greater amount of diatom shell shapes in the Echinocactus brand at 0.01 g/mL concentration, followed by the Gosa brand at 0.01 g/mL concentration, while the brand with the lower diatom shell shapes was Lombriz at 0.0001 g/mL concentration (Figure 2). It was also observed that as the particle concentration increases, the amount of shell shapes increases depending on the brand, as the concentration with the highest mean microalgae shell shape for all brands was 0.01 g/mL concentration, while the lowest mean was 0.001 g/mL concentration.

Diatomaceous earth damage on *G. mellonella* cuticle

The spots as a sign of damage by diatomaceous earth were found in greater amounts under the larvae abdomen. GOSA brand at 0.01 concentration caused 100 % of the spots on the cuticle of *G. mellonella* larvae, followed by Echinocactus brand at the same concentration with 77 %, while Lombriz-MX grand at 0.001 g/mL concentration caused 44 % of the damage (Table 1).

Nevertheless, the correlation analysis between the number of spots observed by microscope field and the percentage of shell shapes present per treatment, showed a weak positive correlation (0.768) between variables, with a determination coefficient of $R^2 = 0.59$ (Figure 3).

***G. mellonella* and *P. brassicae* with *B. bassiana* larvae inoculation and treatments effectiveness**

Damage in larvae cuticle

Statistical differences were found between treatments based on the number of spots found on *G. mellonella* ($P \leq 0.01$) (Figure 4). Bb+DE was the best treatment with a value of 7.22 spots found over the *G. mellonella* cuticle, followed by the polyvinyl alcohol immobilized *B. bassiana* treatment and the diatomaceous earth combination (BbI+DE), while the minimum effective treatment was the TDS with a value of

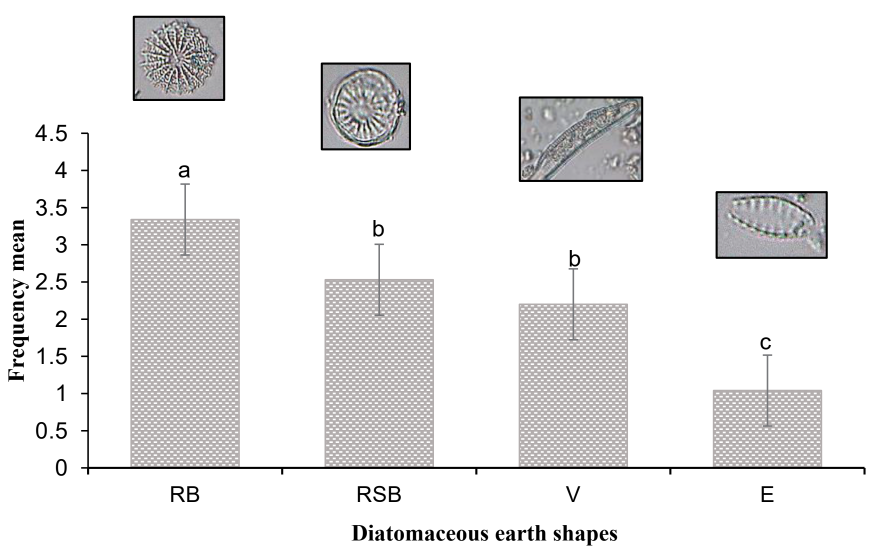

Figura 1. Frecuencia de formas de caparazones encontradas en tierra de diatomea. Medias con la misma letra no son significativamente diferentes (P ≤ 0.05). Radial con bordo (RB), Forma radial sin bordo (RSB), Forma de varilla (V), Forma de elipse (E).

Figure 1. Frequency of shell shapes found in diatomaceous earth. Means with the same letter are not significantly different (P ≤ 0.05). Radial with rim (RB), radial shape without rim (RSB), rod shape (V), ellipse shape (E).

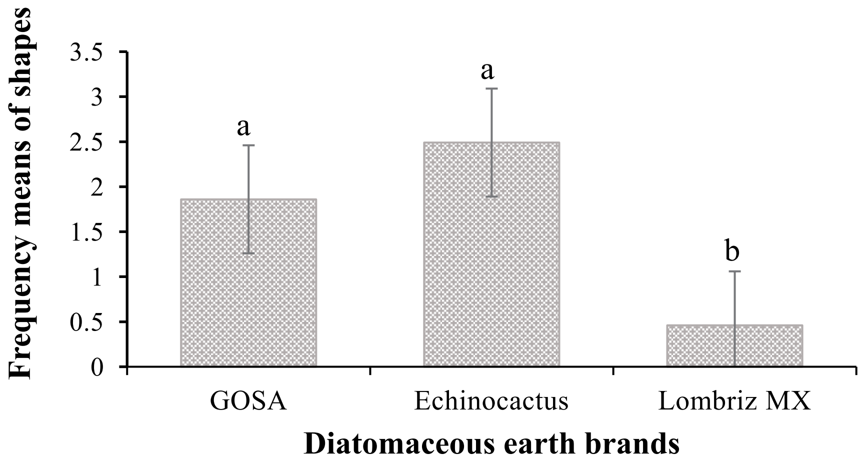

Figura 2. Número de formas de caparazones de algas de acuerdo con la maraca de tierra de diatomea. Medias con la misma letra no son significativamente diferentes.
Figure 2. Number of shell shapes according to the brand of diatomaceous earth. Means with the same letter are not significantly different ($P \leq 0.05$).

Tabla 1. Porcentaje de daño sobre la cutícula de *G. mellonella* a 120 h.
Table 1. Percentage of damage on the *G. mellonella* cuticle at 120 h

Commercial brand	Concentration (g/mL)		
	0.01	**0.002**	**0.001**
GOSA©	100%	77%	66%
Echinocactus©	77%	75%	55%
Lombriz MX©	50%	55%	44%

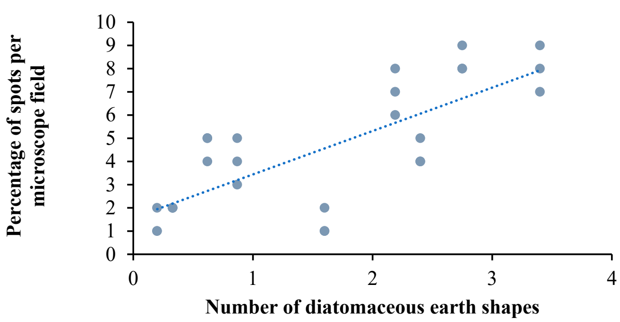

Figura 3. Correlación entre el número de formas de caparazones de algas entre marcas de tierra de diatomea y el porcentaje de manchas sobre la cutícula de *G. mellonella* cuticle. $y = 1.8709x + 1.5705$, $R^2 = 0.5909$.
Figure 3. Correlation between the number of shell shapes in three brands of diatomaceous earth and the percentage of spots on the *G. mellonella* cuticle. $y = 1.8709x + 1.5705$, $R^2 = 0.5909$.

4.56. Also, a significant difference was observed between the counting hours for the number of spots present ($P \leq 0.0001$), and according to the comparison of Tukey means, the time that showed the greatest number of spots was at 72 h, as opposed to 24 and 48 h. It is worth mentioning that no spots were observed on *P. brassicae*.

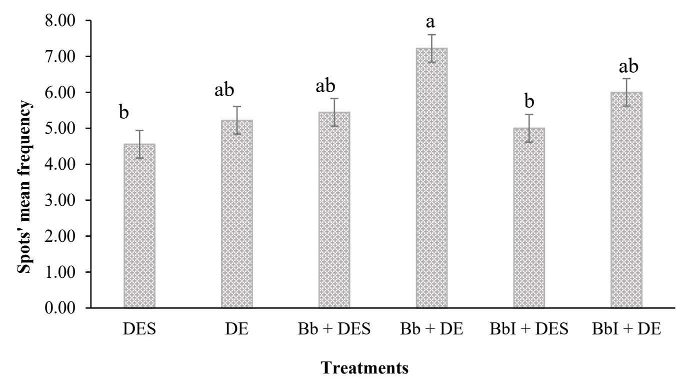

Figura 4. Media de frecuencia de número de manchas provocadas por el daño del tratamiento. Tratamientos a los cuales se les agrega una S fueron aplicados por el método de inmersión, mientras que los que no tienen S fueron aplicados por el método de aspersión. Medias con la misma letra no son significativamente diferentes ($P \leq 0.05$).
Figure 4. Mean frequency of spots caused by treatment damage. To specify that the treatments were applied by immersion, the letter S was added to the treatment key, otherwise, the treatments were applied by spraying. Means with the same letter are not significantly different ($P \leq 0.05$).

Mortality of *G. mellonella* and *P. brassicae*

The treatment that caused the greatest mortality in *G. mellonella* was the solution of conidia in distilled water (Bb) through immersion application with 80 %, which was statistically different from the witness that registered 0 %, while the Bbl+DE treatment reached 40 % through the same application, although when changing the application method, this last treatment increased the larval mortality up to 60 %; at the same time, the best treatment was Bb through immersion method, but when the aspersion method was used, mortality decreased by 10 % (Figure 5).

The observations with *G. mellonella* in the aspersion application showed that the most effective treatment in *P. brassicae* was the Bbl+DE with a mortality percentage of 42

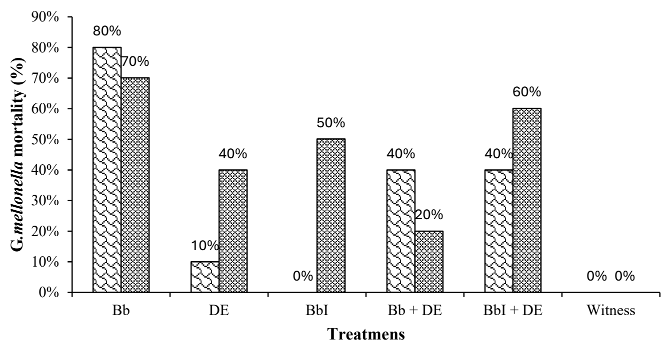

Figura 5. Porcentaje de mortalidad por efecto de los tratamientos en larvas de *G. mellonella* por diferentes métodos de inoculación. Tratamiento por inmersión (▧), tratamiento por aspersión (▩). *B. bassiana* 1x10 ^{6} conidia/mL (Bb), tierra de diatomea 1 mg/mL (TD), *B. bassiana* inmovilizada 6 % (BbI), *B. bassiana* 1 x 10 ^{6} conidias/mL + tierra de diatomea 1 mg/ mL (Bb+TD).

Figure 5. Percentage of mortality due to the effect of the treatments on *G. mellonella* worm by different inoculation methods. Immersion treatment (▧), spray treatment (▩). *B. bassiana* 1 x 10 ^{6} conidia/mL (Bb), diatomaceous earth 1 mg/mL (DE), immobilized *B. bassiana* 6 % (BbI), *B. bassiana* 1 x 10 ^{6} conidia/mL + diatomaceous earth 1 mg/ mL (Bb+DE).

%, while the same treatment used in *G. mellonella* showed 18 % more mortality. On the other hand, the less effective treatment in both species was Bb+DE (Figure 6).

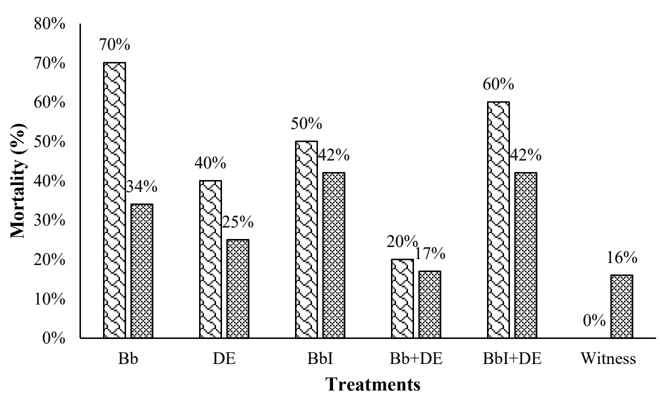

Figura 6. Mortalidad de *G. mellonella* y *P. brassicae* a 120h después de la inoculación por el método de aspersión. *G. mellonella* (▧),*P. brassicae* (▩). *B. bassiana* 1 x 10 ^{6} conidia/mL (Bb), tierra de diatomacea 1 mg/mL (TD), *B. bassiana* inmovilizada en alcohol polivinilico 6 % (BbI), *B. bassiana* 1 x 10 ^{6} conidias/mL + tierra de diatomea 1 mg/ mL (Bb+TD).

Figure 6. Mortality of *G. mellonella* and *P. brassicae* at 120h after inoculation by spray method. *G. mellonella* (▧), *P. brassicae* (▩). *B. bassiana* 1 x 10 ^{6} conidia/mL (Bb), diatomaceous earth 1 mg/mL (DE), immobilized *B. bassiana* 6% (BbI), *B. bassiana* 1 x 10 ^{6} conidia/mL + diatomaceous earth 1 mg/ mL (Bb+DE).

DISCUSSION

According to the microalgae shell shapes from the diatomaceous earth brands, Colín-Garcia *et al*. (2013) indicate that shell patterns are specific and genetically determined, so there is a great variety of shapes in the diatomaceous earth. Hence, there will be greater or lesser effectiveness. Also, Natrass *et al*. (2015) mentioned that the shape variability shown in the diatomaceous earth also depends on the extraction site.

Moreover, regarding the results observed in the different brands of diatomaceous earth and at different concentrations, Arnaud *et al*. (2005) showed similar results when applying diatomaceous earth formulations at different concentrations, confirming that the higher the concentration, the greater the damage caused in the insect.

The correlation analysis between *G. mellonella* spots over the cuticle and the number of shapes present in the diatomaceous earth brand, suggests that the level of damage could also be related to other factors such as quality and purity (Reka *et al*., 2021), as declared by Alves *et al*. (2017) that at greater quality and purity, the effectiveness of diatomaceous earth is greater; in this case, the less pure earth was the one that caused less damage. Although it is not excluded that the shape of particles confirming the diatomaceous earth is related to the damage to the insect cuticule, since it has been observed that the shells with acute shape cause greater damage (Zeni *et al*., 2021; Korunic, 2013), in this study there was a low presence of acute shapes and a greater predominance of radial shapes that could have interfered with the damage percentage; in this regard, Korunic (1998) stated that the effectiveness is related to the contact established between the radial particle surface and the insect cuticule; the more uniform the surface, the greater will be the cuticle adhesion.

According to the results, the damage effectiveness of the treatments over the *G. mellonella* larvae cuticle showed a lower effect with the treatment with diatomaceous earth and *B. bassiana* immobilized in polyvinyl alcohol; Liauw *et al*. (2020) and Angeli *et al*. (2022) mention that polyvinyl alcohol prevents contact between the spore surface and the diatomaceous earth particles, avoiding its function over the insect cuticle when a liquid is applied. Although these authors also mention that dry encapsulation has shown good results in adhesion and conidia retention on surfaces, these formulations promote the abrasive action of the diatomaceous earth particles without being trapped in the liquid formulations. Thus, the treatment with the best results was *B. bassiana* + diatomaceous earth applied by aspersion. According to Wakil *et al*. (2021) and Zeni *et al*. (2021), in these applications, the particles have a greater possibility to touch the insect, and those that are above the surfaces, can be picked up more easily by the insect, unlike the immersion application where only the particles adhered to the insect body will act; besides in immersion applications, there is less time for the particles to adhere to larvae cuticle when submerged in and out of the solution very quickly (Abdou *et al*., 2022), the time is essential to obtain better results. For example, in this study the time showing greater number of spots was at 72 h, although the damage in the insect cuticule also depends on the species, since in this case there were no spots on *P. brassicae* cuticle.

The mortality effectiveness of water-diluted *B. bassiana* has been well documented, unlike for spores covered with polyvinyl alcohol (Ozdemir *et al*., 2020) both in *G. mellonella* and *P. brassicae*, although Nazzaro *et al*. (2012) reported the advantages of polyvinyl alcohol used as an alternative covering material, such as fungal conidia, in which it prolongs its useful life (Wenzel *et al*., 2017). Although in this case, the treatment with immobilized *B. bassiana* in polyvinyl alcohol flasks along with diatomaceous earth (T5) did not cause the expected efficacy when applied by immersion, it has to be considered that the efficacy also depends on the formulation applied. Therefore, additional tests are suggested with *B. bassiana* spores covered with polyvinyl alcohol powder formulated and applied to *G. mellonella* and *P. brassicae*.

The results also showed that, in addition to obtaining a different effect with the treatment and application, the larval mortality effectiveness is related to the larval species susceptibility that receives the treatment as mentioned by Korunic (1998; 2013), with the possible retention of diatomaceous earth particles and *B. bassiana* spores inside the polyvinyl alcohol flask which does not allow them to act in applications by immersion and aspersion. Although, the lack of previous studies on the effectiveness of *B. bassiana* and immobilized diatomaceous earth in polyvinyl alcohol to reinforce these results make it difficult to draw convincing conclusions.

CONCLUSIONS

In this study, the amount and shape of the algal shells present in the diatomaceous earth was observed to cause damage to the *G. mellonella* cuticle; therefore, when using high-purity diatomaceous earth products, efficacy is assured. Also, when immobilizing *B. bassiana* conidia in polyvinyl alcohol vials with diatomaceous earth particles, direct contact of both with the surfaces where they are applied should be avoided, since the effect is less than that observed when applying only *B. bassiana* conidia diluted in water. The above could be show with scanning microscope images, however, there was the limitation of not having the equipment. In adition, it is necessary to work on studies with polyvinyl alcohol powder formulations covering the conidia, leaving the diatomaceous earth particles free to exert their abrasive action on the insect cuticle. It is also suggested to carry out an additional study with at least two species of insects to better detect the effect of the treatments and to obtain reliable results, and decide if the application of these treatments can be made by aspersion or by immersion, since a high concentration of alcohol dissolved in water could limit the aspersion success.

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CONFLICTS OF INTEREST

The authors declare that there is a non-existing conflict of interest.

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