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Efficiency of entomopathogenic bacteria and fungi on *Oligonychus yothersi in vitro* and on *Persea americana* Mill. plants

Eficiencia de bacterias y hongos entomopatógenos sobre *Oligonychus yothersi in vitro* y en plantas de *Persea americana* Mill.

Eficiência de bactérias e fungos entomopatogênicos em *Oligonychus yothersi in vitro* e em plantas *Persea americana* Mill.

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Crop Production

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Abstract

In the germplasm bank of 22 varieties of avocado (*Persea americana* Mill.) belonging to the Fruit Horticultural Institute Investigation, Hermilio Valdizan National University (UNHEVAL)-Peru, it is common to observe a high population of the species *Oligonychus yothersi*, a phytophagous mite harmful to the crop. Controls with commercial acaricides are restricted in place, due to the presence of beehives installed in adjacent plots. The objective of this study was to evaluate the effect of four commercial formulations containing strains of *Metarhizium anisopliae* and *Beauveria bassiana* and the toxins of *Bacillus subtilis*, *Bacillus thuringiensis* var. *kurstaki* (Btk) for the control of *O. yothersi.* The entomopathogenic products were evaluated in the field applying a randomized complete block design with five treatments and three replicates. In the laboratory, 500 adult mites were selected, placing 100 mites per Petri dish with three repetitions per treatment. It was found that the formulation *Bacillus thuringiensis* var. *kurstaki* under field conditions reduced the population incidence of mites by up to 98.07 % in 49 days. In the laboratory, the *B. subtilis* and *M. anisopliae* formulations caused 100 % mortality six days after application proving to be efficient control alternatives.

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Resumen

En el Banco de germoplasma de 22 variedades del palto (*Persea americana* Mill.) perteneciente al Centro de Investigación Frutícola Olerícola, Universidad Nacional Hermilio Valdizan (UNHEVAL)-Perú, es frecuente observar una población alta de la especie *Oligonychus yothersi*, ácaro fitófago perjudicial al cultivo. Los controles con acaricidas comerciales son restringidos en el lugar, por la presencia de las colmenas de abejas instaladas en las parcelas adyacentes. El objetivo de este estudio fue evaluar el efecto de cuatro formulaciones comerciales que contienen cepas de *Metarhizium anisopliae* y *Beauveria bassiana* y las toxinas de *Bacillus subtilis*, *Bacillus thuringiensis* var. *kurstaki* (Btk) para el control de *O. yothersi*. Los productos entomopatógenos fueron evaluados en campo aplicando un diseño de bloques completos al azar con cinco tratamientos y tres replicas. En el laboratorio se seleccionaron 500 ácaros adultos, colocando 100 ácaros por placa Petri con tres repeticiones por tratamiento. Se encontró que el formulado *Bacillus thuringiensis* var. *kurstaki* en condiciones de campo redujo hasta un 98,07 % la incidencia poblacional de los ácaros en 49 días. En laboratorio, los formulados de *B. subtilis* y *M. anisopliae* provocaron el 100 % de mortalidad a los seis días pos-aplicación resultando ser alternativas eficientes de control.

Palabras clave: control biológico, *Beauveria, Metarhizium, Bacillus*, acaropatógenos.

Resumo

No Banco de Germoplasma de 22 variedades de abacate (*Persea Americana* Mill.) pertencenteao Centro de Pesquisa de Frutas Olerícola da Universidade Nacional Hermilio Valdizan (UNHEVAL)-Peru, é comum observar uma elevada população da espécie *Oligonychus yothersi*, um ácaro fitófago prejudicial para a colheita. Os controles com acaricidas comerciaissãorestritosdevido à presença de colméias instaladas em parcelas adjacentes. O objetivo desteestudofoi avaliar o efeito de quatro formulações comerciais contendo cepas de *Metarhizium anisopliae* e *Beauveria bassiana* e as toxinas de *Bacillus subtilis, Bacillus thuringiensis* var. *kurstaki* (Btk) no controle de *O. yothersi*. Os produtos entomopatogênicos foram avaliados em campo utilizando delineamento em blocos casualizados com cinco tratamentos e três repetições. Em laboratório foram selecionados 500 ácaros adultos, colocando 100 ácaros por placa de Petri com três repetições por tratamento. Verificou-se que a formulação *Bacillus thuringiensis* var. *kurstaki* em condições de campo reduziu a incidênciapopulacional de ácaros em até 98,07 % em 49 dias. Em laboratório, as formulações de *B. subtilis* e *M. anisopliae* causaram 100 % de mortalidade seis diasapós a aplicação mostrando-se alternativas de controle eficientes.

Palavras-chave: controle biológico, *Beauveria, Metarhizium, Bacillus*, acaropatógenos.

Introduction

In Peru, the species *Oligonychus yothersi* (McGregor) (Acari: Tetranychidae) plays a limiting role in the agro-export processes of *Persea americana* Miller, commonly known as avocado. In recent years, the country has led the boom in avocado exports, entering 34 international markets (Chávez, 2019), so it is necessary to guarantee compliance with phytosanitary standards as Peru's commitment to the international market.

The pest *O. yothersi* has been reported as a polyphagous and severe species in several countries of the world (Pinto *et al*., 2012; Ceballos *et al*., 2022), plant damage is expressed in the reduction of photosynthetic activity, the consequence of which is excessive defoliation of the plant when attacks are severe (Rioja *et al*., 2018; Rioja *et al*., 2019). Among the most susceptible varieties of *Persea americana* are Hass and Fuerte, with a direct consequence on fruit quality and yield (Ceballos *et al*., 2022). This is the foliar pest with the highest incidence during the autumn and summer season (Yang *et al*., 2015; Bayu *et al*., 2017; Rioja *et al*., 2019) whose symptoms are tanning and leaf fall, because the mite pierces the leaf with its chelicerae in the form of a stylet and sucks the cellular contents (Rioja *et al*., 2018; Chiaradia *et al*., 2021).

The traditional control of *O. yothersi* and other pests in the cultivation of *P. americana* is based on chemical compounds such as abamectins, spirodiclofen and sulfur, harmful to human health, and the environment (Fathipour and Maleknia, 2016; Díaz and Aguilar, 2018; Ramírez, 2018; Borges *et al*., 2021; Tosi *et al*., 2022), in addition to causing resistance and resurgence of mites (Fathipour and Maleknia, 2016). In recent decades, a wide range of microbial pesticides have been developed (Köhl*et al*., 2019) as a resilient strategy in agricultural systems, a challenge of sustainable healthy food production (Balog *et al*., 2017*;* Borges *et al*., 2021).

Among them are entomopathogenic microorganisms with pathogenic capacity towards insects (Solter *et al*., 2012; Solórzano-Acosta *et al*., 2021), capable of causing natural epizootics in populations of mites or other arthropods, in addition to persisting in the absence of their hosts in natural habitats (Meyling and Eilenberg, 2007; Zemek *et al*., 2018; Konopická *et al*., 2022).

The fungi *Metarhizium brunneum, Metarhizium flavoviride*, *Lecanicillium lecanii,* and *Beauveria bassiana* have been proven effective against the different stages of development of the red spider mite (*Tetranychus urticae*) with adult mortality success greater than 80 % (Dogan *et al*., 2017). The fungi, *Akanthomyces lecanii*, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Aschersonia aleyrodis*, after nine days of exposure to red mites (*Panonychus citri*) caused more than 70 % mortality (Qasim *et al*., 2021). Hussein *et al*. (2020) found that the mortality percentage of the mite *Oligonychus afrasiaticus* (McGregor) varied between 73.3 % and 92 %, after seven days of treatment with *B. bassiana*, *Metarhizium acridum*, *Lecanicillium muscarium* and *Isaria fumosorosea*. Among bacteria, *Bacillus thuringiensis* Berliner has been shown to be effective against some mite pests (Erban *et al*., 2009; Alahyane *et al*., 2019; Sánchez-Yáñez *et al*., 2022) against pest insects of the orders coleoptera, diptera, hymenoptera, homoptera, orthoptera, and others (Fang *et al*., 2009; Ferreira-Agüero *et al*., 2020; Sánchez- Yáñez *et al*., 2022). These findings allow us to infer that the pathogenic or endotoxic effects of entomopathogens would prove to be effective against the red spider of the avocado *O. yothersi.* The present study evaluated the effect of the entomopathogens *Metarhizium anisopliae*, *Beauveria bassiana* and the toxins of *Bacillus subtilis*, *Bacillus thuringiensis* var. *kurstaki* in the control of *O. yothersi* in avocado crops.

Materials and methods

The study was developed between November 2019 and February 2020 in the plots of the Hass variety, belonging to the germplasm bank of 22 varieties of the avocado in the Fruit Horticultural Institute

Investigation- UNHEVAL, district of Pillco Marca, Huánuco region, Peru (09° 57′ 03” S, 76° 14′ 79” W), located above 1947 masl, with minimum temperatures of 14.8 °C and a maximum of 26.1°C and relative humidity of 77 % to 81.63 % (National Service of Meteorology and Hydrology of Peru [Senamhi], 2020) during the study period and in the laboratory of agricultural phytopathology, Faculty of Agrarian Sciences- UNHEVAL.

Table 1. Characteristics of the treatments under study.

N°	Treatments	Microorganism	Concentration and dosage
1	*Bacillus thuringiensis* var. kurstaki	Bacterium	$2.5.10^{9}$ CFU.mL, 2 $mL.L^{-1}$
2	*Bacillus subtilis*	Bacterium	$2.5.10^{9}$ CFU.mL, 2 $mL.L^{-1}$
3	*Metarhizium anisopliae*	Fungus	$1.0.10^{10}$ conidia, 2 $g.L^{-1}$
4	*Beauveria bassiana*	Fungus	$1.5.10^{10}$ conidia, 2 $g.L-^{1}$
5	Absolute control	No application	

Methodology in the field

The experimental design was a randomized complete block design, with five treatments and three replicates, because the upper margin of the experimental area borders with the *Coffea arabica* plots and on the right margin with the irrigation distribution points and *Pinus* sp. trees, that fulfill the function of windbreak curtains. The total population of the plants was 180 of the Hass variety (hybrid cross of the Guatemalan and Mexican breeds), of uniform size with 2.82 m of height on average, of five years, established in a frame of four meters between plants, eight meters between lines as recorded in figure 1. In avocado plants highly infested with the mite *O. yothersi*, to determine the initial infestation, a count of mites per leaf was carried out before the application of the products, for which two leaves were identified at random from the lower third, two leaves from the middle third and one leaf from the upper third of each plant.

Application of treatments

Water analysis was necessary to determine the pH and carbonate content, and with the results obtained, the water was neutralized to a pH range of 5.5 since the latter influences the germination of the fungus, delaying the values lower than 5.5 and higher than 7 (Velez *et al*., 1997; Padmavathi *et al*., 2003; National Agrarian Health Service [SENASA], 2014). The sequence for the dosage of the formulations (SENASA, 2014) was as follows:

a) In 5 L of water, 5 mL of pH corrector was added and 30 minutes later, 40 g of the biological product was added and then stirred until a homogeneous mixture was obtained, in the case of entomopathogenic fungi, 20 mL of agricultural oil was also added and then the mixture was left to stand for six hours.

b) In a 20 L backpack sprayer, 15 liters of neutral pH water was incorporated, and this was completed with the 5 L of the previously prepared mixture.

The applications were made in the afternoon (4:00 pm), with a frequency of every 7 days during the first three weeks, and then extended to every 15 days, adding a total of 14 applications in the tree, for the entire trial.

Nine trees were sampled per treatment, the sample unit being 10 leaves per tree, divided into four leaves of the lower third, four of the middle third, and two of the upper third. The number of mites per leaf was quantified every 7 days, making observations at 7, 14, 21, 35, 49, and up to 63 days.

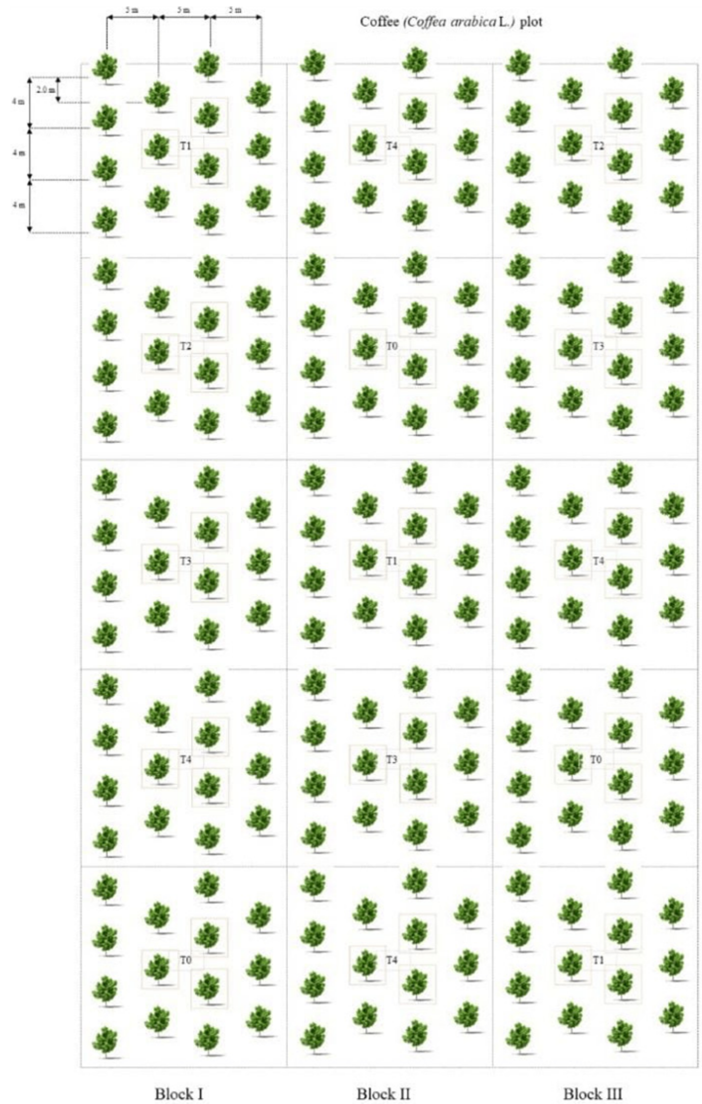

Figure 1. Sketch of the experimental field and distribution of treatments. Coffee plots (*Coffea arabica*). Block I, block II, block III.

Efficiency estimates were made using the formula of Henderson and Tilton (1955).

% Corrected efficacy percentage = [1-(Ca/Ta) * (Td/Cd)] *100

Where:

Ta = Infestation in treated plot before treatment.

Ca = Infestation in control plot before applying the treatment.

Td = Infestation in treated plot after application of treatment.

Cd = Infestation in control plot after applying the treatment.

Data on the number of mites per leaf were subjected to an analysis of variance and Fisher LSD mean comparison test (α= 0.05) using the Info Statstatistical program (Di Rienzo *et al*., 2013).

Laboratory methodology

Five hundred adult mites were selected without being discriminated by sex, which were distributed in 15 units of Petri dishes (90 x 100 mm), each dish with 100 mites and being occupied three dishes for each treatment. Clean and disinfected leaves were placed inside each unit. The preparation of the entomopathogen (SENASA, 2014) was as follows:

a) 250 mL distilled water was available.

b) They were transformed into milligrams or milliliters according to the doses used in the field.

c) The product was dosed in a 60 mL graduated cylinder where 0.06 mL of water corrector and 0.06 mL of agricultural oil (only for the entomopathogenic fungi *M. anisopliae* and *B. bassiana*) and 0.12 g of the biological product were mixed.

d) It was necessary to use a 60 mL Hammer atomizer to pour the distilled water and mix the biological product plus 0.06 mL of water corrector and 0.06 mL of agricultural oil (only for *M. anisopliae* and *B. bassiana*) to finally homogenize the mixture.

e) Efficacy observations were made daily for a period of 7 days, with the response variable being the number of mites killed per dish per day.

f) Efficiency estimates were made using the Abbott formula (1925).

$$\% \text{ Corrected efficacy percentage} = [\, 1-(Ta/Co)]\ *100$$

Where:

Ta = Population in treated plot after treatment.

Co = Population in control plot after treatment.

Results and discussion

Efficiency of entomopathogens in reducing populations of *Oligonychus yothersi* under field conditions

The effectiveness of entomopathogens is shown in table 2. At seven days of pre-application counting, populations ranged from 144 to 177 mites per leaf on average (p>0.05). After the first intervention, populations were significantly reduced to averages between 1.14 mites per leaf with the entomopathogenic fungus *M. anisopliae* and 8.64 mites per leaf with *B. subtilis* and in contrast to the control treatment populations that reached averages of up to 146.5 mites per leaf (p<0.05) in the first fourteen days after application. In subsequent evaluations the number of individuals was low and constant over time with a maximum of 4.08 mites per leaf at 63 days, this being the last evaluation; however, in the control treatment, the mite/leaf averages oscillated over time.

The percentages of efficiency for the population incidence of *O. yothersi* caused by each entomopathogenic product are shown in figure 2, where it is observed that the treatment *M. anisopliae*, during the first 14 days of the trial reduced up to 99.31 %, followed by the entomopathogen *B. thuringiensis* var. *kurstaki* with an efficacy of 97.12 %, leaving with lower percentages the *B. subtilis* and *B. bassiana.*

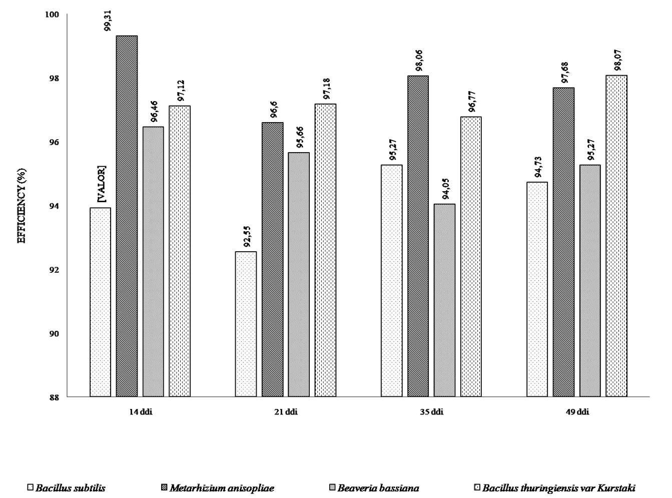

Figure 2. Efficiency (%) in the reduction of mites per leaf in days (ddi) in the avocado crops of the CIFO-UNHEVAL germplasm bank, 2019-2020 season.

Table 2. Incidence of the mite *Oligonychus yothersi* in avocado crop before and after the application of entomopathogens during the 2019-2020 season.

Treatments	Recount Pre-application	Live mites per leaf in days (ddi) ± Standard Error				
		14 ddi	21 ddi	35 ddi	49 ddi	63 ddi
Bacillus subtilis (2 mL.L^{-1})	151.67±10.34 a	8.64±2.64 a	9.28±4.08 a	5.22±3.93 a	7.19±1.85 a	4.08±3.96 a
Bt var *kurstaki* (2 mL.L^{-1})	177.00±10.34 a	5.38±2.64 a	3.67±4.08 a	3.64±3.93 a	3.31±1.85 a	2.98±3.96 a
Metarhizium anisopliae (2 g.L^{-1})	147.33±10.34 a	1.14±2.64 a	3.17±4.08 a	2.14±3.93 a	3.11±1.85 a	2.08±3.96 a
Beauveria bassiana (2 g.L^{-1})	144.00±10.34 a	5.28±2.64 a	5.17±4.08 a	5.56±3.93 a	6.25±1.85 a	2.58±3.96 a
Control	150.67±10.34 a	146.5±2.64 b	119.53±4.08 b	104.83±3.93 b	131.64±1.85 b	128.42±3.96 b
Coefficient of variance (%)	11.63	13.68	25.02	28.08	10.55	22.2

Note: ddi: days after application, different letters in the same column represent significant differences, Fisher LSD test (p < 0.05), independent statistical analysis for each time comparing treatments.

Similar behavior is demonstrated at 21, 35, 49, and 63 ddi, after inoculation, with the percentages of population reduction higher than 90 % and less than 98.07 %, the latter with *Bacillus thuringiensis* var. *kurstaki*. Research by Deka *et al*. (2022) recorded the efficiency of *M. anisopliae* up to 68.2 % in reducing the population incidence of *Oligonychus coffeae* at 14 ddi, although the percentages of effectiveness are lower compared to the present study, and according to the reports of Tahmina *et al*. (2020), native entomopathogenic isolates have higher efficiency than commercial acaricides based on entomopathogens, in the present study it is demonstrated that entomopathogens were efficient possibly favored by environmental conditions that helped dispersion, viability, and incidence (Meyling and Eilenberg, 2006; Meyling and Eilenberg, 2007). Cuatlayotl-Cottier *et al.* (2022) evaluated *B. thuringiensis* spores as a biological insecticide on *Tetranychus urticae*, observing between 60 % and 90 % mortality under field conditions and up to 50 % and 80 % under laboratory conditions. Veloorvalappil *et al*. (2018) demonstrated that the entomopathogen *Bacillus thuringiensis* var. *kurstaki* was efficient in controlling the red mite *Eutetranychus orientalis*.

Efficiency of entomopathogens in the mortality of *Oligonychus yothersi* under laboratory conditions

The evaluations showed that the entomopathogen *M. anisopliae* (1.10^{10} conidia) was the most efficient at the laboratory level, achieving a reduction at a rate of 100; 5.3; 3.7; 0.7; 0,7; 0.7 and 0.00 respectively, during six assessment days, as shown in table 3. Similar behavior was recorded for the treatment *B. subtilis*, of the 100 mites treated on the first day, all were eliminated (death) until the sixth day of evaluation.

With *B. bassiana* and *B. thuringiensis* var. *kurstaki* reductions were close to zero mites per dish, however, no significant differences have been shown between entomopathogenic treatments (p>0.05).

Except for the control treatment where it was observed that, of the 100 mites per dish, 77 mites survived on average up to six days of evaluation.

The percentages of efficiency of entomopathogens in laboratory conditions are shown in table 4, registering for *M. anisopliae* and *B. subtilis* 100 % efficiency on the sixth day after inoculation of the formulations. Followed by *B. bassiana* and *Bacillus thuringiensis* var. *kurstaki* obtaining 98.70 % efficiency in the mortality of mites of the species *O. yothersi.* Slightly higher survival rates were reported by Huanes-Carranza and Wilson-Krugg (2016) with *B. bassiana* and *M. anisopliae* on adults and nymphs of *Oligonychus* sp. Meanwhile, Deka *et al*. (2022) reported that *M. anisopliae* was effective against *O. coffeae* causing a mortality of 78 % four days after application. Mamun *et al*. (2014) verified the efficiency of several entomopathogens against mites of the species *O. coffeae* under laboratory conditions, obtaining 81.83 and 97.24 % mortality with the entomopathogens *M. anisopliae* and *B. bassiana* respectively.

Conclusions

The effect of the entomopathogens *Metarhizium anisopliae*, *Beauveria bassiana* and the toxins of *Bacilluss ubtilis*, *Bacillus thuringiensis* var. *kurstaki*, in the control of *O. yothersi* in avocado crops has been proven, the most promising being *M. anisopliae* and *B. thuringiensis* var. *kurstaki* with high percentages of effectiveness in a short period of time. It is advisable to determine the persistence over time of a single application and verify the efficiency of the two entomopathogens that offered the best results.

Table 3. Number of live mites per Petri dish after the application of entomopathogenic formulations in the laboratory-UHEVAL.

Treatments	Live mites per plate and day (ddi) ± Standard Error					
	1 ddi	**2 ddi**	**3 ddi**	**4 ddi**	**5 ddi**	**6 ddi**
Bacillus subtilis (2 $mL.L^{-1}$)	9.7±2.0 a	8.0±1.9 a	6.0±1.71 a	5.7±1.9 a	2.3±1.8 a	0.0±0.9 a
Bt var. *kurstaki* (2 $mL.L^{-1}$)	3.7±2.0 a	3.7±1.9 a	3.3±1.71 a	2.7±1.9 a	2.0±1.8 a	1.0±0.9 a
Metarhizium anisopliae (2 $g.L^{-1}$)	5.3±2.0 a	3.7±1.9 a	0.7±1.71 a	0.7±1.9 a	0.7±1.8 a	0.0±0.9 a
Beauveria bassiana (2 $g.L^{-1}$)	10.3±2.0 a	8.0±1.9 a	6.3±1.71 a	5.7±1.9 a	4.7±1.8 a	0.7±0.9 a
Control (no application)	98.3± b	96.7±1.9 b	96.7±1.71 b	91.3±1.9 b	84.0±1.8 b	77.0±0.9 b
Coefficient of variance (%)	14.0	13.72	13.1	15.6	16.5	10.1

Note: ddi: days after application, different letters in the same column show significant differences, LSD Fisher test (p < 0.05), independent statistical analysis for each time comparing treatments.

Table 4. Effectiveness of entomopathogens in reducing mites by dish under laboratory conditions.

	Efficiency (%)					
Treatments	**1 ddi**	**2 ddi**	**3 ddi**	**4 ddi**	**5 ddi**	**6 ddi**
Bacillus subtilis (2 $mL.L^{-1}$)	89.80	91.75	93.81	93.41	97.62	100.00
Bacillus thuringiensis var. *kurstaki* (2 $mL.L^{-1}$)	95.92	95.88	96.91	96.70	97.62	98.70
Metarhizium anisopliae (2 $g.L^{-1}$)	94.90	95.88	98.97	98.90	98.81	100.00
Beauveria bassiana (2 $g.L^{-1}$)	89.80	91.75	93.81	93.41	94.05	98.70
Control	......	......	......	......	......	......

Note: ddi: days after inoculation, % effectiveness: calculated according to Abbott's formula (Abbott, 1925), independent statistical analysis for each time comparing treatments.

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