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ORIGINAL ARTICLE

# Susceptibility of *Plutella xylostella* (Lepidoptera: Plutellidae; Linnaeus 1758) to *Beauveria bassiana* Bb9205, *Metarhizium anisopliae* Ma9236 and *Heterorhabditis bacteriophora* HNI0100

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## Abstract

The diamondback moth (*Plutella xylostella*) is a major pest of broccoli worldwide. It mainly causes leaf defoliation and generates annual losses of 80%. In this study we evaluated the susceptibility of *P. xylostella* to entomopathogens *Heterorhabditis bacteriophora* HNI0100, *Beauveria bassiana* Bb9205 and *Metarhizium anisopliae* Ma9236. The methodology was based on the inoculation of third instar larvae of *P. xylostella* with $5x10^1$, $1x10^2$, $3x10^2$, $6x10^2$ and $1{,}2x10^3$ IJs/cm$^2$ of *H. bacteriophora* HNI0100 and evaluated them after 24, 48 and 72 h and $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ con/cm$^2$ of *B. bassiana* Bb9205 and *M. anisopliae* Ma9236, which were evaluated during two weeks. At a dose of $1{,}2\ x10^3$ JIs/cm$^2$, *P. xylostella* had a susceptibility to *H. bacteriophora* HNI0100 of 91,66%. Similarly, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 had a mortality of 95,33 and 99,67% at $1x10^5$ con/cm$^2$. The results suggest that the use of strains of entomopathogenic nematodes and fungi is an innovative alternative for the control of *P. xylostella*. However, studies on the interaction of nematodes and fungi and *Plutella xylostella* are necessary.

**Keywords:** Broccoli; biological control; diamondback moth; entomopathogenic fungi; entomopathogenic nematodes.

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## Introduction

One of the main pests affecting broccoli (*Brassica oleracea*) is the diamondback moth, *Plutella xylostella* (Linnaeus 1758; Lepidoptera: Plutellidae; Bertolaccini et al. 2010, Sáenz 2012). Worldwide, this pest generates losses of over 80% (U.S. $4 to U.S. $5 billion) in annual crop production (**Table 1**) (Verkerk & Wright 1996, Sarfraz et al. 2005, Zalucki et al. 2012).

The diamondback moth has a four-stage life cycle (egg, larva, pupa and adult) of 15 to 40 days depending on regional weather conditions; the entire life cycle takes place on the leaves abaxial surface of the host plant (Sarfraz et al. 2005). The egg-stage lasts 3.2 days at 20 °C. During the 15 days of its larval instars

**Table 1.** Continents where *Brassica oleracea* (Broccoli) is grown and annual insecticide application costs (U.S.$). CI: Cost of insecticide application, and CIPM: Cost of insecticide application with IPM.

| Continent | CI | CIPM | Reference |
|---|---|---|---|
| **Africa** | $ 46,097,772 | $ 11,141,005 | Zalucki et al. (2012) |
| **Australia and the Pacific** | $ 1,169,364 | $ 267,545 | Goodwin & Danthanarayana (1984), Zalucki et al. (2012) |
| **Asia** | $ 695,435,398 | $ 161,195,709 | Honda (1992), Verkerk & Wright (1996), Zalucki et al. (2012) |
| **Europe** | $ 216,137,670 | $ 52,762,140 | Zalucki et al. (2012) |
| **North and Central America** | $ 42,129,738 | $ 10,952,758 | Madder & Stemeroff (1998), Dosdall et al. (2004), Zalucki et al. (2012), Furlong et al. (2013) |
| **South America** | $ 5,644,128 | $ 1,303,107 | Talekar & Shelton (1993), Zalucki et al. (2012) |
| **World total** | $ 1,006,614,070 | $ 237,622,266 | Talekar & Shelton (1993), Zalucki et al. (2012), Furlong et al. (2013) |

(four instars), the pest causes the greatest damage to broccoli crops (Somvanshi & Ganguly 2007). White spots on the leaves evidence surperficial mines that alter the photosynthesis process, and cause a decrease in the size and quality of the product intended for consumption (Franco 2001, Schroer et al. 2005, Chavez & Hurtado 2010). Eighteen days before the adult hatches, the pupa is covered with a silk thread on the abaxial surface of the leaf (Talekar & Shelton 1993, Chavez & Hurtado 2010). Adults are nocturnal; males emerge seeking a partner for copulation and are found on the leaves during the day. The average adult female lays 160 to 360 eggs (Furlong et al. 2013).

This insect pest is controlled through the continued use of agrochemicals. A 99-day crop can be treated three to five times with insecticides such as organophosphates, pyrethroids and carbamates (Sarfraz et al. 2005). The indiscriminate use of these products by farmers has led to excessive application of the same and an increase in resistance issues with this lepidopteran pest (Monzón 2001, Sáenz 2012). For this reason, other alternatives of control have been evaluated such as physical barriers, as light and resistant covers to prevent the pest from accessing the plants (Chavez & Hurtado 2010) or biological control using parasitoids such as *Diadegma insulare* or *Cotesia plutellae* or entomopathogenic fungi and nematodes, which have shown significant advance in managing the diamondback moth (Sarfraz et al. 2005, Schroer et al. 2005, Bertolaccini et al. 2010).

With a 85% mortality, the entomopathogenic nematode, *Heterorhabditis bacteriophora* (Poinar 1976) (Rhabditida: Heterorhabditidae) is one of the species with the greatest potential to control *P. xylostella* (Shinde & Singh 2000, Somvanshi & Ganguly 2007). This nematode species is a cruise-type forager that can enter through the anus, spiracles or cuticle (Sánchez 2002). It is hermaphrodite during the first generation of its life cycle and has a tooth that allows it to perforate any insect cuticle, which gives it a competitive advantage over other biocontrol agents (Sáenz & Lopez 2010).

Entomopathogenic fungi, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Cordycipitaceae) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Clavicipitaceae) have been widely used to control *P. xylostella* (Loc & Chi 2007), producing over 50% mortality (Furlong & Pell 2001, Pucheta et al. 2006,

Hui Wu et al. 2010). Although they do not enter through the mouth or anus, they have the ability to adhere to the insect cuticle and penetrate it using cuticle-degrading enzymes to carry out an effective colonization (Loc & Chi 2007).

The success of using entomopathogenic nematodes and fungi in biological control strategies depends on the strains of natural enemies and their relationship with the pest (Sarfraz et al. 2005). The populations of *M. anisopliae*, *B. bassiana* and *H. bacteriophora* differ genetically and biologically depending on the region in which they are isolated; therefore, it is necessary to assess their virulence and pathogenicity to the pest to be controlled (Furlong et al. 2013).

The objective of this study was to assess the susceptibility of *P. xylostella* to *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 for future studies on the interaction of fungi and nematodes to control diamondback moth.

## Materials and methods

***Entomopathogens: H. bacteriophora* HNI0100, *M. anisopliae* Ma9236 and *B. bassiana* Bb9205.** After screening different strains of Steinernematidae and Heterorhabditidae, entomopathogenic nematode *H. bacteriophora* HNI0100 was used for the laboratory tests (Delgado-Ochica & Sáenz 2012). The Biological Control Laboratory at Pontificia Universidad Javeriana provided the infective juveniles, which were multiplied *in vivo* by infecting last instar larvae of *Galleria mellonella* (Linnaeus 1756) (Lepidoptera: Pyralidae) following the methodology of Kaya & Stock (1997). The entomopathogenic fungi *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 were obtained from the Laboratorio de Control de Calidad de Bioinsumos Agrícolas - Control de Bioinsumos Disciplina de Entomología, Cenicafé, Chinchiná – Caldas – Colombia. The fungi were activated by infecting *P. xylostella*. For the optimal growth and production of conidia, the reactivated strains were grown in PDA (potato dextrose agar) and oatmeal agar for 15 days at 25 °C.

**Entomopathogenic Evaluation:** The susceptibility of *P. xylostella* to *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 was evaluated by transferring one larva of third instar to 2 oz plastic containers, each one containing 5 g of broccoli leaf and 60 g of sterile river sand (**Table 2**). The infective juveniles (IJs) were suspended in distilled water with Tween 80 (0.1 % v/v) and inoculated over the sand and broccoli leaf. Larvae mortality by entomopathogenic nematodes was evaluated during 24, 48 and 72 hours. Subsequently, once dead, the larvae were transferred to White traps to recover infective juveniles. Fungal conidia were suspended in saline solution with Tween 80 (0.1 % v/v) and inoculated over the sand and broccoli leaf. Larvae mortality by entomopathogenic fungi was evaluated during two weeks.

**Statistical Analysis:** *P. xylostella* mortality percentages were tested for variance homogeneity (Levene) and normality (Kolmogorov-Smirnov); the assumptions for parametric data were met, demonstrating that treatment variances were the same and their errors had a normal

**Table 2.** Treatments and doses used for experimental design of *Plutella xylostella* susceptibility to entomopathogens *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236.

| Treatment | *H. bacteriophora* HNI0100 + *P. xylostella* (JIs/cm$^2$) | *B. bassiana* Bb9205 + *P. xylostella* (con/cm$^2$) | *M. anisopliae* Ma9236 + *P. xylostella* (con/cm$^2$) |
|---|---|---|---|
| 1 | 5 x $10^1$ | 1 x $10^4$ | 1 x $10^4$ |
| 2 | 1 x $10^2$ | 1 x $10^5$ | 1 x $10^5$ |
| 3 | 3 x $10^2$ | 1 x $10^6$ | 1 x $10^6$ |
| 4 | 6 x $10^2$ | 1 x $10^7$ | 1 x $10^7$ |
| 5 | 1,2 x $10^3$ | 1 x $10^8$ | 1 x $10^8$ |
| 6 | 0 | 0 | 0 |

distribution. A univariate ANOVA with a factorial arrangement was used to determine whether dose and time means were the same or different in relation to the mortality percentage of each test (entomopathogenic nematodes and fungi) and to determine the interaction of both factors. Subsequently, multiple comparisons using Tukey and Scheffe tests were performed, employing a 95% probability, to identify the dose with the highest mortality of *P. xylostella* larvae, as well as the time during which the highest mortality occurred. The tests were performed using Statistix 10 software.

## Results

Third instar larvae of *P. xylostella* were susceptible to *H. bacteriophora* HNI0100 with significant differences between doses (F=5002.48, p=0.0000) and time (F=2614.75, p=0.0000) (**Figure 1a**). The post-hoc tests indicated that the dose with the highest mortality was 1,2x10$^3$ IJs/cm$^2$ with a percentage of 91,66%. Similarly, the highest larvae mortality occurred at 48 and 72 hours post-inoculation. This experiment replicated the interaction between dose and time (F=205.71, p=0.0000) mentioned by Goettel et al. (1993). Larvae cavaders displayed a yellow-ocher coloration, which is an indicative of a IJs infection (**Figure 2a**). Additionally, the IJs migrated from cadavers (**Figure 2b**).

Third instar larvae were susceptible to *M. anisopliae* Ma9236 (**Figure 2c**) and *B. bassiana* Bb9205 (**Figure 2d** and **2e**), presenting a characteristic fungal symptomatology. Significant differences between doses (F=222.06, p=0.0000) and time (F=274.57, p=0.0000) were found for *B. bassiana* Bb9205 (**Figure 1b**), *M. anisopliae* Ma9236 behaved equally with dose (F=1529.34, p=0.0000) and time (F=11060.65, p=0.0000) (**Figure 1c**).

The highest mortality produced by *B. bassiana* Bb9205 (95,33%) was at a dose of 1x10$^5$ con/cm$^2$ 15 days after inoculation, and by *M. anisopliae* Ma9236 (99,67%) was at a dose of 1x10$^5$ con/cm$^2$ 15 days after inoculation. The interaction between dose and time for *B. bassiana* (F=32.15, p=0.0000) and *M. anisopliae* (F=171.94, p=0.0000) suggests a trend of increasing mortality over time.

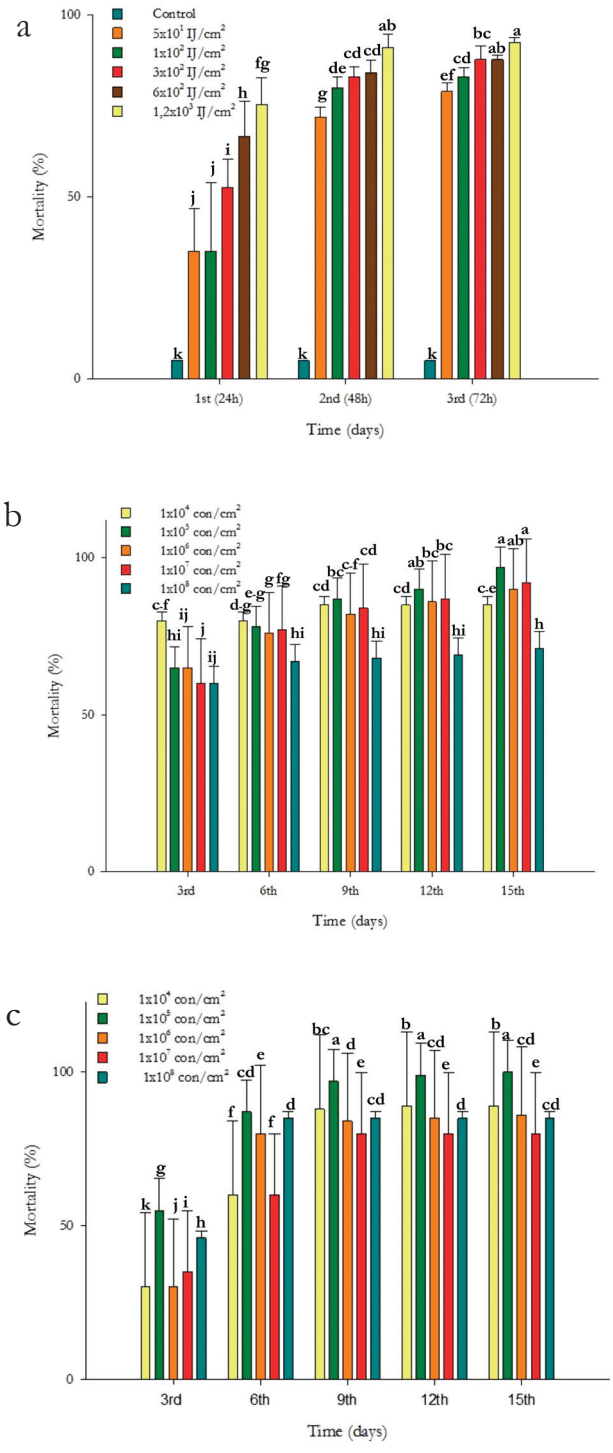


**Fig. 1. a.** Susceptibility of *P. xylostella* to *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 in time. **a.** *H. bacteriophora* HNI0100. **b.** *B. bassiana* Bb9205. **c.** *M. anisopliae* Ma9236. Ratios were calculated for each treatment and used to determine the standard error. Treatments with the same letter had no significant difference (p<0.05).

## Discussion

Third instar larvae of *P. xylostella* were susceptible to *H. bacteriophora* HNI0100. The highest mortality, a percentage of 91,66%, occurred with the dose of $1,2x10^3$ IJs/$cm^2$ at 72 hours post-inoculation. A study by Sáenz (2012) obtained similar data, the mortality rate in this study was of 95%. Ratansinghe & Hauge

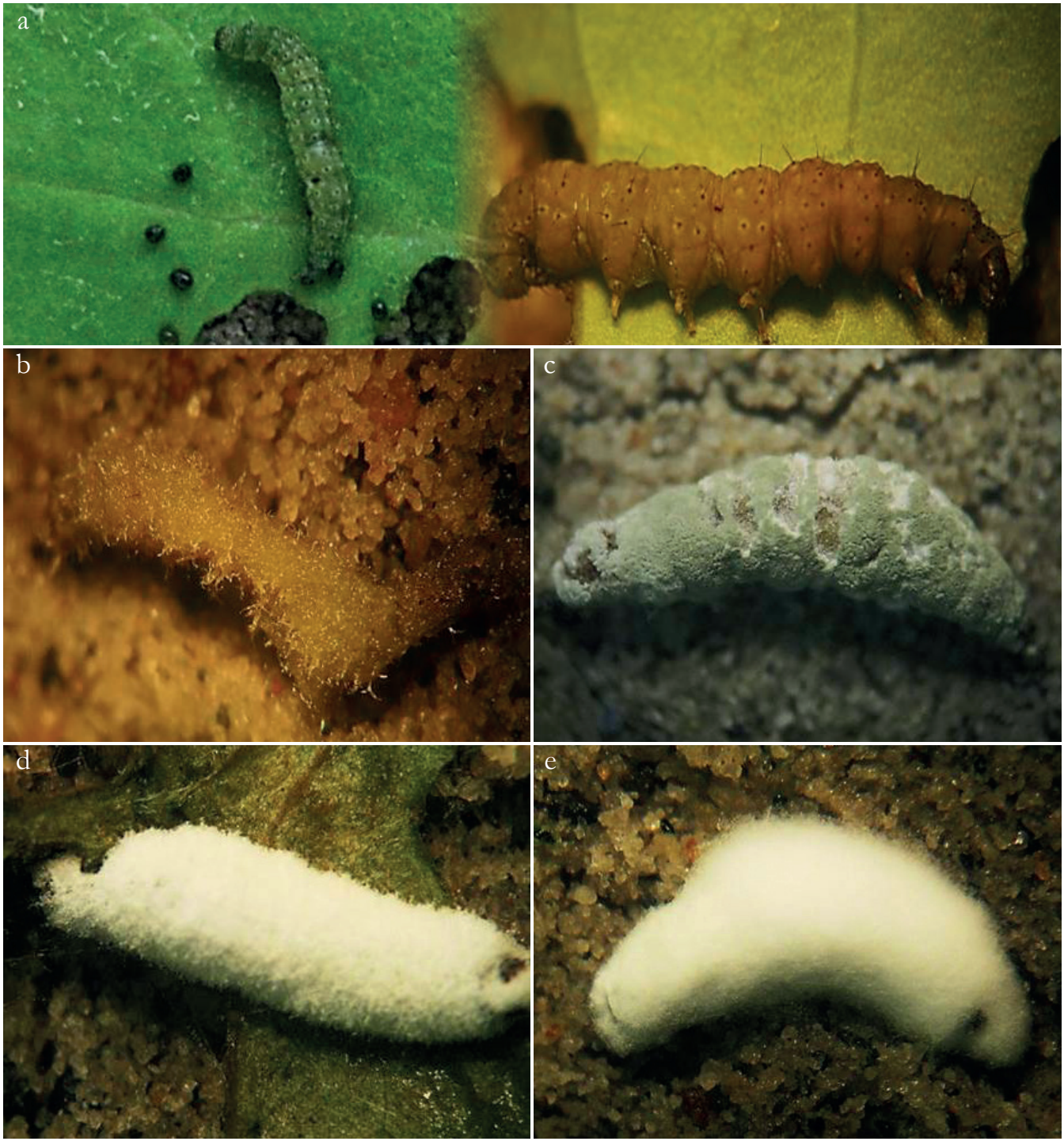


**Fig. 2.** Susceptibility of *P. xylostella* to *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236. **a.** Control larva and larva killed by *H. bacteriophora* HNI0100 with yellow ocher coloration. **b.** Larva killed by entomopathogenic nematodes emerging from host. **c.** Larva killed by *M. anisopliae* Ma9236 with green mycelium. **d.** - **e.** Larva killed by *B. bassiana* Bb9205 with white mycelium.

(1995) and Nyasani et al. (2008 a and b) also obtained mortality rates of 85 to 95% with *H. indica*. The results obtained by Salem et al. (2007) demonstrated that the percentage of mortality increased with time, establishing that the mortality generated in the first 48 hours is due to a mutualistic relationship with *Photorhabdus luminescens* (Poinar 1976), which gives the nematode a competitive advantage allowing it to kill its host quickly (Mason & Wright 2000, Sáenz 2012). According to Mason & Wright (1997) and Baur et al. (1995), the yellow-ocher coloration displayed by *P. xylostella* after infection by *H. bacteriophora* HNI0100 is not the characteristic symptomatology for Heterorhabditidae, which usually display a reddish hue. This divergence is attributed to the insect's original color (green), the concentration of bacterial cells of *P. luminescens* in each infective juvenile that enters the larva (30-200 IJs), the concentration of bacteria in the insect's hemolymph and the refraction of light on the cuticle (Silva et al. 2002, Waterfield et al. 2009). The larvae had a symptomatology characteristic of infection by nematodes, displaying flaccidity and little mobility, as reported by Nyasani et al. (2008).

Third instar larvae were susceptible to *B. bassiana* Bb9205 and *M. anisopliae* Ma9236. Both fungi had the highest mortality at a dose of $1x10^5$ con/$cm^2$ on day 15 after inoculation; this is comparable to results obtained by Franco (2001), who establish that the percentage of mortality increases with time, and is corroborated by studies by Loc & Chi (2007), Thuy (2001) Butt & Goettel (2000), that conclude that fungal pathogenicity is characterized by a gradual increase in mortality with extended exposure time. According to Quesada-Moraga & Vey (2004), Hui Wu et al. (2010) and Anaisie et al. (2011), concentrations of $1x10^5$ con/$cm^2$ are low to cause a mortality percentage exceeding 60% in *P. xylostella*; these authors recommend the use of concentrations in excess of $1x10^9$ con/$cm^2$ to produce a larval mortality of 80%. This study demonstrates that a high mortality can be obtained at low-doses, depending on the species and strain of the entomopathogenic fungus (Roy & Pell 2000, Furlong & Pell 2001, Sun et al. 2002, Furlong 2004). Sarfraz et al. (2005) indicate that the success of biological control strategies depends on the accurate identification of natural enemies and association with the strain; misidentifications can prompt the failure of the pest management program. Ibrahim & Low (1993) and Shelton et al. (1998) state that strains of entomopathogenic fungi produce different responses in mortality of individuals because their virulence and pathogenicity are not the same. By conducting tests on *P. xylostella* with six isolates of *B. bassiana* and two of *M. anisopliae*, Godonou et al. (2009) found that the strain with the highest mortality (94%) was Bba5653, establishing that the percentage and death rate of the pest can be variable. A study by Vandenberg et al. (1998) determined that the survival times for *P. xylostella* larvae, inoculated at different doses, were variable for two strains of *B. bassiana*, pathogenicity decreased with an increased dose, which agrees with our results. They also established that survival times and mortality vary between the two isolates, this is related to strains and their virulence.

The relationship between doses, mortality, biological controllers and pest generally responds to a positive correlation between the dose and the pathogen ability to kill the pest (Pena et al. 1991, Ferron 1981, Butt & Goettel 2000). In this study, the nematodes dose with the highest mortality was the most concentrated ($1,2x10^3$ JIs/$cm^2$), which may suggest a positive correlation between dose and mortality. Contrary, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 showed a possible negative correlation, thus $1x10^5$ con/$cm^2$, the dose with de highest mortality, is one of the lowest doses tested. This result may be explained by Butt & Goettel (2000), who mentioned that for entomopathogenic fungi there is a threshold dose to kill a pest, nevertheless the exact nature of this relationship has not been defined yet. Studies by Goettel et al. (1993) and Vandenberg et al. (1998) report a negative correlation between doses and mortality at the highest concentrations, occurring an autoinhibition; which may explain our mortality results for the fungal strains.

This study is of high importance as it gives way to establish interactions between fungi and nematodes that can generate possible synergy or additivity to pest mortality.

## Conclusion

The inoculations of entomopathogenic nematodes ($1,2x10^3$ JIs/$cm^2$) and fungi ($1x10^5$ con/$cm^2$) cause

mortality rates exceeding 90% for *P. xylostella*, in comparison with the majority of studies using higher concentrations to achieve the same mortality. The use of Colombian strains of *H. bacteriophora* HNI0100, *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 is an innovative alternative for the control of *P. xylostella* and should be considered a management strategy in broccoli production.

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## Conflicts of interest

The authors declare that there are no conflicts of interest regarding the results published in this work.

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**Susceptibilidad de *Plutella xylostella* (Lepidoptera: Plutellidae; Linnaeus 1758) a *Beauveria bassiana* Bb9205, *Metarhizium anisopliae* Ma9236 y *Heterorhabditis bacteriophora* HNI0100**

**Resumen.** La palomilla dorso de diamante (*Plutella xylostella*) es una de las principales plagas del cultivo de brócoli (*Brassica oleracea*) en el mundo. El principal daño es la defoliación de las hojas, generando pérdidas anuales del 80%. El objetivo de este estudio fue evaluar la susceptibilidad de *P. xylostella* a los entomopatógenos *Heterorhabditis bacteriophora* HNI0100, *Beauveria bassiana* Bb9205 y *Metarhizium anisopliae* Ma9236. La metodología se basó en la inoculación de $5x10^1$, $1x10^2$, $3x10^2$, $6x10^2$ y $1,2x10^3$ JIs/cm$^2$ en larvas de tercer instar de *P. xylostella* evaluada a las 24, 48 y 72 h y $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ y $1x10^8$ con/cm$^2$ de *B. bassiana* y *M. anisopliae* evaluadas durante dos semanas. Los resultados mostraron que *P. xylostella* fue susceptible a *H. bacteriophora* HNI0100 con una tasa de mortalidad del 91,66% a dosis de $1,2x10^3$ JIs/cm$^2$. Así mismo, *B. bassiana* Bb9205 y *M. anisopliae* Ma9236 generaron 95,33 y 99,67% de mortalidad con la dosis de $1x10^5$ con/cm$^2$. El uso de nematodos y hongos entomopatógenos es una alternativa innovadora para el control de *P. xylostella*, sin embargo, se requiere estudiar su interacción para el control de este insecto plaga.

**Palabras clave:** Brócoli; control biológico; hongos entomopatógenos; nematodos entomopatógenos; palomilla dorso de diamante.

**Susceptibilidade de *Plutella xylostella* (Lepidoptera: Plutellidae; Linnaeus 1758) a *Beauveria bassiana* Bb9205, *Metarhizium anisopliae* Ma9236 e *Heterorhabditis bacteriophora* HNI0100**

**Resumo.** A mariposa dorso de diamante *Plutella xylostella* é uma das principais pragas do cultivo dos brócolos (*Brassica oleracea*) no mundo. O principal dano é o desfolhamento das folhas, gerando perdas anuais de 80%. O objetivo deste estudo foi avaliar a suceptibilidade da *P. xylostella* aos entomopatógenos *Heterorhabditis bacteriophora* HNI0100, *Beauveria bassiana* Bb9205 e *Metarhizium anisopliae* Ma9236. A metodologia baseou-se na inoculação de $5x10^1$, $1x10^2$, $3x10^2$, $6x10^2$ y $1,2x10^3$ JIs/cm$^2$ em larvas do terceiro instar de *P. xylostella* avaliada às 24, 48 e 72 h e $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ e $1x10^8$ con/cm$^2$ de *B. bassiana* e *M. anisopliae* avaliadas durante 2 semanas. Os resultados mostraram que *P. xylostella* foi susceptível a *H. bacteriophora* HNI0100 com uma taxa de mortalidade de 91,66% com a dose de $1,2x10^3$ JIs/cm$^2$. Desta forma, *B. bassiana* Bb9205 e *M. anisopliae* Ma9236 geraram 95,33 é 99,67% de mortalidade com a dose de $1x10^5$ con/cm$^2$. O uso de estirpes colombianas de nematóides e fungos entomopatógenos é uma alternativa inovadora para o controlo de *P. xylostella*. Ainda se requer estudar a interação entre fungos e nematóides em *Plutella xylostella*.

**Palavras-chave:** Brócolos; controlo biológico; fungos entomopatógenos; nematóides entomopatógenos; mariposa dorso de diamante.