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ORIGINAL ARTICLE

Endophytic *Beauveria bassiana* (Ascomycota: Hypocreales) alters *Helicoverpa gelotopoeon*`s (D.) (Lepidoptera: Noctuidae) life cycle and reproductive parameters

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Abstract

Bollworms comprise the most harmful and economically relevant species of lepidopteran. *Helicoverpa gelotopoeon* (D.) (Lepidoptera: Noctuidae) is native to America and affects many crops. Tobacco is an industrial crop in which methods of pest control rely mainly on the application of insecticides. To develop new eco-friendly strategies against insect pests it is very important to overcome the side effects of insecticides. The utilization of fungal entomopathogens as endophytes is a new perspective that may accomplish good results. The present study aimed to evaluate the ability of endophytic *Beauveria bassiana* (Bals.-Criv.) Vuill. to affect *H. gelotopoeon* life parameters and feeding behavior on tobacco plants. *Beauveria bassiana* LPSC 1215 as an endophyte did not reduce the amount of vegetal material consumed by *H. gelotopoeon* larvae but affected the life cycle period of the plague, particularly the larval and adult stages. Also, egg fertility was affected since adults laid eggs that were not able to hatch. The results of this investigation provide new information on endophytic entomopathogen potential to be incorporated in Integrated Pest Management (IPM) programs.

Keywords: *Beauveria bassiana*, endophytic entomopathogens, fecundity, *Helicoverpa gelotopoeon*, life cycle

Introduction

Bollworms belong to a complex of generalist species that feed on the apical part of plants where meristematic tissues are found causing important losses in crop yields. Bollworms comprise several species primarily in the Noctuidae family that includes the highest number of the most economically relevant species (Herrero *et al.* 2018). *Helicoverpa gelotopoeon* (D.) (Lepidoptera: Noctuidae) is native to America and belongs to this complex. It is a pest which is expanding rapidly in Argentina, affecting many crops such as soybean, cotton, flax, alfalfa and tobacco (Delgado and Fedre 2003; Herrero *et al.* 2018).

Tobacco (*Nicotiana tabacum* L.) is an industrial crop cultivated in many countries such as China, India, Brazil, Zimbabwe and Argentina among others. In underdeveloped countries its cultivation is of great importance to regional economies, sustaining the income of a large part of the urban and rural population in the areas involved (Camara del Tabaco de Jujuy 2008).

Control strategies for lepidopteran pests rely almost exclusively on the utilization of chemical insecticides. Nowadays, the development of new, eco-friendly strategies against insects in order to enhance crop protection and yield has become very important. The utilization of fungal entomopathogens as endophytes is a new perspective that has caught the attention of researchers in recent years (Lacava and Azevedo 2014). Different species of entomopathogenic fungi such as

Beauveria bassiana (Bals.-Criv.) Vuill., *Lecanicillium lecanii* (Zimm.) Zare & W. Gams, and *Purpureocillium* spp. (Ascomycota: Hypocreales) have been introduced as endophytes in a wide range of plants (Vega *et al.* 2008) and are considered as non-clavipitaceous Class 2 type of endophytes since they can be horizontally transmitted (Rodriguez *et al.* 2009). These microorganisms not only may serve as insect control agents but also may play diverse roles when *in planta* promoting plant growth and yield and conferring protection against plant pathogens (Vega *et al.* 2009).

Fungal endophytic entomopathogens are known to colonize several horticultural and agronomic crops, providing protection from herbivore damage and also regulating insect populations. Powell *et al.* (2007) reported damage reduction in tomato by *Helicoverpa zea;* Gurulingappa *et al.* (2011) and Hernawati *et al.* (2011), demonstrated that entomopathogens as endophytes reduced growth and fecundity of aphids. Also, the *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) life cycle and fecundity were affected by endophytic *Acremonium strictum* W. Gams (Ascomycota: Hypocreales) (Jaber and Vidal 2010).

It is necessary to carry out studies on the biology of insect pests and their interaction with entomopathogens and plants while developing Integrated Pest Management (IPM) strategies. The present study was undertaken to evaluate the ability of the endophytic *B. bassiana* strain LPSC 1215 to affect *H. gelotopoeon* life parameters and feeding behavior on tobacco plants.

Materials and Methods

Biological material

Fungus

Beauveria bassiana strain LPSC 1215 (GeneBank MH050802) was isolated from soil associated with tobacco plants and was selected due to its endophytic capacity (unpublished data) on tobacco plants.

Plates containing Potato Dextrose Agar (PDA, Britania® S.A., Buenos Aires, Argentina) were inoculated with the fungal strain and incubated at 25°C in the dark for a week to promote fungal growth and sporulation. Conidia were collected by scraping plate surfaces with a sterile scalpel. Inoculum was prepared by adding 0.1% (v/v) Tween®80. Finally, the concentration was adjusted at 1×10^8 conidia · ml^{-1} with a hemocytometer.

Plants

Nicotiana tabacum L. (variety K394) seeds were provided by Cooperativa de Tabacaleros de Jujuy Ltda. Argentina. These were planted in plastic trays (28 × 50 × 4 cm) with a mix of soil, perlite and vermiculite (1 : 1 : 1) as substrate and grown in a greenhouse with natural lighting at 24 ± 2°C and 75 ± 5% relative humidity (RH). Seedlings were individually transplanted into plastic pots (500 cm^3) at the two-leaf stage using the same substrate. The plants were watered as needed and used for experiments at the five-leaf stage.

For bioassays, treated tobacco plants were inoculated with 2 ml of *B. bassiana* inoculum of adjusted concentration (1×10^8 conidia · ml^{-1}). The technique used for this purpose was leaf aspersion since it has been demonstrated to be the most effective way to introduce *B. bassiana* as endophyte in *N. tabacum* plants (Russo *et al.* 2015).

At the start of each experiment, and 7 days after inoculation, tobacco plants were selected randomly for endophytic colonization assessment. For this, under laminar flow, leaf squares (10 × 15 mm), and 1 cm pieces of stem and roots were cut with a sterile scalpel and were surface sterilized by submerging 2 min in ethanol 70% (v/v), followed by sodium hypochlorite 2.6% (v/v) for 3 min. Subsequently they were washed with three changes of sterile distilled water and finally, dried with sterile tissue paper (Sánchez-Rodríguez *et al.* 2018). Dead tissue edges which resulted from the sterilization process were cut off from every plant piece. Samples were eventually placed on Petri dishes containing PDA, amended with antibiotics (streptomycin and chloramphenicol). The effectiveness of sterilization was ascertained by pressing samples onto the agar surface of PDA according to Schulz *et al.* (1998). Plates were incubated under controlled conditions (24 ± 1°C, 70 ± 5% humidity, dark) and checked every 2 days for endophytic *B. bassiana* emergence. Those plants with colonization rates above 80% were utilized in bioassays.

Insects

Helicoverpa gelotopoeon eggs were provided by Instituto de Microbiología y Zoología Agrícola (IMyZA) INTA Castelar, Buenos Aires, Argentina to establish a laboratory population. Larvae were fed an artificial diet *ad libitum*, according to Patana (1977), and reared under controlled conditions in a climatic chamber (25 ± 2°C and 70% humidity, with a photoperiod of 14L : 10D). Pupae were placed in plastic jars (1,000 cm^3) and moved to similar plastic jars after adult emergence. The adults were offered a water-sugar solution as a food source and were also provided with pieces of paper for egg laying. A pool of newly hatched larvae was reared together until third instar in plastic pots (500 cm^3) and provided with an artificial diet. At that stage they were individualized in plastic Petri dishes. This F1 generation was utilized for laboratory bioassay 1. To study the life cycle and biological

parameters (bioassay 2), 200 eggs were placed individually in plastic Petri dishes.

Bioassay 1: Endophytic *B. bassiana* effect on *H. gelotopoeon* caterpillars` food preference

To determine *B. bassiana* effect on food preference, 3rd instar larvae were placed individually in plastic Petri dishes and were offered two tobacco leaf discs (250 mm diameter), one from inoculated plants and the other one as control (not inoculated and no *B. bassiana* as an endophyte) (Napal *et al.* 2009). Leaf discs were placed over moistened sterile tissue paper to prevent dehydration. Leaf discs were scanned individually before and 24 h after adding larvae. The leaf consumed area for each replicate was calculated utilizing Image J software (Bailer 2006). Three repetitions of 30 individuals were analyzed, and t test performed to find differences between treatments utilizing InfoStat software (2009).

Bioassay 2: Endophytic *B. bassiana* effect on *H. gelotopoeon* biological parameters

Two hundred eggs were placed individually in plastic Petri dishes. After hatching, larvae were fed tobacco leaves. Half of them were provided with inoculated leaves as a food source and the other hundred were fed endophytic free *B. bassiana* leaves.

Food was daily renewed and provided until pupation. Pupae were sexed according to Angulo *et al.* (2008) and couples were placed in clean plastic flasks (1,000 cm^3) until moth emergence. Adults were provided with a sucrose solution by soaking cotton as a food source (Greene *et al.* 1976).

Biological parameters

Development of *H. gelotopoeon* individuals was daily observed. The number of individuals (n_x), days spent, mortality by age (d_x) and survival (l_x) for each of the stages (egg, L1–L5, pupa and adult) were registered.

In the case of the different larval instars, molting was considered when head capsules of the previous stage were observed. Once they reached pupa stage, insects were sexed and placed as couples in plastic jars (1,000 cm^3) and when adults emerged offered a water-sugar solution as a food source. They were also provided with pieces of paper for egg laying.

Dead individuals were placed in a humid chamber (Powell 2007) to promote the development of fungal structures and to confirm the way death had been caused by mycosis.

Fertility and fecundity

The number of days during which females laid eggs, the number of eggs and the number of hatched eggs were registered to determine the ovipositional period, fecundity and fertility, respectively.

Bioassay 2 data sets were analyzed using age stage, two-sex life table analysis (Chi 2013) and when appropriate, t tests were performed using InfoStat software (2009).

Results

Bioassay 1: Endophytic *B. bassiana* effect on food preference

The mean consumed area by *H. gelotopoeon* larvae is shown in Figure 1. Control caterpillars ate an average of 112,906.37 mm^2 ± 25,426.15 of leaves and those fed colonized plants, 120,143.89 mm^2 ± 22,636.59. T-test results indicated that there were no significant differences in food preference between control and treated leaves (t = 0.35, df = 58, p = 0.72).

Bioassay 2: Endophytic *B. bassiana* effect on life table parameters

*Helicoverpa gelotopoeon´*s life table parameters for both control and larvae fed treated leaves are shown in Table 1.

The developmental period for egg and pupa stages did not differ in both cohorts (t = 0.66; df = 198 and t = 0.18; df = 190; p = 0.8549, respectively). Instead, all larval instars showed significant differences between insects fed control and treated plants (L1: t = 2.71, df = 198, p = 0.0073; L2: t = 2.56, df = 198, p = 0.0113; L3: t = 2.66, df = 198, p = 0.0084; L4: t = 2.2, df = 198, p = 0.0289 and L5: t = 2.22, df = 198, p = 0.0277). The adult stage was also significantly longer in individuals fed control leaves than treated plants (t = 4.21; df = 198; p < 0.0001). First instar larvae were the most susceptible in both treatments (d_x = 10; d_x = 11, respectively).

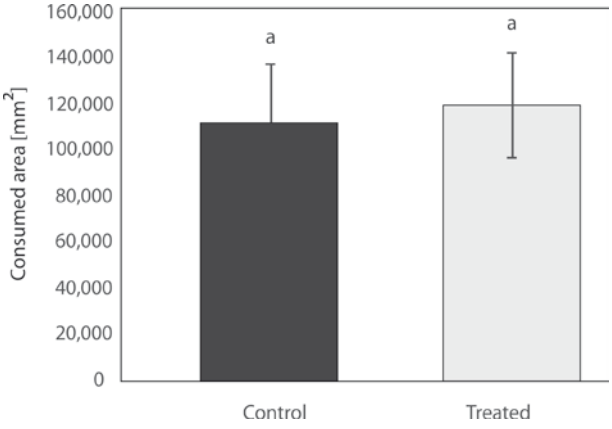

Fig. 1. Mean consumed area by *Helicoverpa gelotopoeon* larvae. Bars indicate ± SD

Table 1. Average duration of each stage (days), number of individuals (n_x), survival (l_x) and mortality (d_x) of *Helicoverpa gelotopoeon* fed tobacco inoculated with *Beauveria bassiana* and not inoculated (control). Different letters within the same line indicate significant differences

Stage	Control				Treated			
	days	n_x	l_x	d_x	days	n_x	l_x	d_x
Egg	5.11 ± 2.04 a	100	1	0	5.3 ± 1.99 a	100	1	0
L1	3.62 ± 1.41 a	90	1	10	3.1 ± 1.29 b	89	0.9	11
L2	4.98 ± 2.03 a	88	1	2	4.26 ± 1.94 b	86	0.9	3
L3	4.7 ± 2.35 a	82	0.95	6	3.87 ± 2.04 b	81	0.85	5
L4	4.58 ± 2.34 a	81	0.9	1	3.88 ± 2.14 b	79	0.8	2
L5	4.08 ± 2.09 a	81	0.85	0	3.43 ± 2.04 b	76	0.75	3
Pupa	7.45 ± 4.09 a	78	0.8	3	7.33 ± 5.11 a	69	0.6	7
Adult	3.84 ± 2.72 a	70	0.8	8	2.34 ± 2.29 b	58	0.6	11
Total cycle	38.36 ± 1.2	–	–	30	33.51 ± 1.54	–	–	42
Sex ratio F : M	1.12 : 1	–	–	–	1.25 : 1	–	–	–

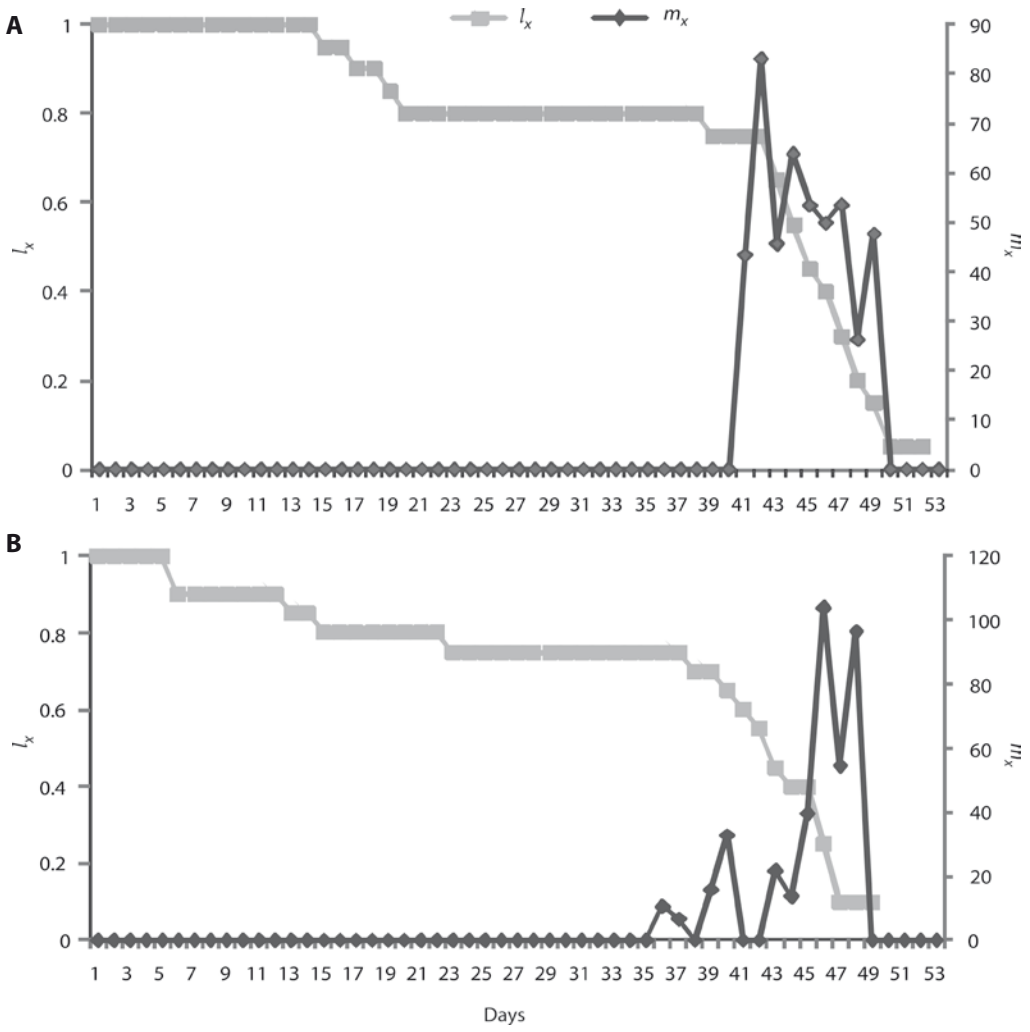

Fig. 2. Survival (l_x) and fecundity (m_x) curves of *Helicoverpa gelotopoeon* in control (A) and individuals fed *Beauveria bassiana* treated plants (B)

For insects provided with treated plants, their life cycle was 33.51 ± 1.54 days, while for control fed insects it was 38.36 ± 1.2 days.

Survival and fecundity curves for control and treated insects are shown in Figures 2A and B, respectively. The survival curve decreased earlier for insects

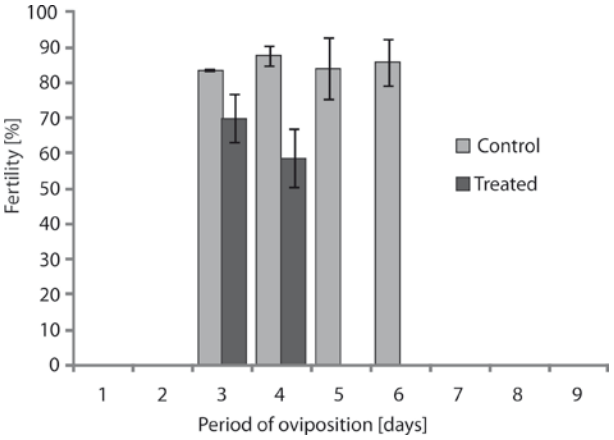

Fig. 3. Percentage fertility values for *Helicoverpa gelotopoeon* fed control and colonized by *Beauveria bassiana* leaves. Bars indicate ± SEM

fed colonized tobacco plants. The reproductive period started earlier in insects fed treated leaves than control ones (days 35 and 39, respectively) and lasted for 14 and 11 days, respectively.

For control insects, the oviposition period was 3.83 ± 1.04 days (Fig. 3) and the mean number of eggs laid per female (fecundity) was 506 ± 86.47. On the other hand, the period of oviposition for larvae fed with treated plants was 2.56 ± 0.51 days (Fig. 3) and the mean number of eggs laid per female (fecundity) was 287.62 ± 75.12. Significant differences between treatments were obtained for fertility ($t = 2.78$; $df = 38$; $p = 0.0085$). Percentage fertility values registered were 85.82 ± 16.46 and 64.73 ± 20.05 for individuals fed control and treated plants, respectively (Fig. 3).

Insect cadavers never showed colonization by *B. bassiana* after placing them in a humid chamber.

Discussion

Tritrophic interactions between the entomopathogen *B. bassiana*, *H. gelotopoeon* and *N. tabacum* under laboratory conditions were studied for the first time.

Beauveria bassiana LPSC 1215 as an endophyte did not reduce the amount of vegetal material consumed by *H. gelotopoeon* larvae but affected the life cycle period of the plague, particularly for the larval and adult stages. Also egg fertility was affected since adults laid eggs that were not able to hatch.

No fungal outgrowth was detected in treated insect cadavers. These results agree with Resquín-Romero *et al.* (2016) and Sánchez-Rodríguez *et al.* (2018). These previous studies hypothesize that when fungal outgrowth is absent, endophytic fungal entomopathogens may cause larval death by secretion of mycotoxins. Mycotoxin secretion by *B. bassiana* LPSC 1215 strain as an endophyte still remains to be confirmed.

Insect deterrence is expected in feeding behavior when insects are provided with plants colonized by endophytic entomopathogenic fungi (Lartey *et al.* 1989; Broza and Halpern 2001) but this was not observed in the present investigation. No differences were registered in food consumption by larvae fed treated and control plants. Although Resquin-Romero *et al.* (2016), evaluated the weight of chewing insects when fed colonized plants, their results agree with ours since they also did not find significant differences between treatments. Furthermore, no effect on larval weight was observed between insects fed treated and control plants by Leckie *et al.* (2008) and Lopez and Sword (2015). In contrast, damage caused by noctuids such as *H. zea* and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) was reduced when plants colonized with *B. bassiana* were offered (Cherry *et al.* 2004; Powell *et al.* 2007). The same was observed by Mutune *et al.* (2016) who found that feeding behavior was negatively affected in *Ophiomyia* sp. Similar results were obtained by Martinuz *et al.* (2012) and Lopez Castillo *et al.* (2014) who performed choice trials and showed that aphids prefer feeding on uncolonized plants.

Haukioja (1980) found that when tests are carried out with processed vegetal material the results are more altered than when performed with fresh plants. Furthermore, Jaber and Vidal (2010) observed that larval feeding and performances were affected when utilizing leaves with mechanical damage (leaf discs), due to the loss of volatiles. This may have been the case in the present study.

This study showed that the entomopathogenic fungi *B. bassiana* as an endophyte negatively affected survival, fertility, larval and adult longevity, life cycle and ovipositional period of *H. gelotopoeon* feeding on inoculated tobacco plants. These results agree with Cherry *et al.* (2014), Lopez and Sword (2015) and Mutune (2016) where colonized plants adversely affected growth and development of the feeding insects.

Beauveria bassiana reduced the number of days of the total cycle (especially the larval and adult stages), ovipositional period and fertility of *H. gelotopoeon.* Several plausible explanations have been given to account for the reduction in growth rates of insects due to endophytic colonization by entomopathogens, such as production of secondary metabolites by the fungus and induction of direct and indirect mechanisms of defense that are triggered by the presence of the endophyte in the plant (Hartley and Gange 2009). Alterations in life cycle duration and fecundity have also been reported by Jaber and Vidal (2010). They observed that *A. strictum* as an endophyte of *Vicia faba* plants produced significant reductions in larval growth and fecundity of *H. armigera*. They attributed these negative effects to indirect endophyte-triggered mechanisms released in the plant by insect foraging behavior. Other

studies have also demonstrated adverse results on herbivore insect fitness due to the presence of endophytic fungi on hosts plants (Crawford *et al.* 2010; Gurulingappa *et al.* 2011; Hernawati *et al.* 2011) but in these cases effects have been attributed to secondary metabolites released by the fungus (Thakur *et al.* 2012). It still remains to be determined which mechanisms are involved in the interaction between *B. bassiana*, *H. gelotopoeon* and tobacco plants.

The results of this investigation provide new information on endophytic entomopathogen potential to be incorporated in IPM programs.

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MFV, SP, MLR and NA conducted laboratory experiments. MFV, MLR and SP analyzed data. MFV and ACS wrote the manuscript. All authors read and approved the manuscript.

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