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ORIGINAL ARTICLE

Interactions between the entomopathogenic fungus *Beauveria bassiana* and the Neotropical predator *Eriopis connexa* (Coleoptera: Coccinellidae): Implications in biological control of pest

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Abstract

Aphids (Hemiptera: Aphididae) are serious pests of crops causing direct damage by feeding and indirect by the transmission of plant viruses. The use of conventional insecticides for controlling aphids has caused different problems and insecticide resistance. Accordingly, there is more interest in alternative control methods such as biological control by natural enemies for sustainable agricultural management. Among biological control agents, entomopathogenic fungi are one of the most significant microbial pathogens of insects. Also, Coccinellidae, as a major group, is a serious natural enemy. Both larval and adult stages of Coccinellidae feed on different soft-body pests, such as aphids. *Eriopis connexa* (Germar) (Coleoptera: Coccinellidae) is a common species in agroecosystems of the Neotropical region where it is considered to be a potential control agent. Pathogens and arthropod natural enemies may contribute to the control of phytophagous pests; however, it is important to assess potential interactions within biological control agents that share hosts (intraguild interaction) to evaluate their combined use for pest control. Therefore, the aim of this study was to evaluate the compatibility and interaction (lethal and sublethal effects) between *E. connexa* and the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales). Both are important biological control agents of aphids. The pathogenicity of *B. bassiana* against larvae, pupae and adults of the predator *E. connexa* was evaluated, and results showed, that *B. bassiana* infected the coleopteran. On the other hand, interaction between *B. bassiana* and the predator was evaluated through infected-prey. The effects of fungus on larvae survival were significantly different when we analyzed the accumulated survival (from first larval instar to adulthood). The daily fecundity was significantly reduced at five days compared to control group. By contrast, no significant differences were observed between the five oviposition days in the rate of hatched eggs. This study shows that despite having received a single dose of the fungus in its life cycle, the population parameters of the predator *E. connexa* are affected. More studies would be necessary to help identify interactions between microbes and natural enemies to increase and enhance opportunities and further develop biological pest control programs.

Key words: *Beauveria bassiana*, biological control, *Eriopis connexa*, pathogen-predator interaction

Introduction

Aphids (Hemiptera: Aphididae) are serious pests in agricultural and horticultural crops through direct damage by feeding and indirect by the transmission of more than 300 plant viruses (Hogenhout *et al.* 2008). The use of conventional insecticides for controlling aphids has caused different problems (e.g. failures in pest control and negative environmental impact) and insecticide resistance by aphids (Foster *et al.* 2007). Therefore, in recent years there has been more interest in other control methods such as biological control by natural enemies for sustainable agricultural management (Lacey *et al.* 2015). Among biological control agents, fungi are one of the most significant microbe pathogens of insects. At least 16 species of fungi are known to infect aphids in nature, and several species frequently cause epizootics among aphid populations (Pell *et al.* 2001; Chen *et al.* 2008). In Argentina, several fungal species associated with aphid pests of horticultural crops were cited: *Conidiobolus obscurus* (Hall & Dunn) Remaudière & Keller, *Entomophthora planchoniana* Cornu, *Neozygites fresenii* (Nowakowski) Remaudière & Keller, *Pandora neoaphidis* (Remaudière & Hennebert) Humber and *Zoophthora radicans* (Brefeld) Batko (Entomophthoromycotina: Entomophthorales) (Scorsetti *et al.* 2007) and *Lecanicillium lecanii* (Zimmerm.) Zare & W. Gams, *L. longisporum* (Petch) Zare & Gams, *L. muscarium* (Petch) Zare & W. Gams (Ascomycota: Hypocreales) (Scorsetti *et al.* 2010, 2012).

Mitosporic entomopathogenic fungi in the order Hypocreales, such as *Beauveria bassiana* (Bals.–Criv.) Vuill., *L. longisporum* and *Isaria fumosorosea* Wize, have been successfully utilized as biological control agents against numerous pests including aphids (de Faria and Wraight 2007; Powell and Pell 2007; Draganova *et al.* 2008). The main biological attributes for a mycoinsecticide are the virulence toward the target insect and that it is not harmful to other beneficial organisms such as parasitoids and predators (Riddick *et al.* 2009).

Coccinellidae make up a major group of natural enemies both the larval and adult stages of this predator species feed on different soft-body pests, such as aphids, whiteflies, mites and mealybugs (Biddinger *et al*. 2009; Obrycki *et al*. 2009). *Eriopis connexa* (Germar) (Coleoptera: Coccinellidae) is a common species in agroecosystems of the Neotropical region where it is considered to be a potential control agent (Sarmento *et al*. 2007; Duarte Gómez and Zenner de Polanía 2009). In Argentina, this species is commonly associated with key horticultural pests such as *Myzus persicae* (Sulzer, 1776), *Aphis gossypii* Glover 1877 (Hemiptera: Aphididae) and *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae) (Fogel 2012).

Pathogens and arthropod natural enemies may contribute to the control of phytophagous pest populations in biological control programs. However, it is important to assess potential interactions between biological control agents that share the same host (intraguild interaction) to evaluate their combined use for pest control. It is well documented that several species of natural enemies may interact synergistically, additively or antagonistically to each other. While a synergistic or additive interaction could accelerate the reduction of insect pest populations, an antagonistic response could interact negatively in controlling insect pests (Roy and Pell 2000).

Intraguild interaction and compatibility between entomopathogenic fungi and other biological control agents of pests have been studied more with parasitoids than predators (Mesquita and Lacey 2001; Rashky *et al.* 2009; Jarrahi and Safavi 2016). In recent studies Mohamed and Hatcher (2017) have reported that the parasitoid *Aphidius colemani* (Dalman, 1820) (Hymenoptera: Braconidae) and *L. muscarium* could be used together for *M. persicae* control. Likewise, *B. bassiana* and *Metarhizium brunneum* Petch (Ascomycota: Hypocreales) results were compatible with *A. colemani* without any negative intraguild interaction between them (Jaber and Araj 2017).

Steenberg and Harding (2009) reported entomopathogenic fungi isolated from the harlequin ladybird predator *Harmonia axyridis* (Palls) (Coleoptera: Coccinellidae) from field samples and observed that the larval stage was the most susceptible to fungal infection, through bioassays with the entomopathogenic fungus *Isaria farinosa* (Holmsk.) (Ascomycota: Hypocreales). In contrast, Bayissa *et al.* (2016) demonstrated that the compatibility between *Metarhizium anisopliae* (Metchnikoff) Sorokin (1883) (Ascomycota: Hypocreales) and the predator *Cheilomenes lunata* (Fabricius) (Coleoptera: Coccinellidae), could provide a sustainable strategy for effective management of aphids on crucifers.

Most studies are focused on the interaction between parasitoids and pathogens. However, there have been few studies on predators. Therefore, the aim of this study was to evaluate the compatibility and interaction (lethal and sublethal effects) between the predator *E. connexa* and the entomopathogenic fungus *B. bassiana*, both, important biological control agents of aphids.

Materials and Methods

Fungal culture

The strain *B. bassiana* LPSc 1067, from the culture collection of the Spegazzini Institute, La Plata, Argentina was chosen based on its laboratory efficacy against several insect pests (Pelizza *et al*. 2012). It was identified

using both molecular and morphological data Gen-Bank (accession number KF500409).

Conidia were obtained from a culture on potato dextrose agar (PDA, Britania S.A., Buenos Aires, Argentina) maintained for 10 days at 25°C in darkness. Conidia were harvested by a cell scraper (Fisherbrand®) and placed in Tween®80 (sodium polysorbate) 0.01% (v/v). Suspensions were adjusted to 1×10^7 conidia · ml^{-1} by a Neubauer hemocytometer. Conidial viability was determined before each stock suspension was prepared by spreading 0.2 ml of a 1×10^4 conidia · ml^{-1} suspension on a slide with PDA and estimating the number of germinated propagules after 24 h at 25°C. Propagules were considered viable when the germ tube length was equal to or longer than the spore diameter when observed under a phase contrast microscope at 400× magnification (Goettel and Inglis 1997).

Insect rearing

The coccinellid *E. connexa* was obtained from organic horticultural crops in the nearest La Plata city, Buenos Aires province, Argentina (34°57'17"S, 57°53'26"W). Insects were kept isolated inside an incubator chamber to avoid the presence of pathogens from the field. Insects used in the bioassays were after the second generation. Adults were kept in polycarbonate cages (14.5 × 14 cm) covered with a fine mesh. Eggs were removed and placed in plastic Petri dishes. Larvae were reared in plastic Petri dishes until pupation. Insects were maintained in a rearing room under controlled conditions [25±2°C, 70±5% relative humidity (RH) and 16 : 8 h L : D].

The aphid *Rhopalosiphum padi* L. (Hemiptera: Aphididae) was used as prey. Aphid colonies were initiated with clones supplied by the technical personnel of the Faculty of Agricultural and Forestry Sciences (National University of La Plata), and were reared on wheat *Triticum aestivum* L. (cultivar Klein Capricornio, Cauda Semillas, Chacabuco, Argentina). Wheat seeds were pre-germinated and planted in a sterilized vermiculite : soil : perlite substrate mix (1 : 1 : 1). Seedlings were aphid infected and maintained in ventilated cages under the same controlled conditions as the predator. Larvae and adults of predator were also feed with a supplement of honey, water and pollen mixture.

Bioassay 1: Pathogenicity test

The pathogenicity of *B. bassiana* against larvae, pupae and adults of the predator *E. connexa* was evaluated by aspersion technique. Treated insects were sprayed with 300 µl of a conidial suspension of 1×10^7conidia · ml^{-1}, with a 35-ml glass atomizer, while the control insects were sprayed with 300 µl of 0.01% (v/v) Tween®80 conidia-free. The experiment consisted of three replicate test groups and three control groups, with all groups containing 10 insects each. Adults used in assays were 10–15 days old.

Insects were treated and placed in a Petri dish with sterile filter paper to dry excess inoculum. Then, they were settled individually in Petri dishes and fed *ad libitum* with *R. padi* aphid as prey. The cumulative mortality was recorded daily for 10 days. Dead insects were surface sterilized and placed in a humidity chamber to allow fungal infection according to Lacey and Brooks (1997). Dead insects were observed until visual signs of mycosis appeared or for 14 days after treatment. The experiments were repeated three times under comparable laboratory conditions. All the bioassays were carried out in a growth chamber with controlled environmental conditions (25±2°C, 70±5% RH and 16 : 8 h L : D).

Bioassay 2: Interaction through prey. Larval survival, development, fecundity and fertility

Interaction between the entomopathogenic fungus *B. bassiana* and the predator was evaluated through infected-prey. For the treatment, aphids were sprayed with a *B. bassiana* suspension of 1×10^7 conidia · ml^{-1} 0.01% (v/v) Tween®80, and placed in a Petri dish with sterile filter paper to dry excess inoculum. Control aphids were applied with 0.01% (v/v) Tween®80 conidia-free.

First larval instar (24 h post-hatched and starved) of *E. connexa* were placed individually in Petri dishes (90 mm) and were provided with 25 infected-aphids. When all infected aphids had been eaten, each larva was fed *ad libitum* with healthy *R. padi* aphid. The experiment consisted of three replicate test groups and three control groups, with all groups containing 10 insects each. Survival and development time of immature stages (from larva to pupa) were checked, every 24 h until adult emergence.

Sublethal effects on fecundity and fertility were assessed on adults emerged from treated and control larvae. Newly emerged adults, both males and females, were placed in plastic cylindrical containers (14 cm diameter and 14.5 cm high) and *R. padi* aphids were provided *ad libitum*. After five days, ten mated females per treatment were randomly selected for the reproduction assessment. Females were placed individually in plastic glasses (5 cm diameter and 10 cm high) with a fine mesh net fixed to the upper opening to allow ventilation. The inner walls of the containers were previously covered with paper as substrate for the oviposition, and *R. padi* aphids were offered *ad libitum*. During the following five days, egg batches were registered and counted daily (Fogel *et al.* 2013). Newly laid eggs were collected and individualized placed individually in Petri dishes. Fecundity (number of laid eggs) and fertility (number of hatched eggs) were recorded for each female.

Insects were maintained in a growth chamber under controlled conditions (25±2°C, 70±5% RH and 16 : 8 h L : D). The experiments were repeated two times under comparable laboratory conditions.

Statistical analyses

Before data analysis, the Shapiro-Wilk test was used to assess data normality. If the assumptions of ANOVA were not met, datasets were transformed (arcsine √x) or a non-parametric test for analysis of data was performed (Kruskal-Wallis test, with the bilateral Dunn test for multiple comparisons in pairs).

The parametric test of analysis of variance (ANOVA) and *a posteriori* Tukey test were used to analyze pathogenicity test data. Repeated measures ANOVA were done to analyze fecundity and fertility data of *E. connexa* adults. Means were separated by the LSD multiple range test among the tested doses ($p < 0.05$). All the analyses were performed using the program XLSTAT (Addinsoft XLSTAT for Excel, Paris, France, 2009).

Results

Pathogenicity test by direct spray on predator

Bioassays of pathogenicity tests conducted with *E. connexa* showed that after 10 days, *B. bassiana* LPSc 1067 infected the coleopteran. There were significant differences between different instar evaluated ($F = 22.02$; $df = 5$; $p < 0.001$). The most susceptible stage was first larval instar, with a 38.8±3.51% of mortality, and the lowest mortality was recorded in pupal stage with 2.22±1.47% (Fig. 1). The average viability of the conidia was over 95%. Fungal growth and sporulation of the fungal isolate was determined on the dead insects following the technique cited by Lacey and Brooks (1997).

Interaction through infected prey

The effects of *B. bassiana* through infected prey on *E. connexa* larvae survival were significantly different when we analyzed the accumulated survival (from first larval instar to adulthood) ($K = 3.84$; $p = 0.029$), but, there were no significant differences in the survival of each individual stage (Table 1).

For the development time (days), statistical analysis showed that there were no significant differences from first larval instar to pupae ($K = 3.84$; $p = 0.092$) being recorded over a total period of 14.8 and 14.9 days in treated and control groups, respectively. However, significant differences were observed in development time in first larval instar ($K = 3.84$; $p = 0.037$), fourth larval instar ($K = 3.84$; $p < 0.0001$) and pupae ($K = 3.84$; $p = 0.019$) between treated and control groups (Fig. 2).

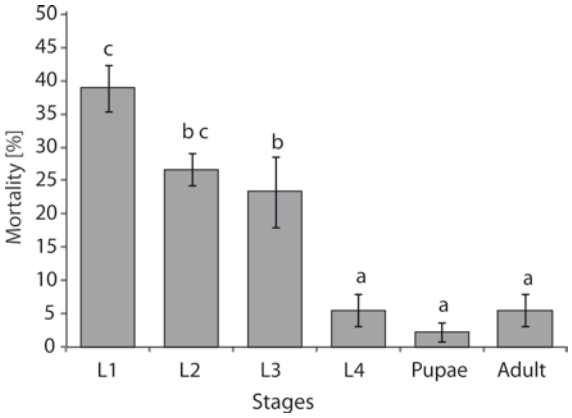

Fig. 1. Mean percent ± SD mortality of *Eriopis connexa* exposed to *Beauveria bassiana* LPSc 1067 by pathogenicity test. The bars indicate standard errors, and different letters denote significant differences between stages according to the Tukey test ($p \leq 0.05$)

Effects of *B. bassiana*, through infected prey, on reproduction of *E. connexa* were assessed on those adults that emerged from treated and control groups. Effects on fecundity and fertility were evaluated according to time factor, for five consecutive days after first oviposition. When analyzing data according to time factor, an increasing tendency in fecundity after the first oviposition day was observed. The daily fecundity was significantly reduced at five days compared to control group ($F = 39.96$; $p \leq 0.0001$; $df = 1, 185$). A mean number of 16.78 against 6.44 eggs/female were laid by control and treated *E. connexa,* respectively (Fig. 3). By contrast, no significant differences in the rate of hatched eggs from treated and control groups were observed between the five oviposition days ($F = 0.145$; $p = 0.704$; $df = 1, 101$), reaching values of 80.45% and 81.30% of treated and control eggs, respectively.

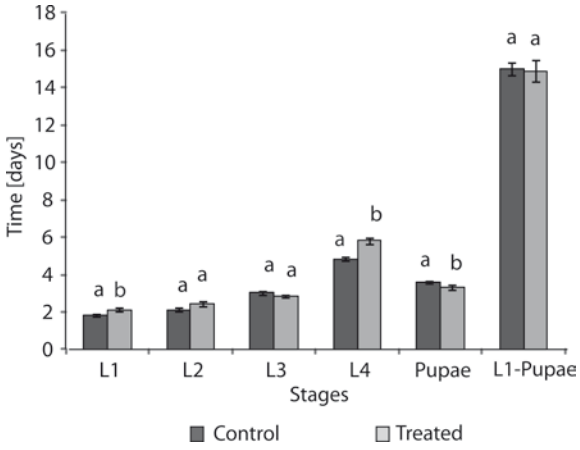

Fig. 2. Effects of *Beauveria bassiana* on the development time of immature stages of *Eriopis connexa* exposed to the fungus by ingestion (treated prey). The bars indicate standard errors, and different letters denote significant differences between treatments according to the Dunn test ($p \leq 0.05$)

Table 1. Effects on the survival of *Beauveria bassiana* through infected prey on immature stages and adults of *Eriopis connexa*

Treatment	L1 [%]	L2 [%]	L3 [%]	L4 [%]	Pupae [%]	Adults [%]	Acumulated survival* [%]
Control	98.3 ± 1.6 a	98.3 ± 1.6 a	100 ± 0 a	100 ± 0 a	100.0 ± 0a	100 ± 0 a	96.7 ± 2.1 a
Treated	91.6 ± 3.0 a	96.4 ± 2.3 a	100 ± 0 a	97.9 ± 2.0 a	98.1 ± 1.8 a	93.9 ± 4.2 a	80.0 ± 5.7 b
	p = 0.083	p = 0.46	n/o	p = 0.31	p = 0.31	p = 0.14	p = 0.02
	df = 1,12	df = 1,12	df = 1,12	df = 1,12	df = 1,12	df = 1,12	df = 1,12
	K = 3.84	K = 3.84	K = 3.84	K = 3.84	K = 3.84	K = 3.84	K = 3.84

The data correspond to means (±SE). Treatments with different letters are significantly different. Kruskal-Wallis test ($p < 0.05$)
*number of adults regarding initial number of Larvae 1 (L1)

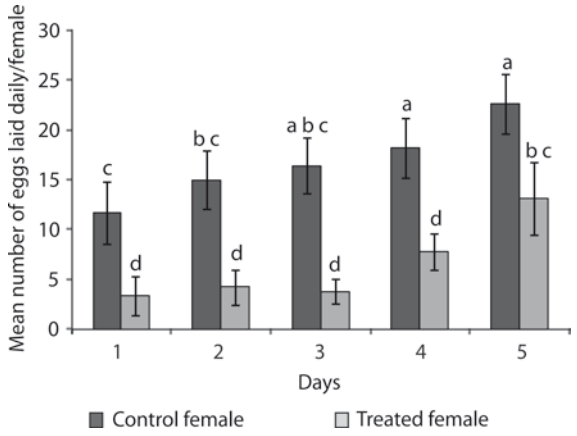

Fig. 3. Number of eggs laid daily by *Eriopis connexa* females from treated and control groups on five consecutive days. The bars indicate standard errors, and different letters denote significant differences between stages according to the Fisher (LSD) test ($p \leq 0.05$)

For personal observation, we recorded three *E. connexa* females that after the oviposition period died. They were conditioned in a humid chamber and the entomopathogenic fungus *B. bassiana* arose from the intersegmental zone of the body.

Discussion

Interspecific competition between natural enemy species is one of the most important factors determining biological control programs (Gonzalez *et al.* 2016). Studies about interactions between entomopathogenic fungi and other natural enemies (parasitoids and predators) have been performed worldwide (Roy *et al.* 2006; Ormond *et al.* 2011; Martins *et al.* 2014; Bayissa *et al.* 2016). However, there is very little information about interactions with other natural enemies in Coccinellidae species in general and *E. connexa* in particular.

Even though many coccinellid species and entompathogenic fungi may occupy the same spatial and temporal habitat, it is curious that so little information exists about the susceptibility and intraguild interactions between the predator species of Coccinellidae and entomopathogenic fungi.

A very small number of entomopathogenic fungi species have been isolated from coleopteran coccinellids collected under field conditions. The fungal species *B. bassiana* was isolated from *Cycloneda sanguinea* L. in Argentina (Toledo *et al.* 2008). The parasitic fungus *Hesperomyces virescens* (Laboulbeniales: Laboulbeniaceae) has been reported to infect several coccinellids, such as *Chilocorus stigma* Say, *C. bipustulatus* L., *Adalia bipunctata* (L.), *H. axyridis*, *Hippodamia convergens* Guérin-Méneville and *E. connexa* (Riddick *et al.* 2009). Even though this fungus invades the cuticle there are no known deleterious impacts on the host (Nalepa and Weir 2007). Cottrell and Shapiro-Ilan (2003) reported that field-collected *Olla v-nigrum* Mulsant was commonly found infected by *B. bassiana*, but *H. axyridis* was never found to be infected by this fungus, nor was mycosis exhibited after death.

Nevertheless, there are few studies about the pathogenicity of entomopathogenic fungi in laboratory bioassays. The present study represents the first study of pathogenicity and provides initial information about the interaction between the fungus *B. bassiana* and the predator *E. connexa.* Cottrell & Shapiro-Ilan (2008) assayed the susceptibility of *Olla v-nigrum*, *Coleomegilla maculata* DeGeer, *Cycloneda munda* (Say, 1835) and *H. convergens* adults to *B. bassiana* isolates under laboratory conditions, showing 76% and 2% mortalities with a LC_{50} rate of 2.5×10^5 conidia · ml^{-1}. In our study, using a higher dose, adults were less susceptible after 10 days. The most susceptible stage was first larval instar and the lowest mortality was found in pupae stage. Also, Scorsetti *et al.* (2012) showed that the entomopathogenic fungus *L. muscarium* at a dose of 1×10^7 conidia · ml^{-1} caused a mortality of 45±5% and 30±5% to L1 and L2 larval instar of *E. connexa*, respectively, under laboratory conditions.

Although laboratory-reared insects are more susceptible to pathogens (Hajek and Butler 2000), the ecological components and behavioral responses of the predators should be taken into consideration in order to explain the low or scarce incidence of entomopathogenic fungi infecting coccinellids under natural field conditions. Ormond *et al.* (2011) observed that both, male and female *Coccinella septempunctata* L.

avoided *B. bassiana* through contact with leaf surfaces, soil inoculated and mycosed cadavers. Also, Pell and Vandenberg (2002) demonstrated that the ladybird *H. convergens* avoided feeding on Russian wheat aphids, *Diuraphis noxia* Mordavilko, infected with *I. fumosorosea*. The ability of coccinellids to detect and avoid entomopathogenic fungi conidia is an adaptation that undoubtedly increases survival and ultimately fitness.

In this study, the method of inoculation to evaluate the sub-lethal effects was through fungus-infected prey aphids, offered one time only. Statistical analyses showed that there were no significant differences between the survivals of both treatments, inoculated and control groups, from larvae to adulthood. The development time (days) was 14.8 and 14.9 days in treated and control groups, respectively. Silva *et al.* (2013) found a total development period of 12.6±0.2 and 14.4±0.2 days for treatment fed different aphids as prey, which is consistent with our study. However, significant differences were observed in development time in stages L1, L4 and pupae between treated and control groups. There were no differences observed on development time, nevertheless, the daily fecundity was significantly reduced at five days compared to control group. These results are consistent with a different study (Simelane *et al.* 2008) in which another coleopteran, *C. septempunctata*, reared on uninfected aphids produced significantly more eggs per day during the observation period than those reared on fungus-infected aphids. Also, 11% of females reared on fungus-infected aphids oviposition stopped within a week and later died. Also, they found that both, male and female *C. septempunctata* reared on *Neozygites fresenii* – infected aphids were smaller than those reared on uninfected aphids.

This study shows that despite having received a single dose of fungus in its life cycle, the population parameters of predator *E. connexa* are affected.

Understanding the ecological consequences of using more than one biological control agent needs closer examination, especially for organic cropping systems that have evolved into complex ecosystems with populations of multiple arthropod natural enemy species. There are a limited number of studies involving microbes and arthropod natural enemies under field conditions. Thus, such studies would be necessary to help identify interactions between microbes and natural enemies to increase and enhance opportunities and further develop biological pest control programs.

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