

IN VITRO* SUSCEPTIBILITY OF THE PEA LEAFMINER *LIRIOMYZA HUIDOBRENSIS* PUPAE TO ENTOMOPATHOGENIC *HETERORHABDITIS INDICA* AND *BEAVERIA* *BASSIANA

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ABSTRACT

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Given the substantial economic losses associated with various aspects of Liriomyza huidobrensis Blanchard feeding on different crops in Lebanon as well as the ability of this pest to rapidly develop resistance to insecticides, the current study attempted to use biological control agents in vitro to manage this pest. For this reason, sensibility of L. huidobrensis pupae was tested against indigeneous entomopathogenic nematode (Heterorhabditis indica) and fungus (Beauveria bassiana). Entomopathogenic nematode solution at 1000 IJs per mL was placed in contact with Liriomyza pupae on one hand and pupae of L. huidobrensis in direct contact with B. bassiana at the rate of 5000, 500, 50 and 5 spores/pupa on the other hand. Results showed a mortality of 53±1.5% for the Liriomyza pupae following the application of entomopathogenic nematodes characterized by a red color and bioluminescence without any emergence of infective juvenile nematodes, one month following the infestation. Treatments with B. bassiana were able to kill 73-97% of the pupae and similarly treatments with B. bassiana and the surfactant Tween 80 were able to kill 73-93% of the pupae. Tween 80 demonstrated to increase the sporulation rate iduring the first 7 days following the application of the spores of B. bassiana.

Keywords: pea leafminer, *Liriomyza huidobrensis*, biological control, entomopathogenic nematode, entomopathogenic fungus, *Heterorhabditis indica*, *Beauveria bassiana*

INTRODUCTION

Blanchard (1926) described for the first time *Liriomyza huidobrensis* Blanchard, known as the pea leafminer, which originated from South America. In 1973, this insect was limited to California in north America (Lange, 1945), but its invasion was reported afterward in central and south America before reaching Europe in 1989 (Sunderland *et al.*, 1992). From

the nineties onwards the species has been spreading with imported ornamentals and have reached the Middle East (Weintraub & Horowitz, 1995). In Lebanon, outbreak of leafminers was noticed on Gerbera plants, leafy vegetables and many other greenhouse and field crops (Abou-Fakhr Hammad & Nemer, 2000). From the order of Diptera, *L. huidobrensis* belongs to the family *Agromyzidae*.

L. huidobrensis can be distinguished from other *Liriomyza* species by their bigger size and dark color. Around 100 eggs are deposited per female in controlled conditions (Hincapié *et al.*, 1993) within leaf tissue (CABI, 2004; Weintraub & Horowitz, 1995). Following their eclosion, larva starts feeding immediately on the spongy mesophyll of the leaf until the third larval instar before emerging to pupate through an exit hole developed on the surface of the leaf. There is also a fourth larval stage (prepupa) between the puparium formation and actual pupation. The life cycle can be completed in 16-43 days at 25 and 15 °C, respectively (Lanzoni *et al.*, 2002), and more than half of the insect's life is spent in the pupal stage.

Considered as a highly polyphagous leafminer, it is capable of inflicting severe damage to the floriculture industry and vegetable crops caused mainly by larval feeding. The mining activity of the larvae can reduce the photosynthetic capacity of the plant. Heavy infestation will cause desiccation and premature fall of leaves. Also, feeding punctures made by the adult females can be invaded by fungi and bacteria (Price & Harbaugh, 1981). To prevent *Liriomyza* attack, sticky traps can be used to monitor adult flies. Crop rotation is an effective pest management tool as it permits varieties which are highly susceptible to avoid leafminer infestations (*e.g.* some chrysanthemums) in greenhouses. Parasitoid wasps, *Diglyphus isaea* (Walker) and *Dacnusa sibirica* Telenga are available for control in greenhouse crops. These parasites will not be effective for vegetables growing in the field. *L. huidobrensis* adults are resistant to conventional insecticides. Also the control of *L. huidobrensis* by chemicals remains a great challenge due to its resistance to insecticides. Also it is difficult to implement biological control methods against *Liriomyza* when the biological control agent is not indigenous (Jayaraj & Rabindra, 1992). Moreover, with the community concern about pesticide residues in food and the desire for a healthy and aesthetic environment, the use of biorational insecticides, in particular the use of entomopathogenic nematodes and fungi, for the control of the leafminer is a primary factor in its management. Entomopathogenic nematodes (EPNs) in the genera *Heterorhabditis* and *Steinernema* (Rhabditida) are obligate parasites of insects (Poinar, 1990) and are therefore considered good biological control agents.

Entomopathogenic fungi (EPF), in particular Deuteromycetes, including *Beauveria bassiana* (Bals.-Criv.) Vuill. are alternative candidates as microbial control agents because of their rapid killing rate in laboratory assays relative to other entomopathogens. The possibility for commercial scale production on artificial media, and their contact route of infection that allows for pest targeting using standard spray applications should be investigated. *B. bassiana* may have less negative impact on native parasitoids than chemicals, and it is one of the genera that have been most investigated for use as a mycoinsecticide (Moore & Prior, 1993). Over 200 species of insects in nine orders, mainly Lepidoptera and Coleoptera, have been recorded as hosts of *B. bassiana* (Feng *et al.*, 1994).

In order to find an environmental and effective means of controlling the pea

leafminer in Lebanon, the current study addresses the efficiency of entomopathogenic nematodes and fungi in vitro for the control of the pea leafminer.

MATERIALS AND METHODS

Source of biological control agents and the pea leafminers

Beauveria bassiana Clade C was isolated from the Cedar Web-spinning Sawfly, *Cephalcia tannourinensis* Chevin, collected in one forest in the North of Lebanon.

Entomopathogenic nematodes were collected from a coastal agricultural field in Lebanon and belong to the species *Heterorhabditis indica*. The nematode was reproduced on last instar greater wax moth larvae (*Galleria mellonella* L.) at 25 °C (Poinar, 1979), stored at 15 °C in Ringer solution and used for the experiments within 2 weeks.

Solanum oleraceum Dunal fields were considered in the present study for collecting pupae of the pea leafminer *L. huidobrensis* from marginal herbaceous and agricultural sites chosen randomly along the coastal area in Lebanon. Samplings were conducted in spring of the year 2012. Infested leaves were transported in bags to the laboratory where *L. huidobrensis* pupae were directly isolated; leaves were also stored at 4 °C for a couple of weeks.

Pathogenicity tests

Effect of entomopathogenic fungus on L. huidobrensis pupae

All bioassays were conducted under laboratory conditions on pupal stage in small Petri dishes (D=5cm) under laminar flow cabinets. Two tests were considered for this experiment, each with four *B. bassiana* concentrations (5000, 500, 50 and 5 spores per pupa) with 30 pupae per treatment. Tests were repeated three times. The first test consisted of applying only 2 µl of the *B. bassiana* solution topically to each *L. huidobrensis* pupa while in the second test, a surfactant, Tween 80, at the rate of 1%, was added to the *Beauveria* solution before its application on the pupa. All conidial suspensions were administered using an Eppendorf micropipette. A control test was added to the experiment in which the *Liriomyza* pupa was only in contact with distilled water. An additional positive control treatment was added in both tests consisting of the commercial fungus *Paecilomyces lilacinus* (Thom) Samson provided by the agricultural company Unifert in Lebanon. Five grams of *P. lilacinus* powder were diluted in 5 mL of sterile water as a recommended use, and was pulverized on 30 pupa in each test.

The Petri dishes were kept in an incubator at 26 °C following the treatments. To ensure high humidity for conidia germination, all dishes were sprayed with a mist of distilled water on alternate days. The emergence of adults and *L. huidobrensis* pupae mortality were assessed at 24, 48 hrs and 5, 7, 9, 11 and 14 days after treatment. In all bioassays, conidia extraction from attacked larvae developing signs of sporulation was cultured on potato dextrose agar (PDA) (Sigma) maintained at 26°C and identified by microscopy and molecular techniques (PCR and sequencing).

Effect of entomopathogenic nematodes on L. huidobrensis pupae

Nematodes were applied on Petri dishes filled with sterilized soil at 1000IJs/mL. Five *L. huidobrensis* pupae were put per Petri dish and were incubated afterward for 15 days at $22 \pm 2^\circ\text{C}$. The experiment was reproduced three times including a control represented by the same number of *L. huidobrensis* in contact with autoclaved and humid soil. Pupae mortality was verified every 48 h during 15 days representing roughly the time needed for the emergence of adults out of the pupae. Bioluminescence of cadavers was observed before placing them on individual white trap (Bedding & Akhurst, 1975). IJs emergence was then assessed during one month and whenever infective juveniles emerged from a cadaver, the life cycle of EPNs were considered successfully accomplished inside the cadaver.

RESULTS AND DISCUSSION

This study showed that *B. bassiana* at the rate of 5000, 500, 50 and 5 spores/pupa caused a mortality ranging from 73 to 97 % (Fig. 1), whereas treatments with *B. bassiana* and Tween 80 caused a mortality range from 73 to 93% (Fig. 2). However, treatments with the commercial entomopathogenic fungus *P. lilacinus* caused a pupal mortality of 76.33%. An acceleration of the mycelial development was observed in the treatment with *B. bassiana* and Tween 80, where more than 50% of the sporulation has occurred in all the treatments on the 7th day following the application. When treated alone with *B. bassiana*, the sporulation rate varied between 13 and 53% on the 7th day following application (Fig. 2). No sporulation was observed in the control treatment. Our results are in line with those obtained by Uziel and Kenneth (1999) and Luz and Batagin (2005) who indicated that Tween 20 accelerates the formation of the germination tube by increasing conidial hydration and thus having an effect on increasing the speed of spore germination.

B. bassiana is exploited in greenhouse and outdoor crops as a tool for the control of many agricultural pest arthropods including whiteflies, aphids, thrips, psyllids, weevils and mealybugs (Shah & Goettel, 1999). Migiro *et al.* (2010) tested *B. bassiana* (Balsamo) against adult pea leafminer in the laboratory and found it pathogenic to the pea leafminer, causing mortality between 40 and 100% at 5 days after exposure. Even though entomopathogenic fungi have not yet attracted attention as leafminer biological control agents, even in greenhouses where environmental conditions can be likely optimized, our experiments confirmed the susceptibility of *Liriomyza* pupae to *Beauveria bassiana in vitro* and encourage further experiments to confirm the reproduction of these results under natural conditions.

Likewise the sensibility of *L. huidobrensis* pupae to the entomopathogenic nematode *H. indica* was relatively high in our experiments. The results showed that $53 \pm 1.5\%$ of the pupae were dead due to treatments, $15 \pm 1.5\%$ emerged into flies, $21 \pm 2.5\%$ were parasitized by *Diglyphus isaea* and $10 \pm 1\%$ were missing (Fig. 3). In the control test, $79 \pm 2\%$ of the pupae emerged into adult flies. All *Liriomyza* pupae cadavers potentially infested by EPNs (53%), showed a reddish color and bioluminescence, two visual characteristics of an insect infested by a *Heterorhabditis* due to the bioluminescence of its symbiotic bacteria (Boemare *et al.*, 1993; Boemare, 2002). However, none of the cadavers showed any emergence of EPNs after one month. Additional experiments are needed to extract bacteria from *Liriomyza* pupae cadavers towards isolating the associated symbiotic bacteria of *H.*

indica. The latter experiments can validate the success of the nematode infestation and would require further methods to study the life cycle of the nematode inside the pupa.

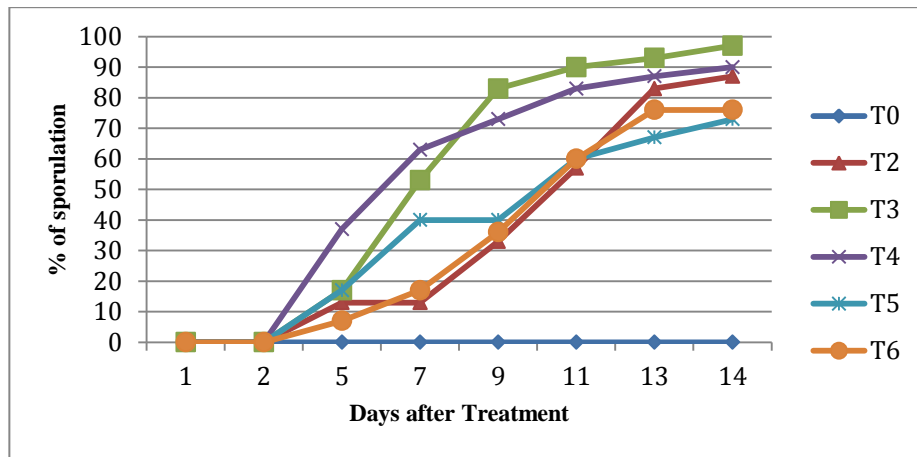


Figure 1. The cumulative percentage of sporulation *Beauveria bassiana* Clade C on pupae *Liriomyza huidobrensis* using different concentrations without the surfactant Tween 80. T0 = Control; T2 = 5000 spores; T3 = 500 spores; T4 = 50 spores; T5 = 5 spores; *Paecilomyces lilacinus* T6 = 1 g / ml.

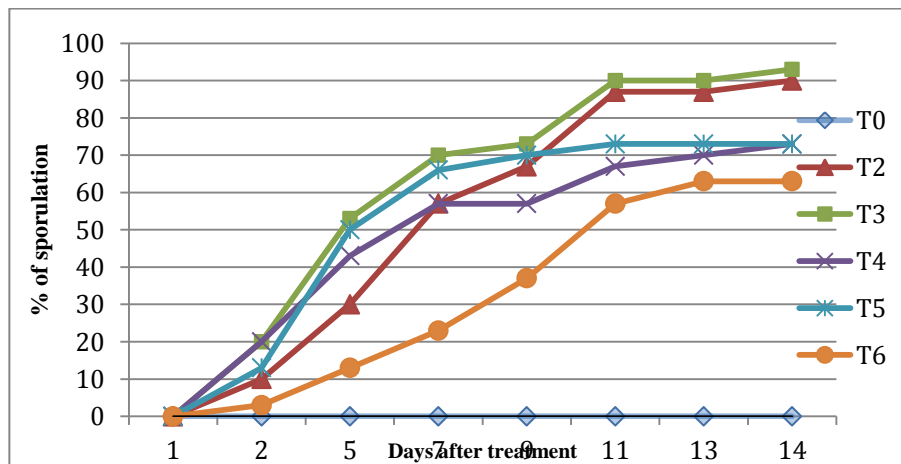


Figure 2. The cumulative percentage of sporulation *Beauveria bassiana* on *Liriomyza huidobrensis* pupae using different concentrations with the surfactant Tween 80. T0 = Control; T2 = 5000 spores; T3 = 500 spores; T4 = 50 spores; T5 = 5 spores; *Paecilomyces lilacinus* T6 = 1 g / ml.

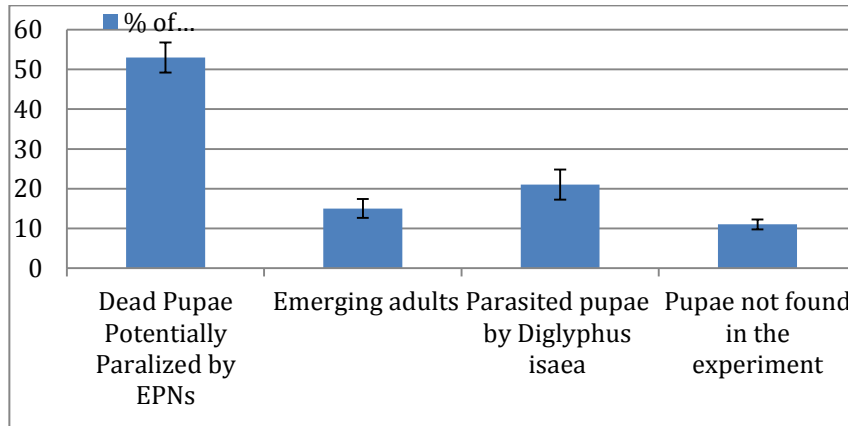


Figure 3. *Liriomyza huidobrensis* mortality following the contact with EPNs.

Steinernema and *Heterorhabditis* nematodes are known for their capacity to control leafminers in the world (Hara *et al.*, 1993; LeBeck *et al.*, 1993; Sher *et al.*, 2000; Tomalak *et al.*, 2005; Williams & Walters, 2000; Harris *et al.*, 1990; Olthof & Broadbent, 1990; 1992). These studies were conducted on *Liriomyza* larvae and mortality rates ranged from 64% to 90% in laboratory trials (Harris *et al.*, 1990; Olthof & Broadbent, 1990) and 53% to 83% in greenhouse trials (Olthof & Broadbent, 1992).

CONCLUSION

In this study, two biological control agents have been tested against *L. huidobrensis* pupae; the entomopathogenic fungus *Beauveria bassiana* and the entomopathogenic nematode *Heterorhabditis indica*. The results obtained on *Liriomyza huidobrensis* pupae under laboratory conditions with both biocontrol agents tested in this study were promising. Replication of the experiment in the field will be needed to assess the efficiency of these entomopathogens against the pea leafminer in order to prepare an effective biological control program against this pest in Lebanon. Studies on the surfactants or oil based formulations of *Beauveria* should be considered for further studies as they may have a role in accelerating the sporulation on the attacked insects. Additional studies are also required on the reproductive life cycle of nematodes inside *Liriomyza* pupae and it is important to note that the incapacity of nematodes to emerge from *Liriomyza* pupae would forbid the emergence of nematodes into the soil, contributing to the stability of the ecosystem (Schroeder *et al.*, 1994) and avoiding ecological risk on non-target species. Further studies should consider testing the combining effect of the entomopathogenic nematodes and fungi on *Liriomyza* pupae. The combined application of these biological control agents may achieve a higher level of control against *Liriomyza* pupae by either acting independently and cause an additive effect, or interact with each other in a synergistic or antagonistic way.

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