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Effect of *Beauveria bassiana* on the invasion and proliferation of the entomopathogenic nematode *Heterorhabditis indica* inside *Galleria mellonella* larvae

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Abstract: In this work, we studied the effect of insect-infection with the fungus *Beauveria bassiana,* on the invasion and proliferation of the insect-pathogenic nematode *Heterorhabditis indica.* Both of these pathogen strains were isolated from Palestine. Results showed that when *Galleria* larvae were infected with nematode Infective Juveniles (IJs) only, the mean percent penetration was 7.37. However, when the larvae were infected with *B. bassiana* for 24, 48, 72, or 96 h before nematode application, the mean percent penetration was significantly reduced to 1.72, 1.40, 1.70, and 0.00 respectively. On the other hand, simultaneous application of both pathogens did not cause any significant decrease in nematode penetration. The total nematode production inside *B. bassiana*-infected Galleria was dramatically reduced compared to the control. When the larvae were infected with nematodes only (control), the number of nematodes collected, after two weeks from insect death, was $106x10^3$ nematodes/larva (14380 nematode/IJ). However, when the Galleria larvae were simultaneously infected with both agents, the total number of nematodes produced was restricted to 35 x 10^3 nematodes/larva (5866 nematode/IJ). A more dramatic effect was observed when larvae were infected with *B. bassiana* for 24, 48, 72, or 96 hours before nematode application; 2.16 x 10^3 nematodes/larva (1256 nematode/IJ) in the case of 24 h preinfection period, while the other preinfection regimes caused a total inhibition of nematode production. Increasing the number of IJs inside preinfected larvae by injection reduced the inhibitory effect of the fungus on the development of IJs to hermaphrodites. The results indicate the occurrence of antagonistic interactions between the two pathogens inside the infected insect. Hence, combining these two biocontrol agents in a short-term biocontrol program may reduce the sustainability of the nematode.

Key words: *Heterorhabditis indica, Beauveria bassiana, Galleria mellonella*, penetration, infective juvenile development

Introduction

Biocontrol agents, such as nematodes, and fungi have been successfully applied to reduce the use of chemical pesticides. Their use involves, in some cases, a dual application of two agents. The applied pathogens may interact during their application resulting in an increased or a decreased efficiency of the biocontrol process. Similar consequence may result from interactions between the applied agent and the naturally occurring organisms (Timper & Kaya, 1989). The nature of such interactions and their effect on the sustainability of each biocontrol agent has not been studied adequately (Barbercheck & Kaya, 1991). Most of the research conducted on the use of combined biocontrol agents focused mainly on their effect on the mortality of the target insect, yet little studies dealt with the reproduction capacity of the applied biocontrol agent. The aim of this work was to study the effect of the fungus

Beauveria bassiana on the penetration, and proliferation of the entomopathogenic nematode *Heterorhabditis indica* inside *Galleria mellonella* larvae.

Materials and methods

IJs of Heterorhabditis indica

Two-weeks old IJs were obtained from a monoxenic culture maintained as described by Lunau *et al.* (1993).

Beauveria bassiana *spores*

Spores were harvested from *B. bassiana* culture as described by Zhang & Watson (1997), and stored at 4 °C till use.

Infecting Galleria *larvae with* B. bassiana *and nematodes*

Appropriate volume of the pathogen suspension was mixed with sand to form a 10 % moistened infecting medium. Last instar *Galleria larvae* were exposed to 1.16 x 10^7 *B. bassiana* spores or 100 IJs in wells of a multi-well plate (one larva per well). After each exposure larvae were washed 3 times with sterile distilled water.

Determination of IJs penetration by pepsin digestion

Infected larvae were dissected and incubated in pepsin solution for two hours at 37 °C at continuous rotary shaking (120 rpm). The pepsin solution contained 8.0 g/l pepsin (Sigma), 23 g/l NaCl, and 940 ml distilled water. The pH was adjusted to 2.0 with HCl. The number of IJs penetrated into the insect was then counted under the microscope.

Determination of IJs recovery to hermaphrodites, and total production of nematodes

At the end of the infection period, larvae were taken from sand, washed and transferred to a Petri dish lined with wet filter paper and left for 72 h at 25 °C. Hermaphrodites were monitored in the dissected larvae under the microscope. For determining the total production of nematodes, each infected larva was washed and placed on a small white trap in the dark at 25 °C for two weeks. At the end of this period, the IJs and the adults in the cadaver were determined under the microscope.

Injection of IJs into Galleria *larvae*

IJs suspended in twenty µl of sterile Ringer solution were injected into the haemolymph of last instar *Galleria* larvae. The number of injected IJs ranged from 27-46 per larva.

Statistical analysis

The results presented in Table 1 are means of two independent experiments. The collected data were statistically analyzed by paired samples t-test using the SPSS 9.0 software. Means were tested for significant difference at *P* value of 0.05.

Results and discussion

Effect of B. bassiana *on the penetration of* H. indica *IJs into* G. mellonella *larvae*

A non-significant effect of *B. bassiana* on penetration was observed when the fungal pathogen was applied simultaneously with the IJs (6.0 compared to 7.37 % in control). However, preinfecting the *Galleria* larvae with *B. bassiana* for periods of 24 h and longer caused a dramatic decrease in the penetration of the nematodes (1.72 % compared to 7.37 % in the control). Furthermore, preinfection for a period of 96 h resulted in a total inhibition of IJs penetration (Table 1). Similar effect of *B. bassiana* was reported by Barbercheck & Kaya (1991). Also, a decreased penetration of IJs into a host that had already been infected with the same nematode was reported by several workers (e.g. Hominick, 1990). They proposed that the infected larvae secrete certain substance that is sensed by IJs and causes them to avoid

penetration into the infected insect. This kind of behavior may have an obvious biological importance in that it prevents overpopulation of the host, which may lead to a detrimental competition on food resources. When the two pathogens are applied simultaneously, the fungus infection might be slower than that of the mobile IJs. The latter may penetrate into the insect before the occurrence of a pathogenic response associated with secretion of the hypothesized repelling substance. Hence, there will be no influence on the penetration of the nematodes.

Effect of* B. bassiana *on the development of IJs to hermaphrodites

Although the simultaneous application of *B. bassiana* and nematodes had no significant effect on the penetration of IJs into the insect, it did influence their development to hermaphrodites. Under this preinfection regime only 52 % of the penetrated IJs could develop to hermaphrodites, compared to 95 % in the control. Similar decrease in IJs development to hermaphrodites was observed when *Galleria* larvae were exposed to *B. bassiana* spores for 24 h before infection with nematodes. However, longer periods of infection with the fungus (48, 72 and 96 h) resulted in a total inhibition of development to hermaphrodites of the small number of IJs that succeeded to penetrate (Table 1). The inhibitory effect of *B. bassiana* on the development of IJs inside the insect could be a consequence of antibiotic activity exerted by the developing fungal mycelium on the nematode symbiotic bacteria. Secretion of toxins and antibiotic substances by *B. bassiana* is a well-documented phenomenon (Krasnoff *et al.*, 1991). When the period of preinfection with *B. bassiana* spores was extended to 48 hours or longer, the well established fungal mycelium may had secreted sufficient amounts of toxins and antibiotic substances that caused a total inhibition of IJs development to hermaphrodites.

Effect of* B. bassiana *on the development to hermaphrodites of the injected IJs

When the number of IJs inside larvae preinfected with *B. bassiana* for 48 and 72 h was increased, by injection, to 24 and 27 fold respectively, the percent recovery to hermaphrodites was dramatically increased compared to that of naturally penetrated IJs (Table 1). While the small number of naturally penetrated IJs failed to develop to hermaphrodites inside the preinfected larvae, their injected counterparts showed 58 % and 47 % development in insects preinfected for 48 and 72 h respectively. These results indicate that an increase in number of IJs, which is accompanied by increased number of symbiotic bacteria released inside the insect, suppresses the inhibitory effect of *B. bassiana* on IJ development. This suppression might be attributed to secretion of antibiotics by the symbiotic bacteria (Akhurst, 1982), which inhibited development of the fungal pathogen.

Effect of* B. bassiana *on the proliferation of the entomopathogenic nematode* H. indica *inside* Galleria mellonella *larvae

The capacity of one infective juvenile to proliferate inside *B. bassiana*-preinfected *Galleria* decreased with the prolongation of the preinfection period. One naturally penetrated IJ was capable of producing 5866 nematodes when it was applied together with the fungal pathogen. This capability was decreased further when the IJ was inside larva preinfected for 24 h with the fungal pathogen and did not exceed 1256 nematodes compared to production of 14380 nematodes in the control (Table 1). These results indicate that as the fungal mycelium becomes more established inside the insect, it exerts a more profound effect on the proliferation of the nematode. The increased suppressive effect is probably due to secretion of greater amounts of antibiotic and toxic substances secreted by the fungus (Krasnoff *et al.*, 1991).

In summary, the above observations indicate that the sustainability of the entomopathogenic nematode *H. indica* as a biocontrol agent might be substantially reduced if applied together with or after application of the entomopathogenic fungus *B. bassiana*.

Table 1. Effect of *B. bassiana* on the penetration, development, and total production of *H. indica*, inside *Galleria mellonella* larvae. The insects were infected with nematodes in wet sand for 24 h. The same lowercase letters indicate statistically not significant differences.

Pathogen	Exposure period to *B. bassiana* (hours)	Mean % penetration of IJs	Number of injected IJs/larva	Mean % recovery of IJs to hermaphrodites		Total nematode production / one naturaly penetrated IJ
				Natural penetration	Injection	
Control		7.37[a]	27.1	94.90[d]	71.90[h]	14380[J]
B. bassiana and IJs	24	6.00[a]	ND	52.00[e]	ND	5866[k]
B. bassiana then IJs	24	1.72[b]	33.7	69.70[f]	78.80[h]	1256[l]
B. bassiana then IJs	48	1.40[b]	34.3	0.00[g]	58.30[i]	0.00[g]
B. bassiana then IJs	72	1.70[b]	46.0	0.00[g]	47.60[i]	0.00[g]
B. bassiana then IJs	96	0.00[g]	36.0	0.00[g]	ND	0.00[g]

* Not determined

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