Antagonistic interactions between the biocontrol agents *Beauveria bassiana* and *Heterorhabditis indica*

Omar M. Darissa and Naim M. Iraki

UNESCO Biotechnology Educational and Research Center (UNESCO BERCEN), Bethlehem University, P.O. Box 9, Bethlehem, Palestine
e-mail: odarissa@bethlehem.edu

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Summary. The effect of the pathogenic fungus, *Beauveria bassiana*, on the invasion and proliferation of the entomopathogenic nematode, *Heterorhabditis indica*, in larvae of the waxmoth, *Galleria mellonella*, was investigated. Simultaneous application of both pathogens did not cause any significant decrease in nematode penetration (6.0%) in comparison to infection with nematodes only (7.4%). However, when the insect larvae were preinoculated with *B. bassiana* for 24, 48, 72, or 96 h before nematode application, the mean percentage penetration was significantly reduced to 1.7, 1.4, 1.7, and 0.0, respectively. When the larvae were infected with nematodes only (control), the number of nematodes collected, after 2 weeks after the death of the insect, was 106,000 nematodes per larva (*i.e.*, 14,380 nematodes per one injected juvenile (InjJ)). However, when the *G. mellonella* larvae were simultaneously inoculated with both agents, the total number of nematodes produced was restricted to 35,000 nematodes per larva (5,866 nematodes per InjJ). A more dramatic effect was observed when larvae were preinoculated with *B. bassiana* before nematode application. The 24 h preinoculation period resulted in the decreased harvest of 2,160 nematodes per larva (1,256 nematode per InjJ), while the other preinoculation regimes caused a total inhibition of nematode production. Increasing the number of nematode juveniles inside preinoculated by fungus larvae by injection reduced the inhibitory effect of the fungus on the development of hermaphrodites. The observed results indicate the occurrence of antagonistic interactions between the two pathogens inside the infected insect.

Key words: infective juveniles' development, penetration of infective juveniles, sustainability of biocontrol agents.

Most of the research conducted on the use of combined biocontrol agents focused mainly on their effect on the mortality of the target insect. Choo *et al*. (1996), for example, used a combination of two entomopathogenic nematodes (EPN) species to control the larvae of western spotted cucumber beetle, *Diabrotica undecimpunetata*. They concluded that no advantage is gained by using such a combination for controlling the insect. On the other hand, a combination of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* caused greater mortality of *Galleria mellonella* than either bioinsecticide alone (Alatorre-Rosas & Kaya, 1991). The same combination of the two nematode species was also used to control the black cutworms and the black vine weevil (Kaya *et al*., 1993).

Fungi such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* were successfully combined with the EPN *H. bacteriophora* and *S. carpocapsae* to increase the mortality of several target insects (Shapiro-Ilan *et al*., 2004; Ansari *et al*., 2008; Schulte *et al*., 2009). Furthermore, the release of *H. bacteriophora* into soil containing *B. bassiana* resulted in higher total mortality of the beet armyworm *Spodoptera exigua* than action of any of the agents alone (Barbercheck & Kaya, 1991). Moreover, in a study by Jabbour *et al*. (2011), a combination of three EPN species (*H. megidis*, *S. carpocapsae* and *S. feltiae*) and the fungus *B. bassiana* resulted in greater mortality of the insects *G. mellonella* and the Colorado potato beetle, *Leptinotarsa decemlineata*, than when using any of the pathogens alone. Although most of the studies on combining biocontrol agents focused on the mortality of a target insect, the possible antagonistic interactions among the biocontrol agents themselves were not adequately studied. The aim of this work was to study the effect of *B.*

bassiana on the survival, penetration, and proliferation of the entomopathogenic nematode *H. indica* inside *G. mellonella* larvae.

MATERIALS AND METHODS

Live specimens. *Heterorhabdis indica* Beth 11 strain was isolated by Iraki *et al*. (2000). Two-week old infective juveniles (IJ) were obtained from a monoxenic culture maintained as described by Lunau *et al*. (1993). The symbiotic bacterium *Photorhabdus luminescens* was isolated from the haemolymph of *G. mellonella* larvae infected with the nematode *H. indica* as described by Ehlers *et al*. (1990) and cultured as reported by Akhurst (1980).

The fungus *B. bassiana* used in this study was isolated from a soil sample from Nablus district, Palestine and identified by Mahassneh (1999). Spores were harvested from *B. bassiana* culture as described by Zhang & Watson (1997), and stored at 4°C until use. The wax moth *G. mellonella* was cultured on an artificial diet (200 g honey, 183 g glycerin, 47 g yeast extract, 4 g nepagin, and 320 g wheat bran). In all experiments last instar larvae were used.

Assessment of the survival of *H. indica* stages in *B. bassiana* liquid culture. One hundred IJ and 50 fourth-stage huveniles (J4) of *H. indica* suspended in 1 ml of Ringer solution (Akhurst, 1980) were incubated in 20 ml of 2-day-old germinated spore cultures of *B. bassiana* (2.0×10^7 spores ml^{-1} of BSA medium). The BSA medium contained (in tap water) 10.0 g l^{-1} nutrient broth (Difco), 10.0 g l^{-1} Tryptic soy broth (Difco), 5.0 g l^{-1} yeast extract (Difco), 5.0 g l^{-1} peptone (Difco), 5.0 g l^{-1} NaCl, 0.35 g l^{-1} KCl, and 0.021 g l^{-1} $CaCl_2$ $2H_2O$. As a control treatment, similar amounts of IJ or J4 were incubated under the same conditions in sterile BSA medium. In all treatments flasks were continuously agitated on a rotary shaker (200 rpm) at 25°C in the dark for 2 days. After 24 or 48 h, three samples of 1 ml each were taken from each treatment and poured into sterile 5-cm diam. Petri dishes to record the survival of nematodes under the microscope. For each treatment, two replicate flasks were included and the whole experiment was repeated twice.

Assessment of the effect of *B. bassiana* on the growth rate of *P. luminescens*. A volume of 50 µl of *B. bassiana* (2×10^7 spores ml^{-1} BSA medium) or 20-h old culture of *P. luminescens* was smeared on 9-cm diam. Petri dish containing solid BSA medium. Filter paper disks (Watman No. 1; 0.6 cm diam.) were soaked in 15 ml of either *B. bassiana* (2×10^7 spores ml^{-1} BSA medium) or 20-h old liquid culture of *P. luminescens*. Part of the disks soaked with *B. bassiana* was placed in BSA medium smeared with *P. luminescens*, while the other part was placed on non-inoculated medium (control). In addition, disks soaked with *P. luminescens* were placed on medium of 0, 1, or 2-day old smear of *B. bassiana*. As a control, *P. luminescens* disks were placed on plates containing the medium only. Each treatment included four plates, each containing four disks. All of the plates were incubated in the dark at 25°C. The growth of the organisms was recorded daily by measuring the diameter of the culture circle surrounding the paper disk. The experiments were repeated three times independently giving 12 plates (48 disks) per treatment.

Inoculation of *G. mellonella* larvae with *B. bassiana* and *H. indica*. Using multi-well plates (well dimensions 1.55 cm diam. × 1 cm depth), *G. mellonella* larvae were buried in construction sand, one larva per well. Buried larvae were exposed to 1.16×10^7 *B. bassiana* spores in BSA medium for up to 4 days before being exposed to 100 IJ suspended in Ringer solution for 24 h. As a control treatment, part of the larvae was incubated with IJ alone for 24 h. After each exposure, 30 larvae were removed from the sand, washed three times with sterile distilled water and then divided into three equal groups. The first group of larvae was used to determine the number of penetrated IJ by pepsin digestion as described in the following section. The second group of larvae was transferred to a Petri dish lined with wet filter paper and left for 72 h at 25°C, then the larvae were dissected and the number of IJ that developed to hermaphrodites was counted under the microscope. For evaluation of the total production of nematodes, each infected larva of the third group was washed and placed on a small White trap (White, 1927) in the dark at 25°C for 2 weeks. At the end of this period, the numbers of IJ and adults in the cadaver were counted under the microscope. All of the experiments were repeated three times independently.

Evaluation of IJ penetration by pepsin digestion. Infected larvae were dissected and incubated in pepsin solution for 2 h at 37°C on a rotary shaker (120 rpm). The pepsin solution contained 8.0 g l^{-1} pepsin (Sigma), 23 g l^{-1} NaCl, and 940 ml distilled water. The pH was adjusted to 2.0 with HCl (Mauleon *et al.*, 1993). The IJ penetrated into the insect were then counted under the microscope.

Recovery of *B. bassiana* from haemolymph of dead larvae. The recovery of *B. bassiana* from dead *G. mellonella* larvae was performed by surface sterilising the dead larvae with 70% ethanol, then inoculates from the haemolymph were streaked on BSA medium and incubated in the dark at 25°C.

Injection of IJ into *G. mellonella* larvae. Infective juveniles, suspended in 20 µl of sterile Ringer solution, were injected with a 1,000 µl syringe into the haemolymph of last instar *G. mellonella* larvae that were preinfected with *B. bassiana* for up to 72 h. The number of injected IJ ranged from 27-46 per larva. The development of IJ into adults was recorded 4 days after injection.

Statistical analysis. The data collected from each experiment in this study were statistically analysed by paired samples t-test using the SPSS 9.0 software. The replicates of each treatment were collected from 2-3 independent experiments. Two treatments were compared at a time until all possible comparable combinations were analysed. Means were tested for significant difference at *P* value of 0.05.

RESULTS AND DISCUSSION

Survival of IJ and J4 of *H. indica* in the presence of *B. bassiana*. The results in Table 1 show that the survival of the IJ was not affected after 48 h of incubation in the *B. bassiana* cultures. By contrast, the survival of the J4 stage was dramatically reduced to 65% in the fungal culture. This is probably because the J4 stage is much more sensitive to *B. bassiana* culture than the IJ, which have a double cuticle that may provide them with better protection from the hydrolytic enzymes secreted by the fungus (Kaur & Padmaja, 2009). It also could owe to the fact that IJ is a non-feeding stage and when it recovers into the subsequent J4 stage it starts feeding immediately on the proliferating symbiotic bacteria. The J4 feeding process may involve ingestion of *B. bassiana* spores and toxins secreted by them which might negatively affect the survival of J4 nematodes.

The above findings may explain the reduced development to adults and proliferation of nematodes in *G. mellonella* larvae preinoculated with *B. bassiana* as discussed below.

Effect of *B. bassiana* on the growth of the symbiotic bacteria *Photorhabdus luminescens*. The growth of the symbiotic bacterium *P. luminescens* on a smear of *B. bassiana* germinating spores was totally inhibited (Fig. 1). This is in accordance with results obtained by Ansari *et al*. (2005) who studied the effect of *P. luminescens* on different fungal species including *B. bassiana*. On the other hand, the growth of *B. bassiana* on a smear of *P. luminescens* was inhibited by 60% as calculated after 3 days of co-incubation, compared to the control treatment where *B. bassiana* disks were mounted on non-inoculated medium (Fig. 1). These results indicate that the development of IJ into adults inside

Table 1. Percentage survival of *Heterorhabditis indica* infective juveniles (IJ) and fourth-stage juveniles (J4) in 2-day-old germinated spore cultures of *Beauveria bassiana*. Figures are means of results from two independent experiments totaling four replicates per treatment. All values with different superscripts are significantly different.

Time (h)	In *B. bassiana*		Control (liquid medium)	
	IJ	J4	IJ	J4
24	97.3^{a}	80.8^{b}	99.2^{a}	94.0^{a}
48	97.1^{a}	65.4^{c}	98.6^{a}	97.7^{a}

Table 2. Effect of inoculation of *Galleria* larvae with *Beauveria bassiana* on the penetration of *Heterorhabditis indica* infective juveniles (IJ) into the larvae and on their development into adults inside the insect. Figures are means of results from three independent experiments. Values with different superscripts in the same column are significantly different.

Inoculation period (h)	Mean % penetration of IJ	Mean % development to adults	Calculated % development of penetrated IJ
no. preinoculation	7.37^{a}	7.00^{d}	94.90
0	6.00^{a}	3.12^{e}	52.00
24	1.72^{b}	1.20^{f}	69.70
48	1.40^{b}	0.00^{g}	0.00
72	1.70^{b}	0.00^{g}	0.00
96	0.00^{g}	0.00^{g}	0.00

Galleria larvae preinoculated with *B. bassiana* might be reduced due to the suppression of the proliferation of the *P. luminescens* (the food of IJ) by the fungus.

The effect of *B. bassiana* on the penetration of *H. indica* IJ into *G. mellonella* larvae. No significant difference on the percentage of IJ penetration was observed when comparing simultaneous application of both pathogens to application of nematodes alone (6.0% compared with 7.4% in control). However, preinoculation of the *G. mellonella* larvae with *B. bassiana* for periods of 24 h and longer caused a dramatic decrease in the penetration of the nematodes (1.7% compared with 7.4% in the control; Table 2). Furthermore, preinoculation for a period of 96 h resulted in a total inhibition of penetration by IJs; a similar effect of *B. bassiana* was reported by Barbercheck & Kaya, (1991). Also, a decreased penetration of IJ into a host that had already been inoculated with the same nematode was reported by

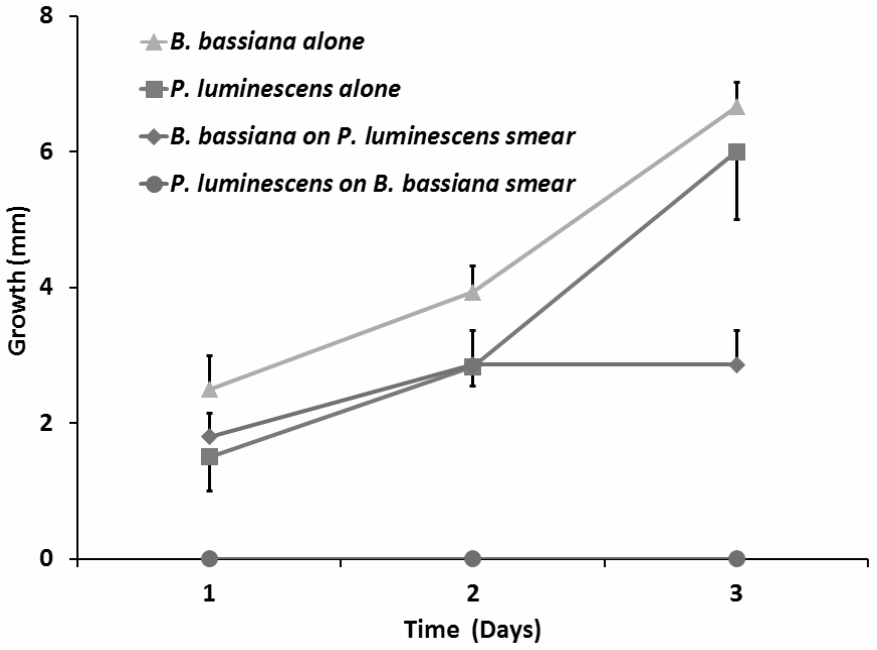

Fig. 1. Growth rates on BSA medium, measured as an increase in culture diameter (mm), of *Photorhabdus luminescens* on a smear of *Beauveria bassiana* and of *B. bassiana* on a smear of *P. luminescens.* Error bars (shown either as a minus or a plus to avoid overlapping) represent the standard deviation of three independent experiments.

Hominick & Reid (1990). It was suggested that the infected larvae secrete a certain substance that is sensed by IJ and causes them to avoid penetration into the infected insect. This kind of behaviour 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 entomopathogens are applied simultaneously, the fungus infection might be slower than that of the mobile IJ. The latter may penetrate into the insect before the occurrence of pathogenic response associated with secretion of the hypothesised repelling substance. Hence, there will be no influence on the penetration of the nematodes.

The effect of *B. bassiana* on the development of IJ to adults (hermaphrodites). When *B. bassiana* and nematodes were applied simultaneously, only 52% of the penetrating IJ could develop to adults, compared with 94.9% in the control (Table 2). A similar decrease in IJ development to adults was observed when *G. mellonella* larvae were exposed to *B. bassiana* spores for 24 h before nematode inoculation. However, longer periods of application of the fungus (48, 72, and 96 h) resulted in a total inhibition of development to adults of the small number of IJ that succeeded to penetrate (Table 2). The inhibitory effect of *B. bassiana* on the development of IJ 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; Khan *et al.*, 2012). When the period of preinoculation with *B. bassiana* spores was extended to 48 h or longer, the well-established fungal mycelium (Fig. 2) may have secreted sufficient amounts of toxins and antibiotic substances to cause a total inhibition of development of IJ to hermaphrodites.

Table 3. Development of *Heterorhabditis indica* infective juveniles (IJ) to adults upon injection into *Galleria mellonella* larvae preinoculated with *Beauveria bassiana* for up to 3 days. The results were observed 4 days after injection of IJ. Figures are means of results from three independent experiments. Values with different superscripts are significantly different.

Inoculation period (h)	Average number of injected IJ per larva-1	Mean % development to adults
no preinoculation	27.1	71.90[a]
24	33.7	78.80[a]
48	34.3	58.30[b]
72	46.0	47.60[b]

Table 4. Reproduction of *Heterorhabditis indica* infective juveniles (IJ) inside *Galleria mellonella* larvae preinoculated with *Beauveria bassiana* for up to 4 days. Figures are means of results from three independent experiments. Values with different superscripts are significantly different in the same column.

Inoculation period (h)	Nematodes reproduction:		*B. bassiana* in haemolymph*	Sporulation of *B. bassiana*
	per larva ($\times 10^3$)	per penetrating IJ		
no preinoculation	106.00^{h}	14380^{L}		
0	35.00^{i}	5866^{m}	ND	–
24	2.16^{j}	1256^{n}	–	–
48	0.00^{k}	0.00^{o}	++	+
72	0.00^{k}	0.00^{o}	++++	+
96	0.00^{k}	0.00^{o}	++++	+

When the number of IJ inside larvae preinoculated with *B. bassiana* for 48 and 72 h was increased, by injection to 34 and 46, respectively (which is 24 and 27 fold higher that number of IJ able to penetrate larva body wall under similar infection conditions), the percentage recovery to hermaphrodites dramatically increased compared to that of naturally penetrating IJ (Table 3). While the small number of naturally penetrating IJ failed to develop to hermaphrodites inside the preinoculated larvae, injected IJ showed 58% and 47% development in insects preinoculated for 48 and 72 h, respectively. These results indicate that an increase in number of IJ, 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 inhibits the development of the fungal pathogen.

Effect of *B. bassiana* on the proliferation of the entomopathogenic nematode *H. indica* inside *G. mellonella* larvae. The capacity of a single infective juvenile to proliferate inside *B. bassiana*-preinoculated *G. mellonella* decreased with the prolongation of the preinoculation period. A single naturally penetrating IJ was capable of producing 5,866 nematodes when it was applied together with the fungal pathogen. When a larva was inoculated with the fungus 24 h before introducing the IJ, the capacity to multiply was decreased to 1,256 nematodes compared to production of 14,380 nematodes in the control (Table 4). Also, when larvae were exposed to the fungus for 48 h or longer before the application of IJ, the nematode could not reproduce at all (Table 4). Associated with this was the detection of pronounced amounts of *B. bassiana* in the insect haemolymph after 48 h or longer of its application (Fig. 2; Table 4). These results indicate the occurrence of antagonistic interactions between

Fig. 2. Streaks from haemolymph of *Galleria mellonella* larvae that were exposed to *Beauveria bassiana* for up to 96 h followed by 24 h exposure to *Heterorhabditis indica* infective juveniles. Top, bottom, right, and left plates are streaks from larvae that were exposed to *B. bassiana* for 2, 4, 1, and 3 days, respectively. The plates were photographed 1 week after infection with *B. bassiana*.

the two entomopathogens inside the infected insect. As the fungal mycelium becomes more established inside the insect (Fig, 2; Table 4) 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 by the fungus (Krasnoff *et al.*, 1991; Jabbour *et al.*, 2011).

Although the above observations reveal the occurrence of antagonistic interactions between the two pathogens inside *G. mellonella* larvae, further studies remain necessary to elucidate these interactions in other target insects and to evaluate the sustainability of the two biocontrol agents when applied together.

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O.M. Darissa, N.M. Iraki. Антагонистические отношения между двумя агентами биологического контроля: *Beauveria bassiana* и *Heterorhabditis indica*.

Резюме. Исследовано воздействие патогенных грибов *Beauveria bassiana* на эффективность заражения энтомопатогенными нематодами *Heterorhabditis indica* личинок *Galleria mellonella* и последующего размножения. Одновременное применение обоих патогенов не привело к существенному снижению доли проникающих в хозяина личинок (6%) в сравнении с заражением только нематодами (7,4%). Однако в тех случаях, когда личинки вощинной моли были заражены спорами грибом *B. bassiana* за 24, 48, 72 и 96 часов до внесения нематод, средняя эффективность проникновения существенно снижалась до 1,7, 1,4, 1,7, и 0, соответственно. Когда личинки были заражены только нематодами (контроль), общее число личинок нематод, собранных через 2 недели, т.е. уже после развития и миграции их из хозяина, составляло 106000 новых личинок на одну личинку моли или 14380 новых личинок на каждую первончально инъецированную инвазионную личинку (InjJ). В тех случаях, когда личинки *G. mellonella* были заражены одновременно обоими агентами биометода, общее число образовавшихся лиичнок снижалось до 35000 на одну гусеницу, или 5866 новых личинок на каждую InjJ. Еще более выраженное воздействие было отмечено при заражении гусениц грибом *B. bassiana* за 24 ч. до введения личинок нематод: 2160 нематод на гусеницу (1256 новых личинок на каждую InjJ) для введения грибов за 24 ч. до нематод. Более длительное воздействие *B. bassiana* на гусениц (48, 72 и 96 ч.) полностью подавляло развитие нематод. Повышение числа инвазионных личинок, вводимых в гусеницу с помощью инъекции, снижало подавляющее воздействие грибов на развитие гермафродитных стадий нематод. Полученные результаты говорят о наличии конкурентных взаимоотношений между двумя этими патогенами внутри пораженных насекомых.