EFFECT OF *PAECILOMYCES LILACINUS* STRAIN 251 ON THE SURVIVAL AND VIRULENCE OF ENTOMOPATHOGENIC NEMATODES UNDER LABORATORY CONDITIONS

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Summary. The effect of the nematophagous fungus *Paecilomyces lilacinus* strain 251 (PL251) on the survival and virulence of the entomopathogenic nematodes (EPNs) *Steinernema feltiae*, *Heterorhabditis bacteriophora* and *H. megidis* was investigated under controlled conditions. In a laboratory assay, EPN survival in sand treated with PL251 was determined at days 1, 7 and 14 after nematode application. In addition, a sand column assay was used to determine the effect of PL251 application on EPN efficacy by measuring insect mortality and nematode penetration rate into *Galleria mellonella* larvae. The survival and biocontrol performance of the three EPN species were not affected by PL251 application. These results indicate the potential of the commercial *P. lilacinus* strain 251 for integration with EPNs in pest management strategies.

Key words: *Heterorhabditis bacteriophora, H. megidis,* nematophagous fungus, *Steinernema feltiae*.

Paecilomyces lilacinus Samson (Eurotiales: Trichocomaceae) is a nematophagous fungus that has been shown to be effective against a wide spectrum of plant-parasitic nematodes (Rumbos and Kiewnick, 2006). The commercial strain 251 (PL251) has recently received US registration as a biological nematicide under the trade name MeloCon® WG, whereas it is undergoing registration procedure as BioAct® WG in Europe (Kiewnick and Sikora, 2006).

Entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae, Heterorhabditidae) are biocontrol agents used for the control of a broad spectrum of soil-dwelling insect pests (Kaya and Gaugler, 1993). Infective juveniles (IJ) of EPNs are applied to soil, where they locate and kill their hosts. EPNs are highly virulent and provide a high level of protection, can be mass-produced, formulated and easily applied with conventional equipment, and are safe to vertebrates, plants and other non-target organisms. Due to these advantages, EPNs have attracted much interest and nowadays a number of species are commercially available and widely used in many crops.

Modern agriculture practices often demand the application of diverse control measures for effective pest and disease control. In many cases, management strategies overlap spatially and temporally, resulting in interactions between the control agents that can affect their efficacy, either synergistically or antagonistically. Information on the compatibility of biological control agents (BCAs) and their interaction if used simultaneously is valuable and lately has stimulated a lot of scientific interest (De Nardo and Grewal, 2003; Krauss *et al.*, 2004). Recent research demonstrated the compatibility of PL251 with important biocontrol agents, such as arbuscular mycorrhiza (Rumbos *et al.*, 2006).

However, only limited information is available concerning possible interactions of PL251 when co-applied with EPNs. Earlier studies, using a previous commercial preparation of PL251, suggested that PL251 might pose a slight risk to EPNs when used at normal field doses (Holland, 2000). Recent advances in solid state fermentation and formulation of PL251 compared to previous products of this strain (Kiewnick, 2001) make it necessary to evaluate the compatibility of the present PL251 formulation with EPNs. Therefore, the objective of the present study was to investigate the effect of the current, commercial preparation of *P. lilacinus* strain 251 on the survival and virulence of *Steinernema feltiae* Filipjev, *Heterorhabditis bacteriophora* Poinar and *H. megidis* Poinar, Jackson *et* Klein used against *Galleria mellonella* L. (Lepidoptera: Galleridae) under laboratory conditions.

MATERIALS AND METHODS

Fungal isolate. *Paecilomyces lilacinus* strain 251 deposited at the National Measurement Institute (NMI), formerly known as the Australian Government Analytical Laboratory (AGAL) (accession number: 89/030550) was used in all experiments. The fungus was commercially formulated as a water-dispersible granule (WG) with a concentration of at least 1×10^{10} viable conidia g^{-1} of product.

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Entomopathogenic nematodes and insect. Commercially formulated infective juveniles (IJs) of *S. feltiae* and *H. bacteriophora* (Nemaplus® and Nematop®, e-Nema GmbH, Raisdorf, Germany), as well as *H. megidis* (Larvanem-M®, Koppert B.V., Berkel en Rodenrijs, The Netherlands), were used in all experiments. The product samples were stored at 4-6 °C according to the manufacturer's recommendation and EPNs were allowed to acclimatize for 8 h in tap water at room temperature before being used in bioassays. Larvae of *G. mellonella* were obtained from Kerf GmbH (Unna, Germany). Last instar larvae were used throughout the study.

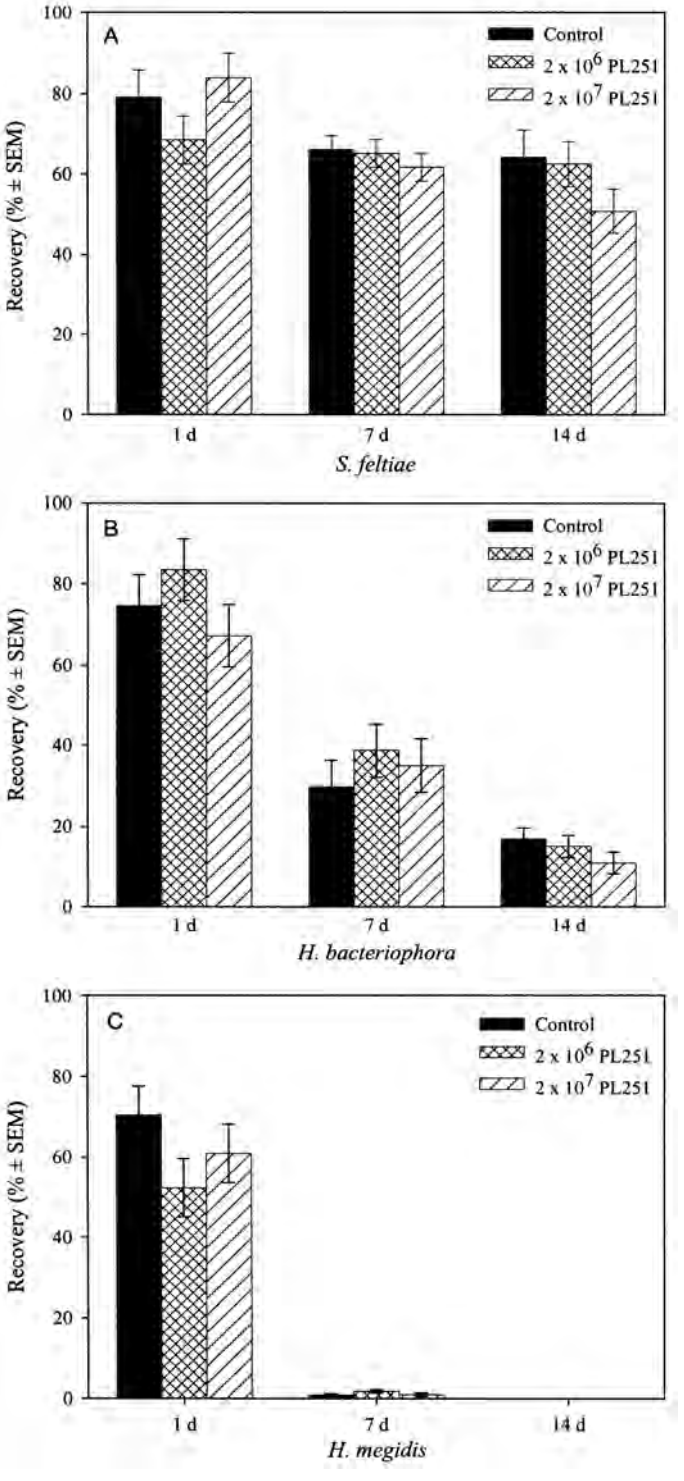

Fig. 1. Recovery (± SEM) of the entomopathogenic nematodes *Steinernema feltiae* (A), *Heterorhabditis bacteriophora* (B) and *H. megidis* (C), 1, 7 and 14 days after application, from untreated sand (Control) and sand treated with *Paecilomyces lilacinus* strain 251 (PL251) at rates of 2×10^6 and 2×10^7 cfu g^{-1} dry sand (n = 6). SEM = Standard Error of the Mean.

Effect of PL251 on EPN survival. The effects of two rates of PL251 conidia on the survival of *S. feltiae*, *H. bacteriophora* and *H. megidis* in sand were determined. An aqueous suspension of PL251 conidia formulated as WG was homogeneously incorporated into autoclaved (60 min, 121 °C) sieved sand (< 2.0 mm diam. particles) to achieve concentrations of approximately 2×10^6 and 2×10^7 colony forming units (cfu) g^{-1} dry sand. Plastic beakers (5 cm diam.) were filled with 100 g of PL251 treated sand to a depth of 5 cm before 5,000 IJs of *S. feltiae*, *H. bacteriophora* or *H. megidis* were applied in 1 ml sterilized tap water. Sand treated with water only served as control. Sand moisture content was adjusted to and maintained during the experiment at 10% (w:w) with sterilized tap water. After 1, 7 and 14 days incubation at 25 ± 1 °C, nematodes were extracted from sand with a modified Whitehead tray (Hooper, 1986). Briefly, the sand from the beakers was placed as a thin layer on a paper tissue, supported by a plastic sieve (1 mm mesh size) in an extraction dish filled with enough water just to touch the bottom of the sieve. After 24 hours extraction, nematodes settled at the bottom of the extraction dish were transferred to a flask and the water volume was adjusted to 25 ml. Two sub-samples of 2 ml of the nematode suspensions were used to determine the number of nematodes recovered using a stereomicroscope (50×) and a counting dish. The experiment was conducted twice with three replicates per treatment.

At all extraction dates, sand samples from the three replicates of each treatment were collected and pooled and a sub-sample was used to determine PL251 densities. In detail, 5 g sub-samples of moist sand were transferred to flasks containing 50 ml of sterile demineralized water and shaken on a horizontal shaker for 20 minutes at 170 rpm. After preparation of the appropriate dilutions (10^2- and 10^3-fold), the numbers of cfu were determined by plating onto OHIO agar (Johnson and Curl, 1972) using a spiral plater (Eddy Jet, IUL Instruments, Germany). Three replicate plates were prepared for each soil sample. Following incubation for 5 days at 25 ± 1 °C, the fungal colonies were counted. After further incubation for 5 days, the number of PL251 colonies per plate was confirmed and the number of cfu g^{-1} dry soil calculated. Identification of PL251 was based on morphological characteristics and on its distinctive colour (pink and later purple).

Effect of PL251 on EPN efficacy against G. mellonella *larvae*. In a sand column assay, the effects of two rates of PL251 on the efficacy of *S. feltiae*, *H. bacteriophora* and

H. megidis against *G. mellonella* was investigated (Ricci *et al*., 1996). PL251 conidia were applied to autoclaved (60 min, 121 ºC), sieved and moist sand (< 2.0 mm diam. particles; 10% w:w moisture) to achieve concentrations of 2×10^6 and 2×10^7 cfu g^{-1} dry sand. Sand treated with water only served as control. Plastic cylinders (5 cm diam.) with one end covered by a metal screen were filled with treated or untreated sand to a depth of 4 cm and placed on lidless Petri dishes filled with moist, sterilized sand. One *G. mellonella* larva was placed in the centre of each Petri dish underneath the cylinder. The screen prevented vertical movement of the insect larva upwards into the cylinder. One thousand IJs of *S. feltiae*, *H. bacteriophora* or *H. megidis* were applied in 1 ml water on the top of each sand column. Following incubation for 2 days at 25 ± 1 ºC, insect mortality due to EPN infection was determined. At the end of the experiment, sand samples from each treatment were analysed to determine PL251 densities, as described above. The experiment was conducted twice with ten replicates per treatment.

As an indicator of EPN virulence, the penetration rate, measured as the percentage of the initial IJ density that invades the insect host, was determined (Caroli *et al*., 1996). *Galleria mellonella* cadavers were rinsed to remove nematodes from their surface, dissected and placed in 15 ml centrifuge tubes containing 4 ml of pepsin solution (2.3 g NaCl and 0.8 g pepsin) (Merck KGaA, Darmstadt, Germany) in 100 ml of deionized water, pH 1.8-2.0). Following incubation for 90 min at 37 °C to digest the insect tissues, the suspension in each tube was diluted with 10 ml of 0.1% Tween 20 (Fluka Chemie GmbH, Buchs, Switzerland) and vortexed for 20 s. The penetration rate was determined from an aliquot using a stereomicroscope (50×) and a counting dish.

Statistical analysis. After verifying homogeneity of variances, data from duplicated experiments were pooled for statistical analysis. Means were tested for significant differences at $P<0.05$ using least significant difference (LSD) tests. The penetration rate was calculated by dividing the number of nematodes found per *G. mellonella* cadaver by the number of nematodes added initially. Two-way analysis of variance (ANOVA) was used to test for the effects of PL251 treatment and nematode species as well as their interaction on EPN survival and virulence.

RESULTS

Effect of PL251 on EPN survival. The recovery of *S. feltiae*, *H. bacteriophora* and *H. megidis* from untreated and PL251 treated sand at day 1, 7 and 14 after nematode application was similar (Fig. 1). However, the nematode species differed significantly ($P<0.001$) in their survival (Table I). The greatest survival over time was observed for *S. feltiae*, with more than 50% of the initial nematode density persisting 14 days after application. *Heterorhabditis bacteriophora* showed intermediate survival with an average of 35 and 14% of the initial population persisting 7 and 14 days after application, respectively. *Heterorhabditis megidis* suffered the greatest mortality with less than 2% of the initial population surviving 7 days after application and no nematodes recovered after 14 days. No statistically significant interaction between the main factors, PL251 treatment and nematode species, was detected for all extraction dates (Table I).

Numbers of cfu of PL251 ranged from 1.83×10^6 to 1.71×10^6 cfu g^{-1} dry sand when PL251 was applied at a low rate and from 1.79×10^7 to 1.26×10^7 cfu g^{-1} dry sand when PL251 was applied at a high rate, 1 and 14 days after application, respectively.

Effect of PL251 on EPN efficacy against G. mellonella *larvae*. The mortality rate of *G. mellonella* was 100% for all three EPN species in the presence or absence of PL251. The penetration rates for the three EPNs ranged from 12 to 16% (Table II). There was no significant effect of the PL251 treatment ($F = 2.13$; $P = 0.122$; $df = 2, 177$), of the nematode species ($F = 0.58$; $P = 0.563$; $df = 2, 177$), or of the nematode-PL251 treatment

Table I. Two-way ANOVA analysis of the effect of *Paecilomyces lilacinus* strain 251 (PL251) treatment and nematode species on the survival of the entomopathogenic nematodes *Steinernema feltiae*, *Heterorhabditis bacteriophora* and *H. megidis* in sand 1, 7 and 14 days after application.

Source of variation	*F* ratio (*P* value)[a]		
	Days after application		
	1	7	14[b]
PL251 treatment	0.64	0.43	2.76
	(0.530)	(0.651)	(0.081)
Nematode species	4.52	162.77	153.36
	(0.016)	(< 0.001)	(< 0.001)
PL251 treatment × Nematode	1.79	0.48	0.51
	(0.148)	(0.749)	(0.608)

[a] PL251 treatment, Nematode species $df = 2, 45$; interaction $df = 4, 45$.
[b] ANOVA was performed only for *S. feltiae* and *H. bacteriophora*, since no *H. megidis* population was recovered; PL251 treatment $df = 2, 30$; Nematode species $df = 1, 30$; interaction $df = 2, 30$.

Table II. Effect of *Paecilomyces lilacinus* strain 251 (PL251) on the penetration rate (± SEM) of the entomopathogenic nematodes (EPNs) *Steinernema feltiae*, *Heterorhabditis bacteriophora* and *H. megidis* into *Galleria mellonella* larvae.

EPN species	Penetration rate (%)[a]			
	Control	PL251		
		2×10^6	2×10^7	
S. feltiae	14.1 ± 6.3	12.1 ± 4.2	15.9 ± 6.8	n.s.
H. bacteriophora	13.7 ± 6.0	11.9 ± 7.5	13.3 ± 5.7	n.s.
H. megidis	11.9 ± 4.1	13.0 ± 5.1	14.5 ± 4.6	n.s.

[a] The penetration rate was determined in untreated sand (Control) and in sand treated with 2×10^6 and 2×10^7 cfu PL251 g^{-1} dry sand, following 2 days exposure to 1000 EPN infective juveniles.
n.s.: not significant at the 95% confidence level according to the LSD test ($P = 0.05$, n = 20).
SEM: Standard Error of the Mean.

interaction ($F = 0.68$; $P = 0.610$; $df = 4, 177$) on the penetration rates.

At the end of the experiment, PL251 population densities were 1.89×10^6 and 2.4×10^7 cfu g^{-1} dry sand when PL251 was applied at a low or high rate, respectively.

DISCUSSION

The survival of EPNs after their application can be affected by abiotic factors, such as temperature, soil water content and texture and biotic factors, such as microbial antagonists (Smits, 1996). Among the antagonists, nematophagous fungi play an important role in reducing the survival of applied EPNs (Kaya and Koppenhöfer, 1996). The trapping fungus *Arthrobotrys robusta* Dudd. caused a significant reduction in *S. feltiae* populations in sand, after the free-living nematode *Panagrellus redivivus* (L.) Goodey was added to induce trap formation (Van Sloun *et al*., 1990). The survival of *S. feltiae*, *S. glaseri* Steiner and *H. bacteriophora* was significantly reduced in soil naturally infested with *Hirsutella rhossiliensis* Minter *et* Brady compared to sterilized soil and this fungus was considered as the major mortality factor (Timper *et al*., 1991).

Holland (2000) investigated *S. feltiae* survival in a sandy loam soil treated with a previous commercial preparation based on PL251 and reported a significant negative dose-related effect of PL251 treatment on nematode survival. However, our data were obtained with the current water-dispersible formulation and suggest that EPN survival is not affected by PL251 application when the two biocontrol agents are applied concomitantly to sand. Even when sand was treated with 2×10^7 PL251 cfu g^{-1}, corresponding to a 10-fold higher concentration than the normal field dose, no adverse effect on the survival of *S. feltiae*, *H. bacteriophora* and *H. megidis* in sand was observed. Whether these contrasting findings are due to the different PL251 formulations used or to differences in the assay design employed in each study needs to be clearly elucidated. Significant differences were observed between the ability of EPN species to survive in sand in the absence of the host. *Steinernema feltiae* showed the highest survival rate, with up to 64% recovery of the juveniles after 14 days. In contrast, neither *Heterorhabditis* species survived for long, confirming that Heterorhabditidae do not survive as long as Steinernematidae (Molyneux, 1985).

Parasites and predators of nematodes may reduce the effectiveness of EPNs by adversely affecting survival in soil or by reducing host detection and infection by the IJs. The impact of *Hirsutella rhosilliensis* on *S. glaseri* and *Heterorhabditis bacteriophora* infection of *G. mellonella* larvae was studied in a one-on-one assay (Timper and Kaya, 1992). It was shown that the LD_{50} of *S. glaseri* was higher in *Hirsutella*-infested than sterilized sand, whereas the LD_{50} of *H. bacteriophora* was not affected, indicating that some EPN species might be more susceptible to fungal antagonists. Moreover, the suppression of *Heterorhabditis marelatus* Liu *et* Berry (formerly *H. hepialus*) infectivity of *G. mellonella* larvae by five nematode-trapping fungi, when larvae were added to the soil 4 days after the EPN, has also been reported (Koppenhöfer *et al*., 1996). In our sand column assay, IJs migrated through sand treated with PL251 to locate and infect the insect host. However, insect mortality was 100% for all three EPNs and was not affected by the fungal antagonist. Furthermore, no significant differences were detected between the penetration rates of the EPN species in untreated or PL251 treated sand, when the fungus was applied simultaneously with the EPNs.

In conclusion, in our study, no adverse effect of the application of PL251 on the survival and virulence of S. *feltiae*, *H. bacteriophora* and *H. megidis* was observed under the laboratory conditions of the tests. Further research is warranted to investigate the potential of the commercial *P. lilacinus* strain 251 for integration with EPNs in pest management strategies.

ACKNOWLEDGMENTS

The authors would like to thank E-Nema GmbH for providing product samples of Nemaplus® and Nematop®

and Koppert B.V. for supplying samples of Larvanem-M®. Prophyta Biologischer Pflanzenschutz GmbH, Germany is acknowledged for providing product samples of BioAct® WG.

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Accepted for publication on 20 July 2007.