LUCA RUIU[a]* - XINGYUE LI[b]- EUSTACHIO TARASCO[c]

ENTOMOPATHOGENIC NEMATODES ASSOCIATION WITH SOIL-DWELLING BACTERIA: THE CASE OF *STEINERNEMA FELTIAE*

[a] *Dipartimento di Agraria, University of Sassari, Sassari, Italy*
[b] *Institute of Plant Protection, Sichuan Academy of Agricultural Sciences, 610066 Chengdu, China*
[c] *Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, University of Bari 'Aldo Moro', Bari, Italy*
* Corresponding Author: lucaruiu@uniss.it

Ruiu L., Li X., Tarasco E. - Entomopathogenic nematodes association with soil-dwelling bacteria: The case of *Steinernema feltiae*

The symbiosis of entomopathogenic nematodes (EPNs) with bacteria in the genera *Photorhabdus* and *Xenorhabdus* is well known. However, other soil-dwelling bacteria can occasionally be isolated from EPNs collected from diseased larvae. Accordingly, through several studies, we have detected the presence of diverse bacterial species including isolates of *Pseudomonas protegens* and *Serratia* spp., in the body of different strains of the entomopathogenic nematode *Steinernema feltiae*. After documenting the presence of such bacteria inside the nematodes, both nematodes and EPN-associated bacteria were cultured, and bioassays were conducted to determine their potential against different insect targets. An isolate of *P. protegens* caused over 60% and 90% mortality of house fly and corn earworm larvae, respectively. These insecticidal effects were dose-dependent. The presence of the bacterium confers virulence to the nematode. According to our studies, it appears that these non-core bacteria can establish occasional or stable associations with *S. feltiae*.

Key Words: Biological control, EPN, bacteria, symbiosis, *Pseudomonas*, soil, bioinsecticide.

INTRODUCTION

The environment in which entomopathogenic nematodes carry out their biological cycle alternates between the soil and the body of host insects (Lewis and Clarke, 2012). Both of these ecosystems are characterized by the presence of a specific microbial community represented by insect body residents and soil-dwelling microorganisms, respectively. Some bacteria in particular have learned during evolution to exploit soil nematodes to be carried into the body of insect hosts, where they find in the haemolymph a rich and suitable environment for their development and proliferation (Goodrich-Blair and Clarke, 2007). A case of intimate interaction, resulting from a very driven evolution, is found in the mutualism between the nematodes belonging to the Steinernematidae family and bacteria of genus *Xenorhabdus*, in which the nematode host bacteria inside its gut, and after entering the body of a host insect, bacteria a released in the haemocoel where they will multiply, counteracting the insect immune system, and producing metabolites and conditions that favour the eventual development and multiplication of nematodes (Nielsen-LeRoux *et al.*, 2012). According to this scheme, infective juveniles of *Steinernema*, leave the insect body toward a life phase in the soil where they will look for other hosts. During this time, nematodes come into contact with several soil microbials including bacteria with entomopathogenic potential. Some of them are occasionally found in the body of nematodes and of insects showing septicaemia after being parasitized by the nematode (Ruiu *et al.*, 2017). While these mostly gram-negative bacteria are considered non-core species, we conducted recent studies supporting a more important role in entomopathogenesis of some *Pseudomonas* and *Serratia* species associated with *Steinernema feltiae* (Ruiu *et al.*, 2022). The results of new experiments corroborating the output of these studies are here summarized and reported.

MATERIALS AND METHODS

Steinernema feltiae isolates

Selected strains of *Steinernema feltiae* from the collection of the University of Bari (Tarasco *et al.*, 2009; Tarasco *et al.*, 2015) were employed in studies aimed at investigating their relationship with different bacterial species isolated from the haemolymph of septicaemic *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae on which the nematode had previously been inoculated. The nematode strains and the associated bacteria are reported on Table 1.

Isolation of bacteria from nematodes and their hosts

As previously mentioned, the first isolation of bacteria involved in this study was from the haemolymph of septicaemic *G. mellonella* larvae. Accordingly, groups of 10 third instar larvae of the lepidopteron were inoculated with a nematode suspension, and incubated for at least 48-72 h before observing larval death followed by tissue liquefaction. At this stage of infection, the insect body was full of nematodes, and haemolymph samples were collected and analysed. After serial dilutions, haemolymph aliquots were full-plate crawled on Luria-Bertani (LB) agar and incubated at 30° C for 24-48 h to isola-

Original scientific contribution presented and discussed at the XIV National Congress of the Italian Society of Nematology (SIN), Napoli - Italy, October 5 - 7, 2022.

Received 15 November 2023 Accepted 20 December 2023

Table 1 – Non-core bacterial species associated with insect host infection by selected strains of *Steinernema fletiae*, with indication of the bacterial detection in the nematode and/or in the insect host.

***Steinernema feltiae* strains**	**Bacterial species**	**Bacterial detection**	
		Insect host	**Nematode**
ALG18	*Alcaligenes faecalis*	+	-
CAST5	*Serratia marcescens*	+	+
CO1	*Pseudomonas protegens*	+	+
OT15	*Enterococcus mundtii*	+	-
RC8	*Serratia nematodiphila*	+	+

te the prevalent bacterial species. This isolation process was repeated several times over time.

In subsequent experiments, nematodes emerging from the larvae treated as previously described, were collected and subjected to analysis in order to check for the presence of the same bacterial species within their bodies. For this purpose, the nematodes were surface sterilized by washing in sodium hypochlorite (1 %) and then processed for total DNA extraction. Nematodes homogenate was used for extracting DNA through the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to manufacturer's protocols. PCR-based analyses were conducted using species-specific primer pairs to detect the presence of the same bacterial species previously identified in larvae treated with a specific *S. feltiae* strain (Ruiu *et al.*, 2022).

laboratory bioassays

Experiments were conducted to assess the insect pathogenicity of the bacterial isolates whose presence was confirmed in the body of nematodes.

Bacteria were cultured in flasks containing Luria Bertani (LB) broth at 30° C shaking at 120 rpm in an orbital incubator. Bacterial cultures were harvested after 72h by centrifugation (10.000 x g) to collect bacterial cells. Cells were resuspended in sterile water to form suspensions of the concentration required in bioassays with insects.

Bioassays on insect species in different orders were conducted either by injection and by ingestion. In the first case, the purpose was to evaluate the affinity of insect haemolymph as a nutrient broth for the development of target bacteria. On the other hand, ingestion bioassays aimed at investigating the ability of bacteria to go through the intestinal barrier to eventually reach the haemocoel, thus expressing their stand-alone entomopathogenic properties. Bioassays were conducted with larvae of the house fly *Musca domestica* L. (Diptera: Muscidae) and the corn earworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) as model species in different orders.

Fly larvae were reared on an artificial diet based on wheat bran (34%), milk powder (1%) and water (65%) (w/w), while *H. armigera* larvae were maintained on tomato leaves until being used in bioassays. Insect injections (1 µl/larva) were conducted with a syringe at the intersegmental membranes of the abdomen, while ingestion bioassays were based on the administration of a treated diet consisting of the rearing artificial diet incorporated with bacterial cells for fly larvae or treated tomato leaves (soaked in a cell suspension) for caterpillars. In all bioassays, the experimental design involved groups of 10 insects and four replicates per treatment. Control insects were left untreated. Insect mortality was assessed daily for 5 days after treatments. All experiments were repeated three times. Bioassay data were processed to calculate means and standard deviation, and variance was analysed by 2 ways-ANOVA (factors: dose and treated species) followed by LSD test for means comparison.

RESULTS

Non-core bacteria detection

Specific bacteria species were isolated from the haemolymph of septicaemic *G. mellonella* larvae treated with different strains of *S. feltiae*. Prevalent species were identified as *Serratia marcescens*, *Se. nematodiphila*, *Pseudomonas protegens*, *Alcaligenes faecalis*, and *Enterococcus mundtii* according to 16S rDNA sequence analysis, as reported in Table 1.

The PCR-based detection using species-specific primers, demonstrated the presence in the body of nematodes of *Se. marcescens*, *Se. nematodiphila* and *P. protegens*, and this association was particularly significant for *P. protegens*. Accordingly, specific bioassays with insects were conducted with the latter species.

entomopathogenicity of EPN-associated *Pseudomonas protegens*

A significant mortality was observed on larvae of *M. domestica* and *H. armigera* injected with *P. protegens* cells, and such effect was dose-dependent. While both species were highly susceptible, a slightly higher susceptibility was observed on *H. armigera* at the higher doses (Fig. I), reaching more than 90% mortality 48 h after the injection of 10^4 CFU/larva. Mortality was significantly affected by treatment dose ($F_{3,88}$= 51.46, P < 0.0001), larval species ($F_{1,88}$ = 557.56, P < 0.0001), and their interaction ($F_{3,88}$ = 9.79, P < 0.0001).

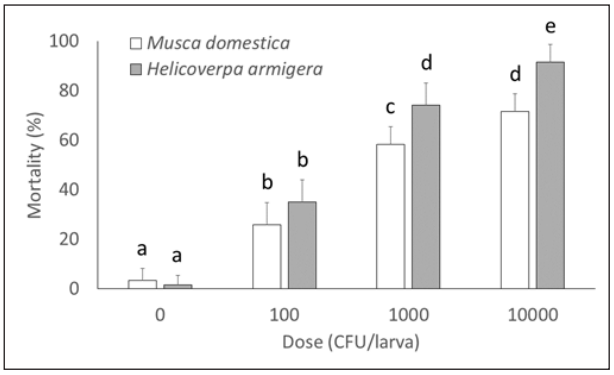

Fig. I - Mortality (mean ± SD) of *M. domestica* and *H. armigera* larvae injected with different concentrations (CFU) of *Pseudomonas protegens*. Different letters indicate significantly different means (two-way ANOVA, followed by LSD test, $P < 0.001$)

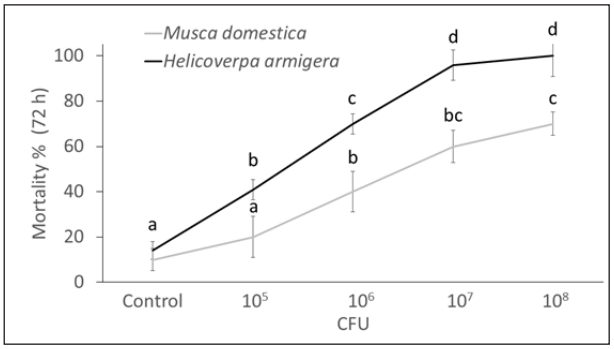

Fig. II - Mortality (mean ± SD) of *M. domestica* and *H. armigera* larvae fed on a diet treated with different concentrations (CFU) of *Pseudomonas protegens*. Different letters indicate significantly different means (two-way ANOVA, followed by LSD test, $P < 0.001$)

Similarly, lethal effects were observed on larvae feeding on a diet treated with *P. protegens* cells, achieving around 70% and over 90% after 5 days in insects exposed to a concentration of 10^8 CFU/g of diet for *M. domestica* and *H. armigera*, respectively (Fig. II). Again, the effect was concentration dependent, but a higher concentration of cells was required to achieve significant mortality in comparison with the control ($F_{4,110} = 215.32$, $P < 0.0001$). A slightly but significantly higher susceptibility of *H. armigera*, compared with *M. domestica* was also in this case detected ($F_{1,110} = 641.47$, $P < 0.0001$).

DISCUSSION AND CONCLUSION

Some soil-isolated strains of *Steinernema feltiae* were found to host non-core gram negative bacteria in their body. These bacteria were involved in the pathogenic action against insects inoculated by *S. feltiae*. While in some case, these bacteria seemed to be only occasionally associated to pathogenesis as opportunistic invaders of the insect body, in three cases their relationship with the nematodes appeared to be more robust with a more significant role in pathogenesis. Such conditions were observed in three *S. feltiae* strains, respectively associated with *Serratia marcescens*, *Se. nematodiphila*, and *Pseudomonas protegens*. Consistently with such role, these bacteria were isolated either from the haemolymph of septicaemic insect larvae previously inoculated with nematodes and directly from the body of surface-sterilized nematodes. Though such behaviour is typically associated with core bacterial symbionts of EPNs, like *Xenorhabdus nematophila*, on several occasions, bacteria in the genera *Serratia* and *Pseudomonas* have been isolated from nematodes (Lysenko and Weiser, 1974). According to a study conducted on *Steinernema*, these soil-dwelling non-core bacteria were observed in the intercellular space under the third-stage cuticle of nematodes (Bonifassi *et al.*, 1999). Regardless of the site in the body where such bacteria are harboured by the nematode, our studies have pointed to their role in the pathogenic process (Ruiu *et al.*, 2017).

Another aspect that emerged from our studies is the observation of an intrinsic entomopathogenic potential associated with these bacterial species, which have, as is known, the ability to produce an insecticidal protein complex, similar to that found in *Xenorhabdus* (McQuade and Stock, 2018). This is the case, for example, with the SEP proteins of *Serratia* (Hurst *et al.*, 2007). In addition, in Pseudomonads, very specific virulence factors against insects, such as FIT proteins, are found, which supports the evolution in these species of a bioinsecticidal capacity of their own (Ruffner *et al.*, 2015). Moreover, these bacteria have shown in our bioassays, a high suitability of insect haemolymph as their substrate for development. In addition, they have shown the ability to cross insect intestinal barriers, which characterizes them as entomopathogenic microorganisms with their own pathogenic capacity following ingestion by the host (Hamze *et al.*, 2023; Ruiu and Mura, 2021; Vesga *et al.*, 2020). Interestingly, such effects are selective respecting non-susceptible species such as some insect predators (Hamze *et al.*, 2022).

So these soil-dwelling bacteria, by establishing a relationship with nematodes, can take advantage of the possibility of being transported directly into the haemocoel where they can reproduce (Spescha *et al.*, 2023). Though we can imagine that this relationship between non-core species and EPNs is to be considered a step further back than the level of specialization we find in the symbiosis between *Steinernema* and *Xenorhabdus*, our findings and the sharing of high levels of gene homology between these bacterial species, documents an important evolutionary step of which other bacteria such as *Xenorhabdus* and *Photorhabdus* might have been protagonists in the past (Péchy-Tarr *et al.*, 2008; Kupferschmied *et al.*, 2014).

The study of these relationships between non-core bacterial species and EPNs may thus provide information on the evolutionary process that in the past characterized the establishment of a very specialized mutualistic symbiosis that we find in core-species, likely involving a common ancestor.

ACKNOWLEDGEMENTS

This study was financially supported by Fondazione di Sardegna, grant 2017, project 'Insect Microbiome Resources'

RIASSUNTO

ASSOCIAZIONE DEI NEMATODI ENTOMOPATOGENI CON I BATTERI DEL SUOLO. IL CASO DI *STEINERNEMA FELTIAE*.

La simbiosi dei nematodi entomopatogeni (EPN) con i batteri dei generi *Photorhabdus* e *Xenorhabdus* è ben nota. Tuttavia, altri batteri che vivono nel suolo possono essere occasionalmente isolati da EPN raccolti da larve malate. Di conseguenza, attraverso diversi studi, abbiamo rilevato la presenza di diverse specie batteriche, tra cui ceppi di *Pseudomonas protegens* e *Serratia* spp. nel corpo del nematode entomopatogeno *Steinernema feltiae*. Dopo aver documentato la presenza di tali batteri all'interno dei nematodi, i nematodi e i batteri associati all'EPN sono stati messi in coltura e sono stati condotti biosaggi per determinare il loro potenziale contro diversi insetti bersaglio. Secondo i nostri studi, sembra che questi batteri non-core possano stabilire associazioni occasionali o stabili con *S. feltiae*.

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