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INFECTIVITY OF MEDITERRANEAN NATIVE ENTOMOPATHOGENIC NEMATODES (STEINERNEMATIDAE AND HETERORHABDITIDAE) FROM NATURAL HABITATS IN RELATION TO TEMPERATURE

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Tarasco E., Oreste M., Li X., Liu Q. – Infectivity of Mediterranean native Entomopathogenic Nematodes (Steinernematidae and Heterorhabditidae) from natural habitats in relation to temperature.

The infectious activity of 15 *Steinernema* and *Heterorhabditis* strains of Entomopathogenic Nematodes (EPNs), belonging to 5 species (*Steinernema feltiae*, *S. affine*, *S. apuliae, S. ichnusae* and *H. bacteriophora*) collected from natural habitats (meadows, coasts and woods) in Southern Italy was compared in laboratory bioassays against *Galleria mellonella* larvae. Infectivity was determined by 2 larval mortality rate assays in relation to different temperature values. In the first experiment the percentage of larval mortality was recorded after a 72-hr exposure period to the EPNs Infective Juveniles (IJs) at 6 temperatures between 10 and 35°C, at intervals of 5°C, using IJs in aqueous suspension. The second bioassay was performed to compare the infectivity at 3 relatively low temperature values (6-10-14°C), using IJs in 2 different suspensions (aqueous and gel); the percentage of larval mortality was recorded every 72 hrs for 12 days after exposure to IJs. In the first experiment *S. feltiae, S. ichnusae* and *S. affine* strains showed the best performances at temperature values 10, 15, 20 and 25°C, while *H. bacteriophora* and *S. apuliae* showed the best results at 30 and 35°C. In the second test *S. feltiae, S. ichnusae* and *S. affine* strains demonstrated a better cold-infectivity (at 6-10-14°C) than *H. bacteriophora* and *S. apuliae* strains. IJs in the water suspension killed the *Galleria* larvae quicker than those in the gel. The gel suspension keeps nematodes more safe and active than the water one.

KEY WORDS: *Steinernema feltiae, S. affine, S. ichnusae, S. apuliae*, *Heterorhabditis bacteriophora,* Mediterranean areas, bioassay.

INTRODUCTION

Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) are obligate parasites of insects (POINAR, 1990) and received great attention as potential biological control agents. Their infectious activity varies with species and strains and it is affected by abiotic and biotic factors, especially temperature (KAYA, 1977; MOLYNEUX, 1985, 1986; BLACKSHAW & NEWELL, 1987; GRIFFIN *et al.*, 1989; KUNG *et al.*, 1991; GRIFFIN & DOWNES, 1991; TARASCO, 1997). Temperature influences the nematodes infectivity as well as their survival, mobility, development and reproduction (MOLYNEUX, 1983; SIMONS & VAN DER SCHAAF, 1986; KAYA, 1990; ZERVOS *et al.*, 1991; MASON & HOMINICK, 1995) and it is one of the most important factors limiting their success (GRIFFIN, 1993). Low temperatures (DOLMANS, 1983; RUTHERFORD et al., 1987; GEORGIS & GAUGLER, 1991) as well as high ones (RAO *et al.*, 1971) may, in fact, restrict their use. It is known that temperatures below 0 °C and above 40 °C are lethal to most nematodes (ULU & SUSURLUK, 2014), although the lethal effect of temperature depends on exposure time (KOPPENHÖFER, 2000). Entomopathogenic nematode species have defined thermal niches (GREWAL *et al.*, 1994). Some species are warm-adapted while others are adapted to cooler environments (HOMINICK & BRISCOE, 1990; WRIGHT, 1992; GREWAL *et al.*, 1994).

According to a "bio-rational approach" for selecting microbial control agents (YEO *et al.*, 2003), selection of EPN strains have to be based not only on their intrinsic virulence to the target host revealed by laboratory bioassays, but also on their ability to operate over the range of abiotic conditions that they could find in the agro-ecosystem. It is necessary, in fact, to select strains that combine the best characteristics for killing the target insects (high virulence against target organisms) and their ability to persist and infect in the environment in which the pest is occurring.

The purpose of this study is to compare the effects of temperature on infectious activity of 15 indigenous Italian EPN strains, recovered from natural habitats (TARASCO & TRIGGIANI, 1997; TARASCO *et al.*, 2015) and comprising 2 species actually isolated only in Italy, determining their thermal niche for optimum infectivity and testing their activity at low temperatures (cold-infectivity). This strain characterization may contribute to select the EPNs for testing in field assays against forest or agricultural pests in relation to different environmental conditions.

MATERIALS AND METHODS

NEMATODES

The 15 isolates of EPNs, belonging to *Steinernema feltiae* Filipjev, 1934 (7 strains: ItS-MSA3, ItS-MF1, ItS-LE1, ItS-CZ19, ItS-CZ23, ItS-TG4, ItS-G16), *S. affine* (Bovien, 1937) (4 strains: ItS-CZ10, ItS-QU3, ItS-ST12, ItS-LP3), *S. ichnusae* Tarasco, Mrá ek, Nguyen & Triggiani, 2008 (2 strains: ItS-SAR4 and ItS-SAR16) *H. bacteriophora* Poinar, 1976 (1 strain: ItH-C6) and *S. apuliae* Triggiani, Mrá ek &

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Reid, 2004 (1 strains: ItS-LD3) (Table 1), were collected using the "*Galleria* baiting technique" (Bedding and Akhurst, 1975) during a soil survey in different habitats in Italy (TARASCO & TRIGGIANI, *L.C.*; TARASCO *et al.*, 2015).

Nematodes were cultured in last-instar *Galleria mellonella* L. (Lepidoptera, Pyralidae) larvae at a temperature of 22°C. To obtain fresh infective juveniles (IJs), 10 wax worms on a 100x10 mm Petri dish with one 90 mm filter paper were treated with ca. 2,000 IJs in 1.5 ml of tap water. Two weeks after the treatment, wax worms were put on modified White traps (White, 1927) for the recovery of new generations of IJs. Collected IJs were kept at 8°C and used within 10 days after harvesting.

INFECTIVITY BIOASSAYS

Infectivity comparison at different temperatures

Plastic boxes (95 x 32 mm) filled with 40 g of sterilized peat (75% degree of humidity) were inoculated with 1,000 IJs in 1 ml of tap water. Each box received 10 *G. mellonella* final instar larvae (100 IJs/larva). There were 3 replicates for each treatment and 3 boxes without nematodes as control. Temperatures ranged between 10°C and 35°C, at intervals of 5°C. The bioassays were repeated 3 times. Larval mortality was recorded after 72-hr of exposure to IJs. Afterwards the dead larvae were removed from the boxes, rinsed in tap water and dissected to determine effective nematode infection.

Cold-infectivity and comparison between water and gel suspension

Petri dishes (90x15 mm) with one 85 mm filter paper were inoculated with 1,000 IJs in 1 ml of tap water or gel (Idrosorb SR 2002 - Nigem® - was used, an acrylic polymer of high molecular weight, which jellified by absorbing water). Each dish received 10 *G. mellonella* (100 IJs/larva). There were 3 blocks of 5 replications at 3 different temperature values (6-10-14°C). Dishes were kept in a container (15x32x40 cm) and wrapped with 2 black plastic bags to minimize desiccation. Larval mortality was recorded every 72 hrs for 12 days after exposure to IJs. The dead larvae were removed from Petri dishes, rinsed in tap water and dissected to verify the nematode infection.

CALCULATION

Data were pooled and analyzed using a general linear model procedure (ANOVA - analysis of variance) and significant differences among means were separated by HSD Tukey's test (Statistix 9.0, 2008). All comparisons were made at 0.05 level of significance.

RESULTS

INFECTIVITY COMPARISON AT DIFFERENT TEMPERATURES

All nematode isolates were able to kill the hosts and no mortality was observed in any of the control treatments.

- 10°C: *Steinernema feltiae* ItS-LE1 gave the best result (26% of larval mortality); other 6 *S. feltiae* strains (ItS-MSA3, ItS-G16, ItS-CZ19, ItS-CZ23, ItS-TG4 and ItS-MF1) followed with percentage values between 16% and 8%. *Steinernema ichnusae* ItS-SAR4 and ItS-SAR16, *S. apuliae* ItS-LD3, the *S. affine* strains (ItS-ST12, ItS-QU3, ItS-LP3 and ItS-CZ10) and *H. bacteriophora* ItH-C6 were statistically different causing very low larval mortality (0-4%) (Fig. I, 1).
- 15°C: *Steinernema feltiae* ItS-CZ19 produced the best larval mortality percentage (64%), followed by 6 *S. feltiae* (ItS-CZ23, ItS-G16, ItS-MF1, ItS-LE1, ItS-TG4, ItS-MSA3) and *S. affine* ItS-QU3 with percentages of larval mortality between 30% and 48%. *Steinernema ichnusae* (both strains), *S. affine* (ItS-ST12, ItS-LP3 and ItS-CZ10), and *S. apuliae* ItS-LD3 gave lower percentages (8-24%) while no larval mortality occurred with *H. bacteriophora* ItH-C6 (Fig. I, 2).
- 20°C: All *Steinernema* strains caused high larval mortality percentages (> 80%), except for *S. ichnusae* ItS-SAR4 (74%), *S. affine* ItS-ST12 (68%) and *S. apuliae* ItS-LD3 (which killed a percentage of *Galleria* larvae approximately of 60%), while *H. bacteriophora* ItH-C6 showed the lowest larval mortality value (33%) (Fig. II, 1).
- 25°C: Almost all steinernematids controlled about 90-100% of *Galleria* larvae while *H. bacteriophora*, *S. apuliae* and *S. affine* ItS-QU3 killed around 80% (Fig. II, 2).

Temperature - 30°C: *Heterorhabditis bacteriophora* gave the highest larval mortality percentage (98%) not statistically different from *S. apuliae* (88%); the other *Steinernema* strains followed with different larval mortality percentages between 60% and 78% (Fig. III, 1).

- 35°C: *Heterorhabditis bacteriophora* presented the highest larval mortality percentage (58%) followed by *S. apuliae* (44%); *S. feltiae, S. ichnusae* and *S. affine* strains were less effective with low larval mortality percentages (4-20%) (Fig. III, 2).

Table 1 – Characteristics of the sites with native Italian EPNs.

Strain	Locality	m a.s.l.	Time	Habitat	Soil texture	pH	Org. Cont.
S. feltiae ItS-LE1	Tricase (LE)	50	Oct 97	Meadows	Silty loam	7.4	0.11
S. feltiae ItS-MF1	Martina F. (TA)	350	Mar 98	Oak	Silty loam	7.3	1.41
S. feltiae ItS-CZ19	Giamberga (CS)	800	May 98	Pine	Sandy loam	6.8	1.63
S. feltiae ItS-CZ23	Lago Cecita (CS)	1100	May 98	Pine	Sandy loam	6.8	3.4
S. feltiae ItS-G16	Gravina (BA)	380	Mar 99	Pine	Silty loam	7.2	2.2
S. feltiae ItS-MSA3	M.S.Angelo (FG)	790	Dec 99	Meadows	Silt	7.4	3.75
S. feltiae ItS-TG4	Torre G. (BR)	20	Jan 00	Swamp	Sandy loam	8	3.5
S. affine ItS-ST12	Santeramo (BA)	400	Apr 98	Oak	Silty loam	7.7	3.25
S. affine ItS-CZ10	S. Paolo Al. (CS)	800	May 98	Oak	Clay loam	7.4	3.16
S. affine ItS-QU3	Quasano (BA)	150	Dec 99	Oak	Silty loam	8	2.9
S. affine ItS-LP3	Lagopesole (PZ)	700	Jun 00	Oak	Silt	6.9	3.6
S. ichnusae ItS-SAR4	Platamona (SS)	10	Jun 00	Sea coast	Sand	8.2	2.7
S. ichnusae ItS-SAR16	Tempio Pausania	250	Apr 09	Oak	Sandy loam	7.0	3.2
H. bacteriophora ItH-C6	Castellaneta (TA)	50	Sep 96	Pine	Sand	7.8	0.97
S. apuliae ItS-LD3	Metaponto (MT)	50	Oct 96	Pine	Sand	7.9	0.34

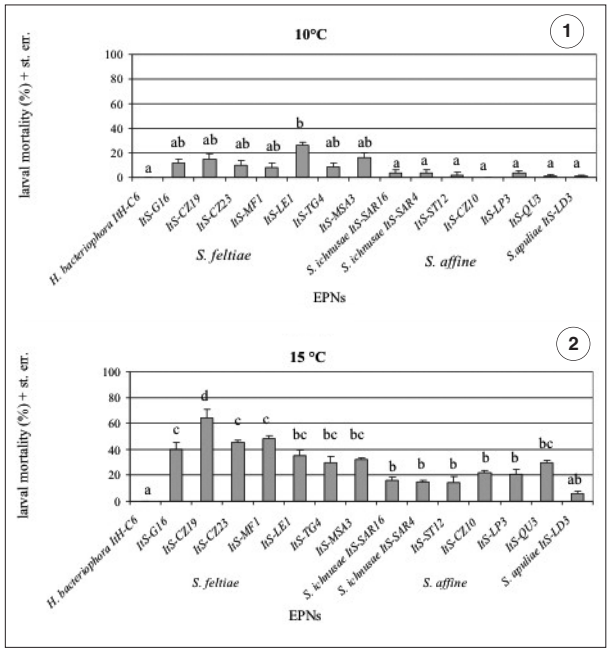

Figure I – Infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 72 hrs of exposure to IJs at 10°C (1) and 15°C (2). Bars with the same letter are not significantly different (P < 0.05).

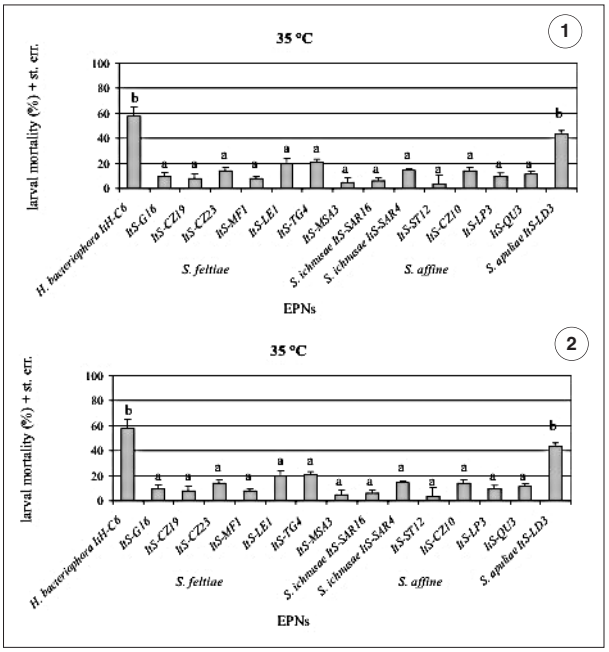

Figure III – Infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 72 hrs of exposure to IJs at 30°C (1) and 35°C (2). Bars with the same letter are not significantly different (P < 0.05).

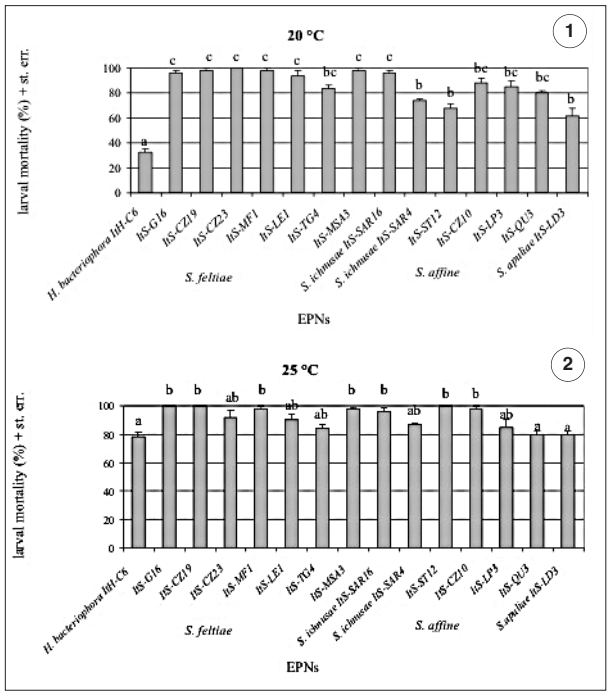

Figure II – Infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 72 hrs of exposure to IJs at 20°C (1) and 25°C (2). Bars with the same letter are not significantly different (P < 0.05).

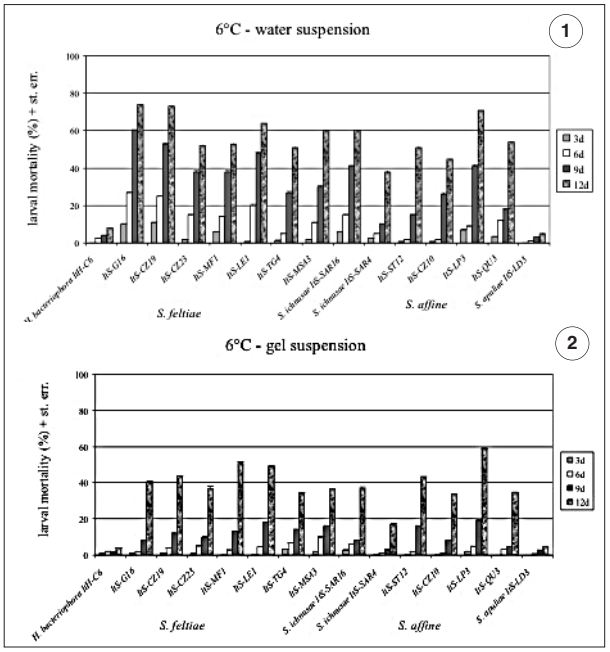

Figure IV – Cold-infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 3-6-9-12 days of exposure to IJs at 6°C in water (1) and gel (2) suspension.

COLD-INFECTIVITY AND COMPARISON BETWEEN WATER AND GEL SUSPENSION

No mortality was observed in any of the control treatments.

– 6°C, water suspension: Larval mortality reached a maximum percentage of >70% with *S. feltiae* ItS-G16 and ItS-CZ19 and *S. affine* ItS-LP3, after 12 days. *Steinernema apuliae* and *H. bacteriophora* presented the lowest values with 5% and 8% respectively, after 12 days. Other strains killed about 45 to 64% of *Galleria* larvae. Considering the test period, there was a quite constant increase in larval mortality during the 12 days for all the strains (Fig. IV, 1).

– 6°C, gel suspension: larval mortality caused by IJs in gel reached lower values in comparison to the IJs in water suspension. *S. affine* ItS-LP3 showed the highest percentage (59%) after 12 days; other *S. feltiae, S. ichnusae* and *S. affine* strains had lower percentages of

34-51% while *S. apuliae* and *H. bacteriophora* killed 4-5% of *Galleria* larvae. All the strains reached the maximum larval mortality percentage after 12 days with a major increment during the 9th and the 12th day (Fig. IV, 2).

– 10°C, water suspension: All the strains killed almost 100% of the *Galleria* larvae after 12 days, except for *S. apuliae* (3.5%) and *H. bacteriophora* (12.5%). The best increase in larval mortality was obtained between the 3rd and the 9th day (Fig. V, 1).
– 10°C, gel suspension: All the strains controlled about 100% of the *Galleria* larvae after 12 days, except for *H. bacteriophora* (21%) and *S. apuliae* (5%). The best increase in larval mortality was seen between the 3rd and the 9th day, as well in the water suspension (Fig. V, 2).
– 14°C, water suspension: All the strains killed 100% of the *Galleria* larvae after 12 days, except *H. bacteriophora* (70%) and *S. apuliae* (10%). The greatest increase in larval mortality was registered between the 3rd and the 6th day (Fig. VI, 1).
– 14°C, gel suspension: All strains killed almost 100% of the *Galleria* larvae after 12 days, except *S. affine* ItS-QU3 (70%), *H. bacteriophora* (32%) and *S. apuliae* (7%). The best increase in larval mortality was obtained between the 3rd and the 9th day (Fig. VI, 2).

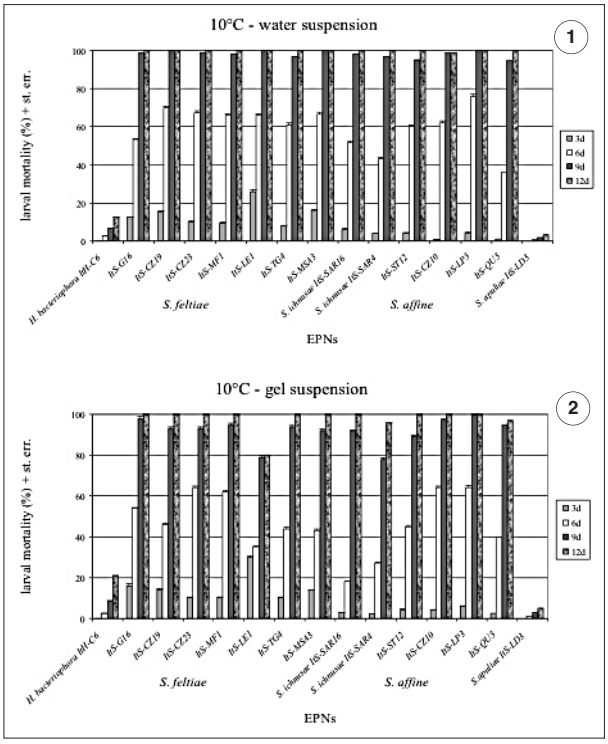

Figure V – Cold-infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 3-6-9-12 days of exposure to IJs at 10°C in water (1) and gel (2) suspension.

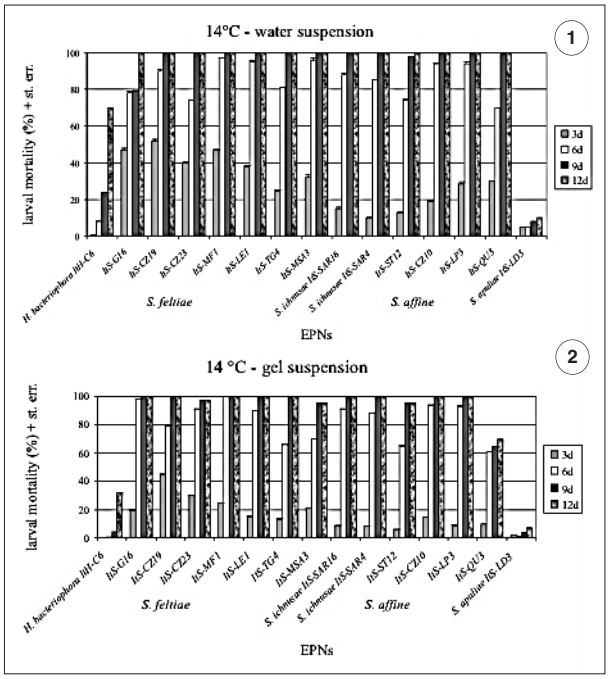

Figure VI – Cold-infectivity comparison among 15 Italian EPN strains: percentage mortality of *G. mellonella* larvae following 3-6-9-12 days of exposure to IJs at 14°C in water (1) and gel (2) suspension.

DISCUSSIONS AND CONCLUSIONS

The most important results of this paper concern the infectivity performances of the endemic species *S. apuliae* and *S. ichnusae*; the infectious behavior at different temperatures of *S. ichnusae* is quite similar to the other 2 steinernematids (*S. feltiae* and *S. affine*), while *S. apuliae* showed a completely different behavior, close to the heterorhabditid strain.

The data obtained from the experiments show that, although the *Galleria* larvae are susceptible to each strain tested, there are wide differences in the pathogenicity of these nematodes at different temperature values. Significant differences exist among the EPN species while similarities and differences occur among isolates of the same species. However, temperature has a direct effect on their infectivity. The data related to *S. feltiae*, *S. affine* and *H. bacteriophora* agree with previous researches. MA et al. (2013) tested the tolerance of thirty-two EPN strains from Northern China to heat, cold and desiccation and they found it differed significantly among and within species. MORTON & GARCIA DEL PINO (2009) found great variability among and within species considering the environmental tolerance of *S. feltiae* and *H. bacteriophora* strains to heat, desiccation, hypoxia and the effect of temperature on infectivity and reproduction and nematode migration in sand columns. Also MUKUKA *et al.* (2010) in their work showed a high variability among strains of *H. bacteriophora, H. indica* Poinar, Karunakar & David 1992 and *H. megidis* Poinar, Jackson & Klein, 1987 considering the heat tolerance.

The thermal preference of EPN strains seems to be correlated with the geographical origin of strains (MASON & HOMINICK, 1995; ULU & SUSURLUK, 2014) although some authors do not completely agree with this assumption. MUKUKA *et al.* (2010) showed that the influence of the strain origins on their heat tolerance is less important since the soil temperatures have much lower variability than air temperatures. GREWAL *et al.* (1994) stated that each nematode species has a well known thermal niche which is not affected by climatic conditions. Our results from the first experiment indicate that *S. feltiae, S. ichnusae* and *S. affine* strains are more effective at 10, 15, 20 and 25°C than *S. apuliae* and *H. bacteriophora*. Also, regarding the best pathogenic behavior, strains of *S. feltiae* performed better than the others at 10 and 15°C,

but at 20 and 25°C temperatures there are no differences between *S. feltiae, S. ichnusae* and *S. affine* strains. *S. apuliae* and above all *H. bacteriophora* gave better results than the other species at 30 and 35°C. Significant differences among the strains for each nematode species were also found (*S. feltiae, S. ichnusae* and *S. affine*) especially at low temperatures. Considering the sites of collection, these Italian strains showed a correlation between the thermal preference and their geographical origin: *Steinernema apuliae* ItS-LD3 and *H. bacteriophora* ItH-C6 were collected in see coast habitats and resulted as warm-adapted nematodes, while the other steinernematids collected in different inner zones showed, in addition to a wider distribution, even greater thermal range with also a quite good adaptation for lower temperatures. The findings on the temperature preference of *S. feltiae* and *H. bacteriophora* match with the published literature (MOLYNEUX, 1986; WRIGHT, 1992; GRIFFIN, 1993; LONG *et al.*, 2000; HAZIR *et al.*, 2001) while data on *S. affine,* and above all *S. ichnusae* and *S. apuliae* represent a contribution to the knowledge of their infectious behavior in relation to temperature variations.

The most significant findings are those related to the second experiment on cold-infectivity: *S. feltiae, S. ichnusae* and *S. affine* strains gave the best results, with significant differences in comparison to *S. apuliae* and *H. bacteriophora* which killed a low number of *Galleria* larvae. High cold-infectivity (10-15°C) of *S. feltiae* against *Cydia splendana* (Hübner) and *Curculio elephas* Gyll. was found also by KARAGOZ *et al.* (2009), while *H. bacteriophora* was the most effective at 20 and 25°C. LACEY et al. (2006) showed that *S. feltiae* was more effective in controlling the codling moth in apple and pear orchards during the cold seasons than the less cold-active species such as *S. carpocapsae* (Weiser, 1955). Numerous researches showed that *S. kraussei* is efficient at low temperature (from 6 to 10°C) (LONG *et al.*, 2000) particularly in controlling the black vine weevil *Otiorhynchus sulcatus* Fabricious while some other species (*S. carpocapsae*, *S. feltiae* and *H. megidis*) have not shown satisfying efficiency (LONG *et al.*, 2000; WILLMOTT *et al.*, 2002; HAUKELAND, 2007). BROWN *et al.* (1996) studied the cold tolerance of steinernematid and heterorhabditid nematodes and they found that *S. feltiae* had the lower lethal temperature and the higher survival after prolonged freezing at -4 degrees.

Regarding nematode suspension, the pathogenic data showed that nematodes in gel suspension killed larvae more slowly than those in water suspension at each temperature value. Gel was more resistant to dehydration than water and released nematodes more slowly; this means that gel suspensions "keep" the nematodes safe and active longer than water suspension, confirming results obtained by TRIGGIANI & TARASCO (2000a). The type of suspension, like the temperature variation, is related to the pathogenic behavior of the nematodes.

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