GIULIA TORRINI (*) - SILVIA LANDI (*) - CLAUDIA BENVENUTI (*) - FRANCESCA DE LUCA (**)
ELENA FANELLI (**) - ALBERTO TROCCOLI (**) - EUSTACHIO TARASCO (**) (***)
PAOLO BAZZOFFI (*) - PIO FEDERICO ROVERSI (*)

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION
OF A *STEINERNEMA CARPOCAPSAE* (NEMATODA STEINERNEMATIDAE)
STRAIN ISOLATED IN VENETO REGION (ITALY)

(*) *Consiglio per la ricerca e la sperimentazione in agricoltura – Research Centre for Agrobiology and Pedology, via Lanciola 12/A, 50125 Firenze, Italy; Corresponding author: Giulia Torrini,* e-mail: giulia.torrini@isza.it
(**) *Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche, Via Amendola 122/D, 70126 Bari, Italy*
(***) *DISSPA, Facoltà di Agraria, Università degli Studi di Bari "Aldo Moro", Via Amendola 165/A, 70126 Bari, Italy*

Torrini G., Landi S., Benvenuti C., De Luca F., Fanelli E., Troccoli A., Tarasco E., Bazzoffi P., Roversi P.F. – Morphological and molecular characterization of a *Steinernema carpocapsae* (Nematoda Steinernematidae) strain isolated in Veneto region (Italy).

A new strain of *Steinernema carpocapsae* (Weiser, 1955) was isolated from soil collected in a lagoon plain in Veneto region (North-East Italy). This new strain was named ItS-CAO1. Molecular and morphological analyses were performed. The ITS region and the 18S rRNA gene were amplified and sequenced. The ITS products were then digested with six restriction enzymes in order to unequivocally identify this species. Nematode virulence was tested against last instar of *Galleria mellonella* (L.) using different laboratory assays. Insect mortality of this new strain is very high in penetration (100%) and sand column assay (93.3%) and the percentage of penetrating infective juveniles was 57.6 and 42.9, respectively. Larval mortality in one-on-one quality assay was 50% and in exposure time assay it was 50% at 19 minutes. With the results of infectivity assays we can evaluate the possibility to use this new strain in biological control programs.

INTRODUCTION

Entomopathogenic nematodes (EPN) in the genera *Heterorhabditis* and *Steinernema* are capable of controlling a wide variety of economically important insect pest worldwide (KAYA *et al.*, 2006). In the entomopathogenic nematode infection process, infective juveniles (IJs) penetrate into the insect haemolymph and release the symbiotic bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) that kill the insect host, usually in short time.

EPN have been used successfully as biological control agents to suppress insect populations (SHAPIRO-ILAN *et al.*, 2002). At present, mainly non-native strains have been used in nematode applications, but these strains may not be well adapted to environmental conditions, whereas native species are adapted to local climatic conditions and have therefore more likely to survive after application.

During a survey in Veneto region, a new steinernematid strain of *Steinernema carpocapsae* were found in soil samples collected. The objectives of this study were (i) to isolate and identify EPN, (ii) to characterize at molecular level the Italian isolate, (iii) determine their survival and infectivity to *Galleria mellonella* larvae using different types of laboratory bioassays. This information will serve as basic knowledge to develop new biological control agents from native insect parasitic nematodes.

MATERIALS AND METHODS

ISOLATION OF EPN FROM SOIL

A survey was conducted on September 2013 in a site located at farm Vallevecchia of Veneto Agricoltura (45°37'51.21"N-12°58'10.29"E), in Caorle (Venezia) in lagoon plain at 1 m a.s.l. The mean annual air temperature of the site is 13.5°C with average maximum temperatures in July-August (28.4°C) and average minimum temperatures in January (-0.8°C). The mean annual precipitation is 806 mm concentrated in October (109 mm). The soil texture was classified as silt-loam with 19.5% (standard error 0.38) of sand, 63.4% (0.19) of silt and 17.1% (0.23) of clay. Until 2008, a rotation based on maize and sorghum was applied. Three different set-aside regimes were established in 2008. The total area of three plots was 1.5 ha (0.5 ha per plot) with ecotonal zone on one side for each. The fields of the three set-aside managements were covered by mowing on May, on July or none, respectively.

For each location, in each plot, 6 scores were randomly sampled and then mixed to form one composite sample of approximately 2 Kg each. The soil was transported in sterile polythene bags to the laboratory and 5-6 *Galleria mellonella* L. (Lepidoptera, Pyralidae) larvae were inserted in a long-handled tea infuser in the middle of each sample (TARASCO & TRIGGIANI, 1997). Then the samples were incubated at 25°C for 7 days in the laboratory of Namatology of the C.R.A. – Research Centre for Agrobiology and Pedology, Florence (Italy). Then died

Galleria larvae were placed individually in modified White traps (KAYA & STOCK, 1997) and juveniles emerged from the cadavers were collected from water solution and established in a laboratory culture in 50 ml plastic tubes. Some of them were transported to the Institute of Sustainable Plant Protection (IPSP)-CNR, Bari (Italy) for molecular characterization, and some infective juveniles were used to fresh *G. mellonella* larvae to produce nematodes (IJs and males) used for identification.

MORPHOLOGICAL IDENTIFICATION

Nematodes were heat-killed in warm water at 60°C and fixed in triethanol-amine-formalin (TAF) (COURTNEY *et al.*, 1955), processed to glycerin by a modification of a glycerin-ethanol series of Seinhorst's (1959) rapid method and then permanently mounted in anhydrous glycerin on microscope slides.

Specimens were examined and measured with a Leitz ORTHOPLAN light microscope at up to 1000 magnification. IJs and males were identified using morphological and morphometric characters based on NGUYEN & SMART (1996) and NGUYEN & HUNT (2007).

MOLECULAR ANALYSIS

Specimens of *S. carpocsae* for molecular analysis were collected from *Galleria* larvae and directly processed. Genomic DNA was extracted from fifteen individual nematodes as described by DE LUCA *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified by using the forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT -3') (JOYCE *et al.*, 1994) for the ITS region, and by using the 18SnF (5'-TGGATAACTGTGGTAATTCTAGAGC-3') and 18SnR (5'-TTACGACTTTTGCCCGGTTC-3') for the 18S rRNA gene. PCR cycling conditions used for amplification were: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50s, annealing at 55°C for 50s and extension at 72°C for 1 min and a final step at 72°C for 7 min. The size of the amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 l on a 1% agarose gel.

PCR products of the ITS containing region and the 18S rRNA gene from three individual nematodes were purified for sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sequenced in both directions. A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed using to confirm their nematode origins and species (ALTSCHUL *et al.*, 1997). The newly obtained sequences for the ITS were aligned using ClustalW (LARKIN *et al.*, 2007) with default parameters with corresponding published gene sequences of *Steinernema*. Sequence alignments were manually edited using BioEdit in order to improve the default parameters of the multialignment. Outgroup taxa for each dataset were chosen according to the results of previously published data. Phylogenetic trees, obtained for both datasets, were performed with Neighbour-Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods using MEGA version 6 software (TAMURA *et al.*, 2013). The phylograms were bootstrapped 1,000 times to assess the degree of support for the phylogenetic branching indicated by the optimal tree for each method.

The newly obtained sequences were submitted to GenBank with the following accession numbers LN624756-LN624757 for the 18S rRNA gene, LN624758-LN624759 for the ITS region .

For Restriction Fragment Length Polymorphism (RFLP) analysis, ten µl of each product, containing the ITS region, from two individual nematodes of *S. carpocsae* isolate were digested with the following restriction enzymes: *Alu* I (Roche), *Ava* II, *Bam* HI, *Dde* I (Roche), *Hinf* I (Roche), and *Rsa* I (Roche) (5 U of enzyme for each digestion) at 37°C overnight. The digested DNA fragments were loaded onto 2.5% agarose gel and visualized by gel red staining gel. All gel images were stored digitally.

INFECTIVITY ASSAYS

The pathogenicity of the new strain was assessed using a method modified from that proposed by GLAZER & LEWIS (2000). Penetration, exposure time, one on one and sand column assays were performed using last instar *G. mellonella* larvae, with an average weight of 0.2-0.4 g. IJs were stored at 12°C for 3 weeks before use. The first three assay, indicated above, were performed in 12-well plates (COSTAR®, Corning, New York) with filter paper (Whatman No. 1) at the bottom of each one of wells. Sand column assay were performed using plastic containers (40 mm height x 45 mm diameter) contained moist sterile sand (autoclaved). In all bioassays, the incubation periods were carried out in the dark at 24°C.

Penetration assay

A nematode suspension of 4000 IJs ml^{-1} was prepared and with a micropipette a 0.5 µl aliquot was transferred to each one of twelve wells followed by a single last instar *G. mellonella* larva. Larval mortality was recorded daily. After an additional 48h in 6-cm Petri dishes with moist filter paper, cadavers of larvae were dissected. Using a stereomicroscope the number of nematodes established in the host was counted. The penetration rate was expressed as the percentage of the initial IJ inoculum that had invaded the insect host (CAROLI *et al*, 1996).

Exposure time assay

Every single *G. mellonella* larva was exposed to nematodes (400 IJs in 0.5 µl /well) for periods of 20, 40, 60, 120 and 180 minutes (twelve replicates per treatment). At the end of each exposure period, insects were rinsed and transferred to 6-cm Petri dishes with moist filter paper. The mortality was recorded 48 h after initial nematodes exposure and the ET$_{50}$ values (the exposure time of nematodes to insects that are required to achieve 50% insect mortality) was calculated using probit analysis (SPSS 15.0).

One-on-one assay

This assay was conducted using two 12-well plates. In every well was placed only one IJ in 50 µl and one *G. mellonella* larva (MILLER, 1989). Twelve wells with 0.5 µl of deionized water were used as control. The mortality was recorded after 72 h of incubation.

Sand column assay

Fifteen plastic containers (40 mm height x 45 mm diameter) were used for this assay. One last instar *G. mellonella* larva was placed in the bottom and then the container was filled with moist sterile sand. A suspension of 100 IJs in 100 µl was added to the top of each column.

After 24 h, the larvae were removed from the sand and incubated in 6-cm Petri dishes with moist filter paper for a further 3 days. Larval mortality and penetration rate was recorded.

RESULTS

The ItS-CAO1 strain was found in set-aside no mowing regime.

MORPHOLOGICAL IDENTIFICATION

Morphometric data of first generation males and infective juveniles are in table 1.

Males: Body C-shaped when heat-relaxed. Cuticle smooth. Stoma shallow. Pharynx with cylindrical procorpus, slightly enlarged metacarpus, followed by an isthmus and a basal bulb with valve. Nerve ring surrounding isthmus. Excretory pore anterior to nerve ring. Reproductive system monorchic, testis reflexed. Spicules paired, symmetrical, slightly curved. Gubernaculum length *ca* 65% of spicule, boat-shaped in lateral view. Tail short, tip with a mucron and *ca* 70% of anal body diam. long. Bursa absent.

Female: Variable in size (n=10, L: 6493.7 ± 971). Body in form of spiral on heat-relaxation. Anterior region similar to male. Reproductive system amphidelphic, reflexed. Vulva in form of transverse slit. Tail short, conical.

Infective juvenile: Body slender, enclosed in a sheath. Pharynx long and narrow, often degenerate, with valvate basal bulb. Nerve ring localized at 68% of pharyngeal length. Tail elongate and conical.

MOLECULAR ANALYSIS
PCR-RFLP and sequencing analyses

Amplification of the ITS region in *S. carpocapsae* from Italy produced a fragment of approximately 0.8 kb in length for all specimens. A restriction profile of the ITS region from the Italian isolate of *S. carpocapsae* is shown in Fig. I. The restriction patterns with *Alu* I, *Dde* I, *Hinf* I and *Rsa* I enzymes resulted the same of those reported by REID *et al.*, 1997 and SUSURLUK & TOPRAK, 2006. *Alu* I enzyme yielded two polymorphic bands that were not present in the restriction profiles of other *S. carpocapsae* isolates identifying specifically the Italian isolate of *S. carpocapsae* (HOMINICK *et al.*, 1997; NGUYEN, 2003).

The sequences of the entire ITS of *S. carpocapsae* were determined for 2 specimens for the Italian population. No intraspecific differences were found in the sequences of these specimens. The ITS sequences of *S. carpocapsae* from Italy showed an intra-specific variability with the

Table 1 – Morphometrics of Infective juveniles and first generation males of *Steinernema carpocapsae* (ItS-CAO1). All measurements are in μm: mean ± s.d. (range).

Character[a]	Male	Infective Juveniles
n	20	20
L	1608.96 ± 143.3 (1339.5-1845.1)	559.0 ± 23.8 (505.8-597.0)
a	13.8 ± 1.1 (11.3-15.5)	21.3 ± 2.0 (18.7-27.1)
b	11.4 ± 1.0 (9.1-13.9)	4.7 ± 0.4 (4.2-5.5)
c	49.9 ± 4.4 (42.1-59.0)	10.7 ± 0.4 (10.2-11.5)
W	117.2 ± 12.5 (102.3-156.5)	26.4 ± 2.6 (20.1-30.4)
ES	141.9 ± 5.8 (130.9-151.1)	118.5 ± 9.0 (99.7-139.0)
EP	68.0 ± 5.5 (60.2-78.2)	36.2 ± 3.4 (28.2-40.3)
NR	101.0 ± 6.2 (93.3-114.4)	81.3 ± 5.5 (70.0-90.0)
T	32.3 ± 2.0 (29.5-36.1)	52.5 ± 2.7 (48.8-57.0)
ABD	47.1 ± 3.0 (41.8-54.2)	13.4 ± 0.5 (12.7-14.2)
Testis reflex	525.9 ± 59.3 (396.0-624.0)	–
SL	69.4 ± 4.3 (54.8-75.2)	–
Spicule width	13.8 ± 1.3 (11.4-15.1)	–
GL	45.4 ± 1.8 (42.1-48.2)	–
SW	1.5 ± 0.1 (1.3-1.6)	–
GS%	65.7 ± 5.3 (58.3-79.6)	–
D%	48.0 ± 4.2 (41.7-55.1)	30.7 ± 3.7 (23.9-40.1)
E%	211.6 ± 24.1 (166.8-265.1)	69.1 ± 6.4 (53.6-79.0)

[a] L = body length, W = max. body diam, ES = pharynx, EP = excretory pore, NR = nerve ring, T = tail length,
ABD = anal body diam., SL = spicule length, GL = gubernaculum length.
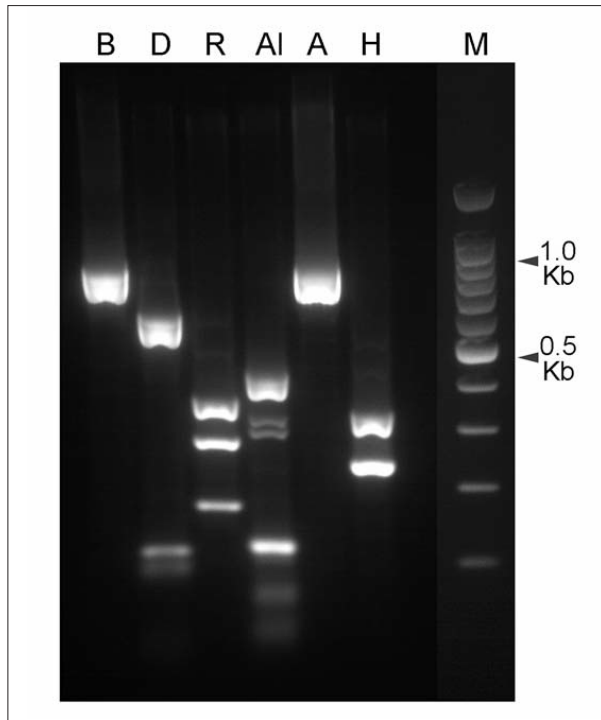Ratios: a = L/W, b = L/ES, c = L/T, SW = SL/ABD, GS% = GL/SL x 100, D% = EP/ES x 100, E% = EP/T x 100

Fig. I - Restriction fragments of amplified ITS containing region of *Steinernema carpocapsae*. Al: *Alu*I, A: *Ava* II, B: *Bam* HI, D: *Dde*I, H: *Hinf* I, R: *Rsa* I and M:100 bp ladder.

corresponding sequences of *S. carpocapsae* present in the database ranging from 0 to 2% (5-16 bp different).

The sequencing of the 18S rRNA gene produced a sequence of 1633 bp. The partial 18S sequence was 99% identical to *S. carpocapsae* present in the database, 1627/1630 identities and 0 gap and 99% similar to *S. monticolum* (FJ040423), 1585/1589 identities and 1 gap.

For phylogenetic analysis of the ITS region, the corresponding sequences of *S. carpocapsae, S. feltiae, S. surketense, S. nepalese, S. tami, S. siamkayai, S. minutum* and *Steinernema* sp. from the database were aligned along with the ITS sequences obtained in this study. Phylogenetic trees generated by Neighbour-Joining, Maximum Parsimony and Minimum Evolution methods showed no significant conflict in branching order and support level, so only ML tree is shown (Fig. II). ML tree supported five clades within *Steinernema*. The Italian isolate of *S. carpocapsae* formed a well supported clade (90% support) with all sequences of *S. carpocapsae*, despite the high sequence variability as reported by SPIRIDONOV *et al.* (2004). The *S. carpocapsae* cluster resulted closely related to *S. surketense, S. nepalese* and *Steinernema* sp. (clade II). Clade III included only one taxon, *S. scapterisci*. Clade IV grouped *S. tami, S. siamkayai* and *S. minutum*. Clade V contained only one taxon, *S. feltiae* from the database, located at the basal position of the tree. Most clades were supported by high bootstrap values giving strong support to these associations.
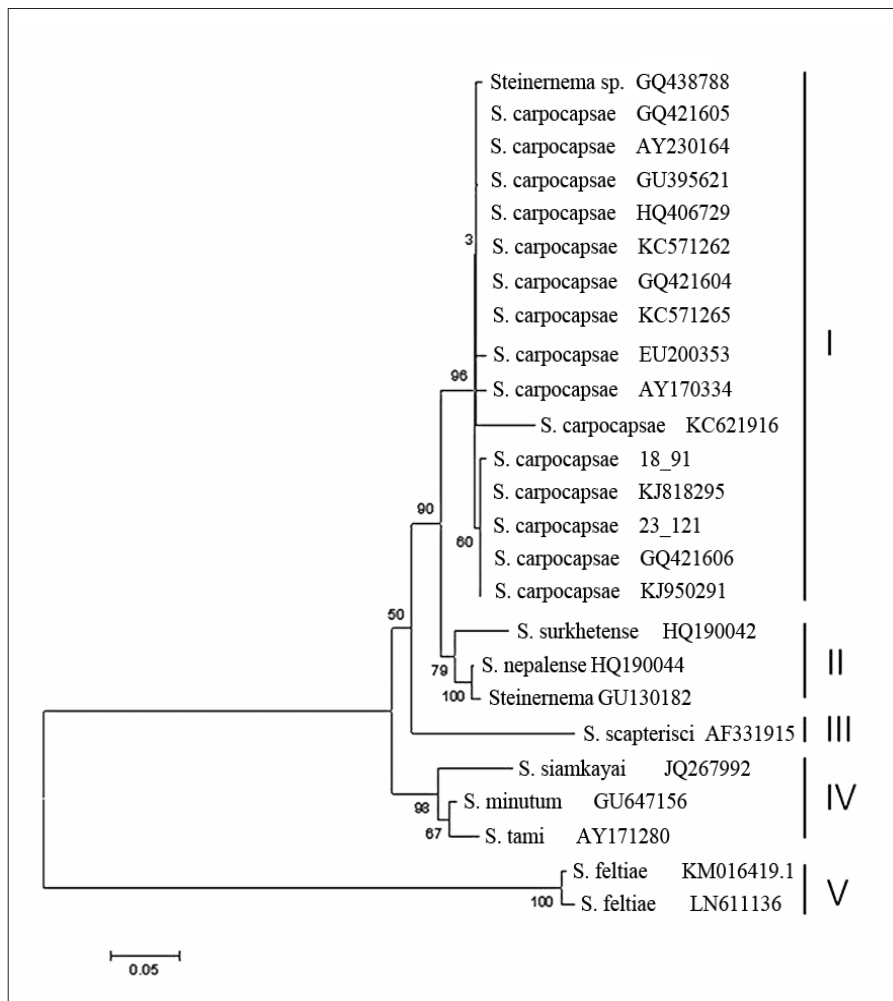

Fig. II - Phylogenetic trees of ITS containing region of *Steinernema carpocapsae* and the closest species. Sequences were analysed using Maximum Likelihood method. Numbers at nodes indicate bootstrap values.

Table 2 – Results of different infectivity assays with *Steinernema carpocapsae* strains.

Bioassay strain	Penetration	Sand column		One-on-one		Exposure time	
	ItS-CAO1	ItS-CAO1	Uk[c]	ItS-CAO1	All[c]	ItS-CAO1	All[c]
Insect mortality (%)	100	93.3	42	50 (33.3-66.6)	50 (45-85)		
Days to die	1.25 ± 0.5 (1-2)	2.3 ± 0.5 (2-3)					
P[a]	57.6 ± 9.5 (40-70)	42.9 ± 8.0 (31-63)	1.5				
ET_{50} (min)[b]						19	65 (50-70)

[a] P: penetration rate (percentage of initial nematodes that invades an insect host)
[b] ET_{50}: exposure time in minutes that is required by nematode to acheive 50% insect mortality
[c] Data from GLAZER & LEWIS (2000)

INFECTIVITY ASSAY

ItS-CAO1 strain caused high levels of mortality of the insect tested, achieving the 100% after 48 hours from the nematodes exposure (Table 2). The ItS-CAO1 penetration rate is higher compared to that reported by CAIROLI *et al.* (1996) at the same conditions. In the exposure time ET_{50} was lower than *S. carpocapsae* All strain. In the one-on-one quality bioassay, ItS-CAO1 virulence was the same to All strain (50%). In sand column bioassays, ItS-CAO1 showed insect mortality (93.3%) and penetration rate greater than UK strain.

DISCUSSION

S. carpocapsae was previously isolated from soil sample in Veneto region (EHLERS *et al.*, 1991) but it was not morphologically or molecularly characterized.

Molecular identification of *Steinernema* species is a prerequisite for proper classification, biodiversity study and their potential use in biological control programs.

DNA sequence and phylogenetic analyses of entomopathogenic nematodes provide additional criteria for identifying and delimiting species within *Steinernema*. Sequence analyses of the ITS and the 18S rRNA gene well supported the identification of *S. carpocapsae* isolate from Italy. In particular, sequence analyses of the ITS containing region has allowed to assess the heterogeneity among isolates of *S. carpocapsae*, and among *Steinernema* species, even for those that are closely related (HOMONICK *et al.*, 1997). This result was also confirmed by RFLP profiles that were in accordance with the patterns reported previously for *S. carpocapsae* (Fig. I) (SPIRIDONOV *et al.*, 2004; SUSURLUK & TOPRAK, 2006). The alignment of the optimised ITS sequences from different species of *Steinernema* spp. revealed multiple insertion and deletion events (indels) among taxa of varying size (1-20 bp) and the longest indels were localized in the ITS1 (data not shown). Furthermore, the multiple alignment constructed revealed several conserved sequence motifs characteristic for each *Steinernema* species.

The phylogenetic analyses with ML, NJ and MP methods yielded congruent phylogenetic trees. Notably, one highly supported group was evident in both analyses, consisting of all *S. carpocapsae* sequences (Fig. II) closely related to *S. surkhetense, S. nepalense, Steinernema* sp. and *S. scapterisci*.

In conclusion, the ITS sequences allowed clear separation of *Steinernema* species in spite of the high intra-specific variability. The alignment revealed small species-specific DNA sequences suitable for the construction of potentially useful species-specific primers or for a more promising approach for DNA barcoding of entomo-pathogenic nematodes.

Screening of EPN species is an important step before initiating mass production or field evaluation. Laboratory studies are essential for maximising the chances of success in field experiments (BEDDING, 1990). The bioassays used in this study examined nematode activity in the infection process. Insect mortality with ItS-CAO1 strain is very high in penetration and sand column assay (100% and 93.3% respectively). Nevertheless, the penetration ability is an important characteristic for EPNs. High penetration rates would increase the infection rates, which in turn increase toxins produced by developing nematodes and their symbiotic bacteria (BURMAN, 1982) and so will enhance septicaemia and kill the insect host more rapidly. In ItS-CAO1 strain Infective juveniles' penetration was 57.6% in penetration assay and 42.9% in sand column assay.

The exposure time assay indicated indirectly how quickly insects were infected by the nematodes (ET_{50} 19 min). In one-on-one assay mortality at 72h was 50%. With the results of infectivity assays we can evaluate the possibility to use this new strain in biological control programs.

ACKNOWLEDGEMENTS

REFERENCES

ALTSCHUL S.F., MADDEN T.L., SCHAFFER A., ZHANG J., ZHANG Z., MILLER W., LIPMAN D.J., 1997- *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs*. - Nucleic Acids Research, 25: 3389-3402.

BEDDING R.A., 1990 - *Logistics and strategies for introducing entomopathogenic nematodes* Neoaplectana *spp* and Heterorhabditis *spp.* - Annals of Applied Biology, 140: 117-120.

BEDDING R.A., AKHURST R.J., 1975 - *A simple technique for the detection of insect parasitic rhabditid nematodes in soil*. – Nematologica, 21: 109-110.

BURMAN M., 1982 - Neoaplectana carpocapsae: *toxin production by axenic insect parasitic nematodes.* – Nematologica, 28: 62–70.

CAROLI L., GLAZER I., GAUGLER R., 1996 – *Entomopathogenic nematode infectivity assay: Comparison of penetration rate into different hosts.* – Biocontrol Sci. Technol., 6: 227-233.

COURTNEY W.D., POLLEY D., MILLER V.I., 1955 - *TAF, an improved fixative in nematode technique.* - Plant Disease Reporter, 39: 570-571.

DE LUCA F., FANELLI E., DI VITO M., REYES A., DE GIORGI C., 2004 - *Comparison of the sequences of the D3 expansion of the 26S ribosomal genes reveals different degrees of heterogeneity in different populations and species of Pratylenchus from the Mediterranean region.* - European Journal of Plant Pathology, 111: 949-957.

EHLERS R.U., DESEÖ K.V., STACKERBRANT E., 1991 – *Identification of* Steinernema *spp. and their symbiotic bacteria* Xenorhabdus *spp. from Italian and German soil.*- Nematologica, 37: 360-366.

GLAZER I., LEWIS E.E., 2000 – *Bioassys for entomopathogenic nematodes.* In: Bioassys of entomopathogenic microbes and nematodes. Navon A. and Ascher K.R.S. Ed., CAB International, Wallingford, UK, pp. 229-247.

HOMINICK W.M., BRISCOE B.R., del PINO F.G., HENG J., HUNT D.J., KOZODOY E., MACREK Z., NGUYEN K.B., REID A.P., SPIRIDONOV S., STOCK P., STURHAN D., WATURU C., YOSHIDA M., 1997 - *Biosystematics of entomopathogenic nematodes: current status, protocols and definitions.* - Journal of Helminthology, 71: 271-298.

JOYCE S.A., REID A., DRIVER F., CURRAN J., 1994 - *Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes.* In: Burnell, AM., Ehlers, R.U. and Masson, J.P. (Eds). Cost 812 Biotechnology: genetics of entomopathogenic nematodes-bacterium complexes. Proceedings of symposium and workshop, St Patrick's College, Maynooth, County Kildare, Ireland. Luxembourg, European Commission, DGXII, pp.178-187.

KAYA H.K., AGUILLERA M.M., ALUMAI A., CHOO H.Y., DE LA TORRE M., FODOR A., GANGULY S., HAZIR S., LAKATOS T., PYE A., WILSON M., YAMANAKA S., YANG H., EHLERS R.U., 2006 - *Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world.* - Biological Control., 38: 134–155.

KAYA H.K., STOCK S.P., 1997 - *Techniques in insect Nematology.* In: Techniques in Insect Pathology. Lacey, L.A. Ed., Academic Press, London, pp. 822-831.

LARKIN M.A., BLACKSHIELDS G., BROWN N.P., CHENNA R., MCGETTIGAN P.A., MCWILLIAM H., VALENTIN F., WALLACE I.M., WILM A., LOPEZ R., THOMPSON J.D., GIBSON T.J., HIGGINS D.G., 2007 – *ClustalW and ClustalX version 2.* - Bioinformatics, 23 (21): 2947-2948.

MILLER R., 1989 – *Novel pathogenicity assessment technique of steinernematids and heterorhabditis entomopathogenic nematodes.* - Journal of Nematology, 21: 574.

NGUYEN K.B., 2003 – *ITS sequence of* Steinernema *cut by 22 enzymes with numbers of cuts and fragments lengths.* In: http://kbn.ifas.utl.edu/MyHTML.htm

NGUYEN K.B., HUNT D.J., 2007 – *Entomopathogenic nematodes: Systematics, Phylogeny and Bacterial Symbionts. Nematology monographs and perspectives.* Brill E.J. Ed., Vol. 5, 816 pp.

NGUYEN K.B., SMART G.C., 1996 – *Identification of entomopathogenic nematodes in the Steinernematidae and Heterorhabditidae (Nemata: Rhabditida).* - Journal of Nematology, 28: 286-300.

REID A.P., HOMINICK W.M., BRISCOE B.R., 1997 - *Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of ITS region of the ribosomal DNA repeat unit.* - Systematic Parasitology, 37: 187-193.

SEINHORST J.W., 1959 - *A rapid method for the transfer of nematodes from fixative to anhydrous glycerin.* – Nematologica, 4: 67-69.

SHAPIRO-ILAN D.I., GAUGLER R., TEDDERS W.L., BROWN I., LEWIS E.E., 2002 - *Optimization of inoculation for in vivo production of entomopathogenic nematodes.* - Journal of Nematology, 34: 343-350.

SPIRIDONOV S.E., REID A.P., PODRUCKA K., SUBBOTIN S.A., MOENS M., 2004 - *Phylogenetic relationships within the genus* Steinernema *(Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8S-ITS2 region of rDNA and morphological features.*- Nematology, 6: 547-566.

SUSURLUK I.A., TOPRAK U., 2006 - *Molecular identification of three entomopathogenic nematodes from Turkey by PCR-RFLP of the ITS regions.* – Phytoparasitica, 34: 17-20.

TARASCO E., TRIGGIANI O., 1997 – *Survey of* Steinernema *and* Heterorhabditis *(Rhabditida: Nematoda) in Southern Italian soils.* – Entomologica, 312: 117-123.

TAMURA K., STECHER G., PETERSON D., FILIPSKI A., KUMAR, S., 2013 - *MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.* - Molecular Biology and Evolution, 30: 2725-2729.