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Biocontrol potential of nematophagous fungi against *Meloidogyne* spp. infecting tomato

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Abstract Root-knot nematodes are the most economically damaging group of plant parasitic nematodes. Intensive agriculture on sandy soil of Moroccan agroecosystems results in a prevalence and wide distribution of *Meloidogyne* spp., limiting both conventional and organic fruit and vegetable production. The aim of this study is to assess the nematicidal potential of twelve local isolates of nematophagous fungi in organic tomato production in laboratory and greenhouse conditions. Fungal isolates were of seven genera: *Paecilomyces*, *Purpureocillium*, *Trichoderma*, *Fusarium*, *Talaromyces*, *Arthrobotrys*, *Dreschslerella*, and *Monacrosporium*. In vitro assays screened the isolates for their ability to immobilize *Meloidogyne javanica* juveniles using 96-well tissue culture plates at a concentration of 10^6 spores.ml^{-1}. The same isolates were tested for their potential to reduce *M. javanica* populations and galling on tomato roots in pot experiments with infested soil, applied upon transplantation at 10^7 spores.ml^{-1}. Average mortality rate of second-stage juveniles of *Meloidogyne* spp. in vitro ranged from 11 to 42%, with a maximal range of 64 to 73% mortality. The highest rates of mortality were recorded after 72 h using *Purpureocillium lilacinum* and *Arthrobotrys oligospora*. In pot experimentation, the reproduction rate of root-knot nematodes ranged from 176 to 5920% with the gall index varying from 2.7 to 4.9 in treated pots. This study identified *Paecilomyces* and *Arthrobotrys* direct nematicidal effect against *Meloidogyne* spp., in laboratory conditions. To achieve successful control, further studies should be conducted to identify the optimal range of environmental factor practices which lead to the enhancement of biocontrol activity of these NF in the field.

Keywords Nematicidal activity · *Paecilomyces* · Root-knot nematodes · *Arthrobotrys*

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Introduction

Root-knot nematodes *Meloidogyne* spp. are among the most economically damaging genera of herbivorous nematodes causing serious losses to vegetables crops on a global scale (Gálvez et al. 2019), due to agricultural intensification and poor agronomic practice (Atandi et al. 2017). Furthermore, root-knot nematodes are ranked highly in the ten most pervasive genera of plant parasitic nematodes (Ebadi et al. 2018; Saxena 2018) and are of greatest importance in organic crops (Hallmann 2013). In fact, organic farming is confronting the same plant parasitic Nematode concerns as conventional farming, because of the limited management options and the ban of synthetic pesticides (Briar et al. 2016). Among the group of plant parasitic nematodes in Moroccan agroecosystems, root-knot nematodes are the most abundant genera and include three species: *M. javanica, M. incognita*, and *M. arenaria*. In the Souss region (Central west of Morocco), *M. javanica* has been reported to be the predominant species in the vegetable cropping systems in over 80% of cases (Janati et al. 2018). Infection by second-stage juveniles of root-knot nematodes leads to formation of giant cells known as galls. The development of root galls disrupts plant nutrition and thus decreases the quality and yield of crops and may interfere with plant disease resistance (Singh et al. 2019).

Organic farming optimized by biological control is one of the most promising alternatives for controlling root-knot nematodes (Abd-Elgawad and Askary 2018; Muneret et al. 2018). Improvements in soil quality and pest control with biological agents reduce the environmental impacts of intensive agriculture (Birkhofer et al. 2008). Natural enemies of nematodes include fungi, bacteria, nematodes, mites, and viruses (Stirling 2014). Due to their potential as biocontrol agents against plant parasitic nematodes, nematophagous fungi have been the subject of interest for many researchers (Nordbring-Hertz et al. 2001) and show potential for application in organic agriculture. Among all natural enemies of nematodes, nematophagous fungi offer the most diverse proliferation of antagonistic organisms. They belong to different taxonomic groups within the fungal kingdom including four main groups which are categorized by their mode of action: nematode trapping, endoparasitic, egg and female parasitic, and toxin-producing fungi (Dong and Zhang 2006; Stirling 2014). Many studies have been carried out to evaluate the nematicidal potential of nematophagous fungi, and their use as biocontrol agents has been demonstrated by numerous researchers. Jamshidnejad et al. (2013) showed that *Arthrobotrys oligospora* and *Trichoderma harzianum* can be efficient bioagents against *M. javanica*. *A. oligospora* is also reported to be an effective potential biocontrol agent against *M. graminicola* (Singh et al. 2012). Furthermore, Kiewnick and Sikora (2006) have found that a strain of *Paecilomyces lilacinus* provided significant control of *M. incognita* on tomato. The fungus *Pochonia chlamydosporia* has been widely assessed for biological control against plant parasitic nematodes and causes a significant mortality to the eggs of *Meloidogyne* spp. (Dalla Pasqua et al. 2020; Nasu et al. 2018).

It is suggested by Elshafie et al. (2006) that few fungi are ideal as biological agents against nematodes with among 70% of fungal genera and 160 species being associated with nematodes. Consequently, a thorough understanding of several factors affecting the efficacy of the chosen fungi and their use as biological control agents (BCA) in soil is necessary. The nature of host-parasite interactions, effect on shelf life, root colonization capacity, soil ecosystem properties (moisture, pH, structure, and temperature), and the specific habitat and target of the biocontrol agent (Spiegel and Chet 1998) require particular attention. Isolation and identification of locally suitable isolates are recommended for nematode management approaches rather than the use of foreign bioagents that are less adapted to local climates and conditions which result in a limited success and highly variable results (Radwan et al. 2012).

The present study aims to evaluate the nematicidal activity of some nematophagous fungi previously isolated from olive nurseries (Aït Hamza et al. 2017) in both in vitro and in vivo settings.

Materials and methods

Molecular characterization of fungi

To establish a taxonomic profile of selected strains, sequence analysis of the ITS (internal transcribed spacer) region in the ribosomal RNA gene cluster were carried out. Mycelia were harvested from petri dishes of fresh strains, and the genomic DNA was extracted using the NucleoSpin®Plant II Genomic DNA Purification Kit (Promega®) according to the manufacturer's

instructions. The ITS rDNA gene cluster was amplified using the primers ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′) (White et al. 1990). The PCR amplification was carried out using the GeneAmpR PCR System 9700 (Applied Biosystems®). The length, quality, and quantity of PCR products were confirmed using gel electrophoresis (1% w/v). The same ITS primers were used to sequence PCR products. Species identification and verification or genes affiliation of collected isolates was performed by BLAST similarity search in the non-redundant nucleotide database of the GenBank (Altschul et al. 1997). The phylogenetic tree was obtained by using data from one of three equally parsimonious trees through 1000 bootstrap replicates.

Nematode inoculum preparation

The inoculum of the root-knot nematode *M. javanica* was obtained from pure culture raised by single egg mass and maintained on roots of tomato plants in the Laboratory of Biotechnologies and Valorization of Natural Resources, Ibn Zohr University. Egg masses were hand-picked from infected roots using a Binocular microscope (× 40) and then crushed with a bamboo sliver, and the J2 were collected after 48 h of filtration through a paper sieve, and the suspension of J2 was then adjusted to 50 larvae/200 µl for immediate usage.

Fungal inoculum preparation

The 12 isolates of fungi were obtained from the Laboratory of Microbial Biotechnology and Plant Protection, Ibn Zohr University, and were previously isolated by Aït Hamza et al. (2017). The fungi were cultured on Potato Dextrose Agar (PDA) at 25 °C for mass production over 7 to 10 days. After incubation, spore suspension was prepared and spores were separated from mycelia by sieving through two-layered gauze. Spore concentration was determined with the aid of a haemocytometer and adjusted to 10^6 spores.ml^{-1} with sterile distilled water.

In vitro pathogenicity tests

A volume of 200 µl of *M. javanica* suspension containing approximately 50 s stage juveniles (J2) was pipetted into each well of 96-well culture plate containing 100 µl of a spore suspension, already incubated for 7 days and 100 µl of water-agar medium (2% w/v). The control treatment comprised only J2 of *M. javanica* incubated in water-agar medium. Each treatment was replicated five times with one control for each plate. The culture plates were incubated at 25 °C. The number of trapped and immobile J2 was counted as dead after 24, 48, and 72 h from the day of incubation. The mortality percentage of J2 in each plate was calculated according to the Abbott formula (Abbott 1925):

$$MP\% = (MPt–MPc)$$

- MP: Corrected percentage of second-stage juvenile mortality
- MPt: Percentage of second-stage juvenile mortality treated with fungus (treatment)
- MPc: Percentage of second-stage juvenile mortality in water (control treatment)

In vivo pathogenicity tests

Fungal isolates were tested in greenhouse experiments in order to compare the effect of fungi in two different sets of conditions (in the laboratory with optimal conditions and in greenhouse soil environments with a variety of physical, biological, and chemical factors).

Pathogenicity test of selected fungal strains was performed using the concentration of 10^7 spores.ml^{-1} for each strain. A mixture of sterile peat and naturally infested sandy soil (pH: 7.5, OM: 2.53%, EC: 344.4dS/m, N: 1.1%) (2:1, v/v) sampled from an infested tomato greenhouse was prepared. The infested substrate was distributed in plastic pots of 1000 cm^3 filled to 2/3 of the height. A single 3-week-old grafted tomato (*Solanum lycopersicon*, cv. Calvi) seedling with 3–4 true leaves was transferred to each pot which received a volume of 350 ml of each fungal suspension or water for the control. The treatments were as follows: (i) soil treated with fungal suspensions, (ii) negative control (infested soil without fungal treatment), and (iii) chemical control (infested soil with chemical nematicide Solvinova, abamectin (4 l/ha)). Five replicates for each treatment were performed, and each replicate was represented by two plants (as one experimental unit). A total of 140 pots were arranged in a randomized block design. The experiment was conducted in an

experimental greenhouse, where temperature and luminosity were not controlled, for a period of 5 months. The biocontrol potential of nematophagous fungi was assessed at the end of the experiment based on root galling index GI (0–5) according to Taylor and Sasser (1978) and root-knot nematode reproduction rate. Nematodes extraction was performed using a modified Baermann funnel.

Statistical analysis

Data from in vitro pathogenicity test and the greenhouse experiment were analysed, and the variances were tested for their homogeneity and subjected to analysis of variance (ANOVA) using STATISTICA software version 6.1. Mean values of gall index and reproduction rate were compared using Duncan tests at $p < 0.05$, and box plots for in vitro pathogenicity were prepared using R language (R3.5.1 version, Readxl, base and survival packages).

Results and discussion

Molecular characterization of the fungi

The selected fungi for the tests were characterized molecularly, and a phylogenetic tree was formed. The BLAST test showed that the ITS sequences of all sequenced strains were at least 99% similar to the corresponding GenBank reference sequences (Altschul et al. 1997). The chosen species of this investigation are shown in Table 1. The phylogenetic analysis including ITS sequences of the selected isolates revealed ten distinct species belonging to seven genera: *Paecilomyces*, *Purpureocillium*, *Trichoderma*, *Fusarium*, *Talaromyces*, *Arthrobotrys*, *Dreschslerella* and *Monacrosporium* (Fig. 1).

In vitro pathogenicity test

Addition of the juveniles of *Meloidogyne* spp. to the fungal culture resulted in significant reduction of living juveniles after 3 days of inoculation (Table 2). The average percentage of observed mortality ranged from 11 to 42%, and the low percentage ranged from 0 to 6% including 7 isolates of fungi. Two isolates including one strain of *Purpureocillium lilacinum* and one strain of *Arthrobotrys oligospora* parasitized a high percentage of *M. javanica* (Fig. 2) and led respectively to 73.50% and 65.45% as mortality percentage after 72 h of incubation. The most effective fungus was a strain of *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) known as the most investigated biological control agent against nematodes (Kiewnick et al. 2011). It is one of the important nematode egg parasitic fungi reported to parasitize species of *Meloidogyne* spp., *Globodera* spp., and *Heterodera* spp. (Cannayane and Sivakumar 2001). *P. lilacinus* is considered to be a nematode egg parasite able to destroy the egg shells of the root-knot nematodes and infect juveniles (J2) (Bonants et al. 1995; Holland et al. 2003; Khan et al. 2004). In vitro study of this fungal isolate showed high larvicidal potential with a corrected mortality percentage of 73%. This result concords with the in vitro bioassay of *Paecilomyces* 6029 culture filtrate tested by Sharma et al. (2014) against *M. incognita* and Huang et al. (2016) who evaluated the ovicidal and larvicidal effect of *P. lilacinum* and other organisms against *M. incognita* on cucumber. Moreover, even with the specificity of parasitizing egg of *Meloidogyne* spp., this *Paecilomyces* isolate had an effect against J2 of *Meloidogyne*, which relates to its capacity of producing nematicidal metabolites (Cayrol et al. 1989; Degenkolb and Vilcinskas 2016; Li et al. 2007). *Arthrobotrys oligospora* ranked as the second most efficient fungus in this in vitro study. It is considered to be a nematode trapping fungus with characteristic organs for capturing nematodes (Niu and Zhang 2011) such as adhesive hyphae, branches, nets and knobs, and non-constricting and constricting rings (Cumagun and Moosavi 2015). After 24 h of inoculation, traps of *A. oligospora* were induced and the three dimensional hyphal nets were observed capturing J2 of *M. javanica*. After 72 h, 65.45% of J2 were trapped/killed, concurrent with Singh et al. (2012), Jamshidnejad et al. (2013), and Mostafanezhad et al. (2014). The trapping fungus (*A. oligospora*) is also known to produce active secondary metabolites able to (i) spread out the nematicidal activity or (ii) control the formation of trapping organs (Degenkolb and Vilcinskas 2016).

In vivo pathogenicity test

Comparison of untreated tomato roots and after those after 150 days of fungal inoculation, none of the treatments showed significant effects on the nematode gall index or on final population densities even though they

Table 1 BLAST results of ITS rDNA sequences of the nematophagous fungi selected

Code UIZ	GenBank reference strains				
	Species	Strain	GenBank accession no.	Number of nucleotides	Maximum similarity (%)
UIZFSA-31	*Talaromyces assiutensis*	KF147920	ph721	537	99
UIZFSA-55	*Monacrosporium thaumasium*	MTU51972	U51972	549	99
UIZFSA-2	*Paecilomyces lilacinus*	MY683	GU980015	541	99
UIZFSA-18	*Arthrobotrys scaphoides*	CBS 226.52	KF494006	565	99
UIZFSA-15	*Purpureocillium lilacinum*	SBTPl-001	KF766523	625	99
UIZFSA-97	*Arthrobotrys brochopaga*	ABU72609	U72609	525	100
UIZFSA-102	*Trichoderma hamatum*	ARC2	MN533707	550	100
UIZFSA-27	*Trichoderma harzianum*	SZMC 20965	KP316410	567	100
UIZFSA24	*Trichoderma asperellum*	SI14	KJ432865	548	100
UIZFSA-5	*Purpureocillium lilacinum*	DF58	KT582081	713	99
UIZFSA-100	*Arthrobotrys oligospora*	AOZ1	X94121	497	100
UIZFSA-103	*Trichoderma asperellum*	SD-5	KY807766	542	100
UIZFSA-100	*Arthrobotrys oligospora*	AOZ1	X94121	497	100

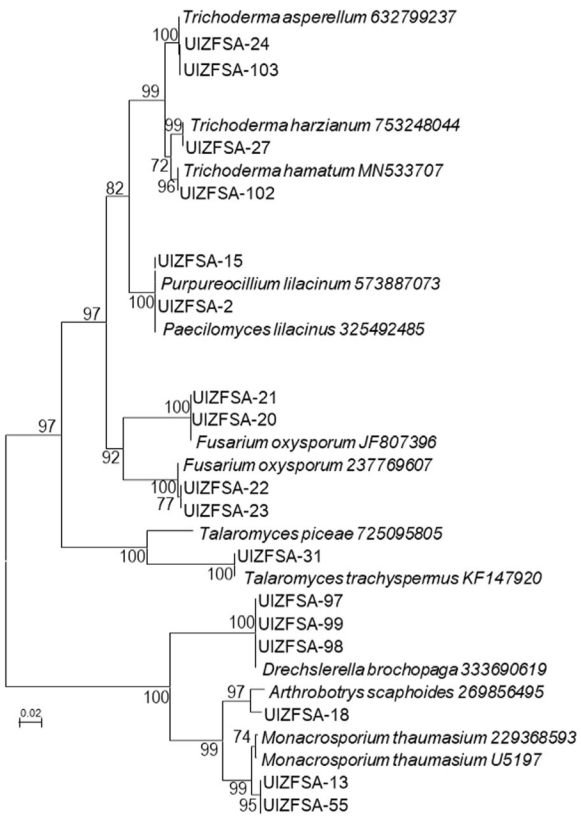

Fig. 1 The selected nematophagous fungi and their closest GenBank match with accession numbers. The numbers represent bootstrap values

ranged between 0 and 4.90 for the gall index and from 176 to 5920 for the reproduction rate. The only significant result was the gall index of the chemical control ($p < 0.05$), which shows that the chemical nematicide in our study was more effective than the fungal treatments (Table 3). The present study is, to our knowledge, the first investigation in Morocco testing endogeneous nematophagous fungi both in the well culture plates and in the greenhouse conditions on a tomato crop. The non-effectiveness of nematophagous fungi treatments may be due to the following: (i) the short period between nematophagous fungi application and transplantation; an increase of this period will allow the fungi sufficient time to grow, invade soil, and reduce nematodes population in soils. Timing of inoculation is crucial for an important nematicidal effect, and Spiegel and Chet (1998) have confirmed this in an experiment in which juveniles of *M. javanica* were exposed to *Trichoderma* spp. for 18 days before planting with maximum nematicidal efficacy. Moreover, Kiewnick et al. (2011) demonstrated that the application of *Paecilomyces* strain PL251 to the soil 6 days before transplanting tomato resulted in a high level of *M. incognita* control; and (ii) soil biodiversity including other microorganisms which could have antagonistic effect against the nematophagous fungi added to the soil. In fact, according to Cooke (1968), the chance of establishing a balance to the introduced species of

Table 2 The one-way ANOVA table of pathogenicity test in vitro

Source of variation	DF	Sum of squares	Mean square	F-statistic	*p* value
Strain	13	31,422	2417	14.95	0.000
Error	37	5982	162		
Total	50	37,404			

nematophagous fungi in the soil is small. Furthermore, fungi are poor saprobic competitors in the soil, and other soil organisms display a highly antagonistic action against them (Swe et al. 2011).

A promising strategy for efficient control by nematophagous fungi consists of simultaneous application of two or more compatible nematophagous fungi that may be effective in synergy as recommended by Hashem and Abo-Elyousr (2011). Mixing of *P. lilacinus* and *Pochonia chlamydospora* resulted in 75% mortality of juveniles of *M. incognita* and *M. mayaguensis* (Ortiz Paz et al. 2015). Huang et al. (2016) have reported that the combined use of the filamentous fungi *Syncephalastrum racemosum* and *P. lilacinus* was more effective at controlling *M. incognita*.

In our study, in vitro results showed the high efficiency of the two fungi *P. lilacinum* and *A. oligospora*; however, the in vitro study performed by Aït Hamza et al. (2017) showed that *Talaromyces assiutensis* is the most effective fungus, although it only represented 10% of mortality in the present experiment. This demonstrates that the activity of the nematophagous fungi can change under specific environmental conditions that could affect the stability of fungal biocontrol efficacy. Although the antagonistic metabolites were not tested, it is highly likely that they were involved in

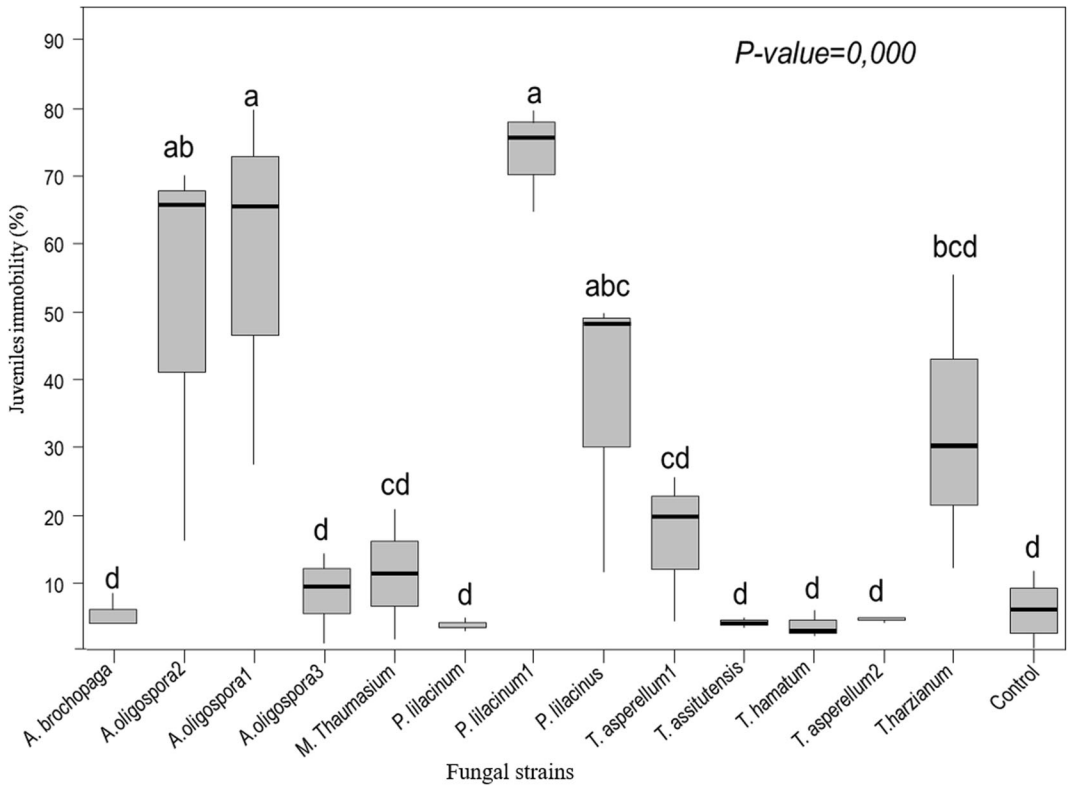

Fig. 2 Corrected juvenile mortality (%) of fungal isolates tested in vitro (treatments with the same letters are not significantly different, $p < 0.05$)

Table 3 Effect of fungal isolates on root galling and reproduction rate on tomato in the greenhouse pot experiment

Treatments	Fungus	Gall index (0–5)	Reproduction rate
HT8	*Talaromyces assiutensis*	2.70[b]	176[a]
HT22	*Monacrosporium thaumasium*	2.80[b]	230[a]
HT30	*Paecilomyces lilacinus*	3.20[b]	458[a]
HT4	*Arthrobotrys oligospora*	3.30[b]	1112[ab]
HT32	*Purpureocillium lilacinum*	3.40[b]	1940[ab]
HT24	*Arthrobotrys brochopaga*	3.70[b]	2079[ab]
HT58	*Trichoderma hamatum*	3.90[b]	3499[ab]
HT13	*Trichoderma harzianum*	4.00[b]	3589[ab]
HT14	*Trichoderma asperellum*	4.00[b]	4379[ab]
HT21	*Purpureocillium lilacinum*	4.10[b]	4774[ab]
HT1	*Arthrobotrys oligospora*	4.20[b]	11,312[ab]
HT61	*Trichoderma asperellum*	4.70[b]	12,089[b]
Negative control	–	4.90[b]	592,086[ab]
Positive control	–	0.00[a]	0[a]

Superscript data are means of five replicates. Values reported in columns followed by the same letter are not significantly different at $P < 0.05$

juvenile mortality, due to nematophagous fungi metabolites synergistic interactions as a whole. In vivo results were non-significant, and this may have been due to the experiment being conducted under greenhouse conditions in summer, where plants were exposed to high temperature, favourable for the development and damage potential of root-knot nematodes, which plays a role in the interaction between biocontrol agents and nematodes. Greenhouse temperature is one of the factors that affects the efficacy of the fungi in soil in our experiment. In soil parameters of this study, soil pH was normal (7.5), and it has been shown that acidic conditions enhance conidia production, mycelia growth, and antagonistic activity of some fungi (Duffy et al. 1997). The low amount of nitrogen (1.1%) and organic matter (2.53%) led to a decreased antagonism of fungi and thus influenced its performance (Duffy et al. 1997; Widmer et al. 2002). Furthermore, the strains used in this study were isolated from an olive soil ecosystem and tested against root-knot nematodes infesting tomato plants. Stirling et al. (1979) found that even if the fungus *Dactylella oviparasitica* was responsible for reduction in the root-knot nematode (*M. javanica*) populations and was the primary factor responsible for the nematode-suppressive soil, it was ineffective in control of the same root-knot nematode species when it occurred on roots of tomato and grape. Apparently, similar to our experiment, the higher reproductive ability of *M. javanica* in its preferable host negates fungal efficacy (Sayre and Walter 1991).

Conclusion

Among the tested nematophagous fungi, *Paecilomyces lilacinus* and *Arthrobotrys oligospora* equate an efficient direct nematicidal potential against *Meloidogyne javanica*. However, application of such fungi as bioagents does not guarantee their effectiveness to control bioagressors in the soil in protection of tomato. Loss of efficiency results under some environmental conditions and agronomic practices. Thus, specific conditions leading to optimal benefit from these fungi as a viable component in control of root-knot nematodes, especially in organic vegetable farming, should be known. Further studies aiming at the evaluation of the selected nematophagous fungi against root-knot nematodes in greenhouse conditions should (i) identify optimal environmental conditions including the dynamic of soil physicochemical characteristics, air/soil particle density, and humidity and temperature range for the host plant and its microbiome; (ii) determine the suitable practices promoting nematophagous fungi growth and effectiveness such us the period between soil treatment with fungi and transplantation; and

(iii) determine synergetic/antagonistic effect when selected nematophagous fungi strains are in combination together or with other biological control agents or soil microbiota.

Code availability Not applicable.

Data availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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