

Diversity of nematophagous fungi in Moroccan olive nurseries: Highlighting prey-predator interactions and efficient strains against root-knot nematodes

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21 Abstract

Plant-parasitic nematodes are major pests in agriculture. Microbial antagonists such as 22 nematophagous fungi are of great interest for developing alternative biocontrol strategies to 23 replace nematicide use. This study aims to isolate, identify and assess the diversity and the *in* 24 vitro predatory activity of nematophagous fungi from various olive nurseries in Morocco. A 25 total of 305 soil samples were collected in 25 nurseries in Moroccan olive-producing areas 26 (Jbala, Guerouane, Haouz and Souss). Seventy-three pure fungal strains from nematophagous 27 fungi were recovered from nursery substrates infested with root-knot nematodes. Diversity 28 indices showed that fungi were more numerous in southern regions, whereas fungal 29 communities were more numerically alike in northern regions, partly depending on the 30 occurrence of root-knot nematodes. One fungal strain, Talaromyces assiutensis, killed all 31 Meloidogyne javanica juveniles during in vitro predation tests. Orbiliaceae (Arthrobotrys 32 33 spp., Drechslerella brochopaga, Monacrosporium spp.) trapped 50 to 80% of juveniles. Purpureocillium and Trichoderma species killed 30 to 50% of juveniles. The diversity of 34 35 nematophagous communities could be affected by the north-south climate gradient of Morocco, by their nematode prey expansion, and, to a lesser extent, by the habitat origin of 36 the substrates used. Talaromyces assiutensis was the most efficient nematophagous species 37 against *M. javanica*. The use of native nematophagous fungi is a promising alternative 38 strategy for nematode control in olive nurseries and to prevent nematode introduction into 39 olive orchards. 40

41 **Keywords:** Culture substrate, Ecology, Fungal antagonists, Olive tree, Root-knot nematode.

43 1. Introduction

The olive tree (*Olea europaea* L.) is widely distributed throughout the Mediterranean Basin. In Morocco alone, the olive-growing area is estimated at 650,000 ha and produces 120,000 tons of oil and 150,000 tons of table olives per year (Ministry of Agricultural and Marine Fisheries, 2009). Planting material comes from several nurseries distributed throughout the olive-producing areas and olive plantlets are certified free of pathogens (e.g., *Verticillium dahlia*) and parasites (e.g., plant-parasitic nematodes). Nevertheless, standard health practices are not applied in all nurseries and seasonal and informal nurseries coexist.

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52 Several plant-parasitic nematode species (PPN) have been found to be associated with olive trees (Castillo et al., 2010; Ali et al., 2014; Hamza et al., 2015), including root-knot 53 nematodes (RKN, *Meloidogyne* spp.) that were shown to impact olive growth (Afshar et al., 54 2014). RKN have been reported to be major pests on olive trees, mainly in nurseries where 55 irrigation conditions are favorable to their multiplication (Nico et al., 2002; Sanei and 56 Okhovvat, 2011). Moreover, most RKN infestations in olive orchards result from 57 contaminated plant material produced in uncertified nurseries (Nico et al., 2002). To protect 58 nurseries, vegetative propagation of olive is a crucial step to prevent plant pest dispersal. 59 60 While some industrial olive-producing countries use synthetic substrates to cultivate olive trees, other countries use more traditional olive cultivation techniques, planting roots in soil 61 substrates. In countries that have implemented large-scale planting programs (e.g., 1,220,000 62 ha in the Morocco Green Plan, 2015), the "nursery risk" is a concern, with two main 63 consequences: (i) the weakening of olive plants before their transplantation into orchards; and 64 (ii) the introduction of pathogens into orchards. The nematode risk is now controlled in 65 nurseries, either by multiplication of resistant varieties (Palomares-Rius et al., 2012) or by 66 67 inoculating microbial antagonists in the substrates, such as mycorrhizae (Castillo et al., 2006).

Thus, in view of the large-scale development of olive cultivation in Morocco, sanitization of 68 the substrates must be a priority. Some previous studies highlighted that certain substrates can 69 be suppressive depending on their composition, either because of microbial antagonisms or of 70 toxicity. For example, the addition of forest residues provides organic matter that could 71 contribute to PPN suppression by increasing soil microflora (Rodriguez-Kabana, 1987). 72 Composted dry cork, especially in nurseries, could be effective for *M. incognita* suppression 73 due to the toxicity of the products released (e.g., ammonia, phenolic compounds) (Nico et al., 74 75 2004). Moreover, the introduction of microbial antagonists into substrates would strengthen the sustainability of preventive techniques and help introduce biocontrol agents into orchards. 76

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Microbial antagonists of PPN include nematophagous fungi (NF), raising expectations for 78 their use in integrated pest management (Waller, 2006; Larsen, 2006; Maingi et al., 2006). 79 80 These fungi act by antibiosis, parasitism or predation (Imerglik, 1981; Gaspard and Mankau, 1986). Fungi trap nematodes using adhesive networks or buttons. Some NF capture their prey 81 82 with constrictor rings before strangling them and others with hyphae networks that produce 83 sticky compounds. The specific enzymes then digest the nematodes. Recognition and attachment of the mycelium to the nematode cuticle are mainly due to compatible 84 85 glycoproteins such as lectins (Nordbring-Hertz et al., 2006).

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Nevertheless, edaphic factors such as soil pH, temperature, moisture and structure influence
their efficiency (Brown and Kerry, 1987). Fungal diversity can increase in stressed or polluted
sites (Korol et al., 1994) in order to adapt to changing environments (West et al., 1999). In *Arthrobotrys oligospora*, genetic diversity varies according to environmental conditions.
Their strains could generate recombinant genotypes by crossing with native strains, thus
enhancing their environmental adaptability and parasitizing ability (Zhang et al., 2013).

However, the reproductive strategies of fungi like *A. oligospora* can change depending on the
population. According to Cook (1993), the search for microorganisms from the rhizospheric
soil of a specific crop could lead to the isolation of effective antagonists against pathogens
that could be adapted to the plant species as well as to particular environmental conditions.

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Considering that olive seedlings in Morocco are generally cultivated in non-sanitized 98 substrates consisting of soil material from various habitat origins, the recovery of native NF 99 100 from substrates may be of great interest in order to develop RKN biocontrol agents adapted to nursery conditions. In this context, this work aims to (i) evaluate the nematode populations in 101 substrates from olive nurseries in Morocco; (ii) isolate and characterize NF able to control 102 RKN (Meloidogyne spp.); (iii) determine the occurrence and diversity of nematode-associated 103 NF and discuss prey-predator interactions; and (iv) investigate their in vitro predatory 104 105 potential towards M. javanica juveniles and eggs, and their usefulness as biocontrol agents for olive protection in nurseries and in orchards. 106

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2. Materials and methods

109 2.1. Site description and olive plantlet sampling

Soil samples were collected in spring 2013 and 2014 from 25 commercial olive nurseries located in the main olive-producing areas in Morocco (Fig. 1 and Table 1): the Jbala, Guerouane, Haouz and Souss regions. The nurseries were selected for their plantlet production, the cultivars grown and the rearing substrates used.

Olive plants are grown in 2-to-3 liter plastic bags filled with solid substrates from different sources (alluvial sandy soils, forest soils, loamy open-field soils) supplemented with different proportions of sand, peat fertilizer and animal manure. Plants are first grown in plastic greenhouses and then outside. They are watered by sprinklers and fertilized with Osmocote® 118 (Everris CompanyTM).

In each nursery and for each variety, five olive plantlets (*Olea europaea* subsp. *europaea*) growing in plastic bags were sampled. Information about the origin and the preparation of the growth substrates and about the cultivars was recorded. A total of 305 olive plantlets were collected and maintained in the laboratory and kept under greenhouse conditions (12 h light at 25 °C; 12 h dark at 20°C).

124 2.2. Nematode extraction and quantification

A 250-cm³ substrate subsample was removed from the rhizosphere of each olive plantlet and used for nematode extraction using the Oostenbrink (1960) elutriation procedure (ISO 23611-4). Free-living nematodes (FLN) and plant-parasitic nematodes (PPN), which specifically exhibit a stylet for plant-cell feeding, were enumerated in 5-cm³ counting chambers (Merny and Luc, 1969) under a stereomicroscope (×60 magnification). Nematode population levels were expressed as the number of individuals per dm³ of fresh substrate. Among PPN, rootknot nematodes belonging to the *Meloidogyne* genus (Mai and Mullin, 1996) were counted.

132 2.3. Isolation of nematophagous fungi

NF were isolated from solid substrate samples infested with RKN using the soil sprinkling 133 134 technique (Duddington, 1955) as modified by Santos et al. (1991). The direct soil powdering on media was preferable to the modified Baermann method (aqueous soil suspensions) 135 (Hernández-Chavarría and Avendaño, 2001) because it provided more concentrated material 136 137 for fungal isolation. Moreover, isolation success was increased by sprinkling cold soil (stored at 4°C) on culture medium at 37 to 40°C (Davet and Rouxel, 2000). Substrate aliquots from 138 each olive plantlet were spread on a tray to be air-dried. One gram was then sprinkled on the 139 surface of Petri dishes containing water-agar (WA 2% w/v) supplemented with antibiotics 140 (0.05% streptomycin-sulphate and 0.05% chloramphenicol). Three replicates were done per 141 142 olive plantlet. A 1-mL suspension containing approximately 3,000 M. javanica second-stage

juveniles (J2) and 10 eggs produced in the laboratory was added as fungal bait, according to 143 the procedure used by Drechsler (1941). The Petri dishes were sealed with Parafilm and 144 incubated at room temperature (20-27°C) in darkness for two months. Petri dishes were 145 examined daily during the first week of incubation and weekly thereafter. Fungi that had 146 developed were isolated under a microscope (x60 magnification). Conidia or pieces of agar 147 bearing either an NF mycelium or an infected nematode were transferred onto Petri dishes 148 containing Corn Meal Agar (CMA) or Potato Dextrose Agar (PDA) supplemented with 149 150 antibiotics (0.05% streptomycin-sulphate and 0.05% chloramphenicol). The fungi were transferred to a new culture medium plate for purification after reaching full growth. Pure 151 cultures were maintained on PDA or CMA plates at 4°C in darkness for a maximum of six 152 months. 153

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2.4.

Morphological and molecular characterization of the fungi

The initial identification of the fungi was based on colony morphology and microscopic characteristics. Slide sub-cultures from pure NF cultures were observed under a dissecting microscope (up to x100 magnification). Genera and species were assigned according to specialized morpho-taxonomical keys (Cooke and Dickinson, 1965; Haard, 1968; Barron, 1977; Yu et al., 2014; Philip, 2002).

Sequence analyses of the ITS (internal transcribed spacer) region in the ribosomal RNA gene 160 cluster were performed to confirm the identity of the NF species. The DNA was extracted 161 from 50-200 mg of mycelium (fresh weight) using the NucleoSpin[®]Plant II Genomic DNA 162 Purification Kit (Promega[®]) according to the manufacturer's instructions. PCR reaction was 163 performed according to the method described by White et al. (1990): the ITS rDNA gene 164 cluster was amplified using the primers ITS1 (5'TCC GTA GGT GAA CCT GCG G 3') and 165 ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3'). The PCR amplification was carried out 166 using the GeneAmpR PCR System 9700 (Applied Biosystems[®]). Twenty µl of reaction 167

mixture contained 2 µl (10 ng) of template DNA, 1 µl of each ITS1 and ITS4 primer (10
mM), 4 µl PCR buffer, 2.4 µl MgCl₂ (25 mM), 0.6 µl dNTPs (10 mM), 0.1 µl BSA (0.1
mg/ml) and 0.2 units of GoTaq[®] DNA polymerase.

PCR cycling conditions consisted of an initial denaturation step at 94°C for 3 min, 35 cycles
of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 10
min. PCR products were checked for length, quality and quantity by agarose gel
electrophoresis (1% (w/v) in 0.5x Tris-Acetate-EDTA (TAE).

PCR products were sequenced from both ends by Eurofins MWG GmbH (Ebersberg, 175 Germany), using the same ITS primers. CHROMAS LITE v2.1.1 (Technelysium Pty Ltd.) 176 software was used to edit and assemble DNA sequences. BLAST similarity searches were 177 performed in the non-redundant nucleotide database of GenBank (Altschul et al., 1997) to 178 identify/verify species or genus affiliation of collected isolates. Sequences were aligned with 179 180 ITS sequences of reference strains obtained from GenBank. Subsequently, the alignment was used to perform the phylogenetic tree with PHYLOGENY.FR (Dereeper et al., 2008) and 181 182 Mega 6 (Tamura et al., 2007) using the neighbor-joining method (Saitou and Nei, 1987) and the Jukes-Cantor correct distance model (Jukes and Cantor, 1969). The phylogenetic tree was 183 obtained from data using one of three equally parsimonious trees through 1,000 bootstrap 184 replicates (Felsenstein, 1985) with a heuristic search consisting of 10 random-addition 185 replicates for each bootstrap replicate. 186

187 2.5. Diversity of the fungal communities

Four diversity indices were calculated to assess NF communities: total number of isolates found in each soil sample (*N*); richness (*S* = number of species in the community); Shannon– Wiener local diversity index ($H'=-\Sigma$ (p_i.lnp_i), where p_i is the proportion of isolates with the species *i*); and evenness (E = $H'/\ln(S)$), which quantifies the numerical equality of populations in communities.

193 2.6. Pathogenicity analyses

194 An olive population of the RKN M. javanica (detected in 72% of the nurseries surveyed and dominant in orchards) (Ali et al., 2016) was reared on RKN-susceptible tomato (cv. Roma) in 195 a greenhouse (12 h light at 25°C; 12 h dark at 20°C). Fungal strains that exhibited trapping, 196 adhesive or encysting organs were sub-cultured on WA in 9-cm-diameter Petri dishes. One 197 week later, 100 second-stage juveniles (J2) of *M. javanica* (the only free form in the soil) 198 were washed five times with 0.05% streptomycin-sulfate in sterilized distilled water, 199 200 introduced into each fungal sub-culture and maintained at 25°C in darkness. Three Petri dishes per fungal strain were considered as replicates. Fungal predation structures were 201 202 observed and predated/dead nematodes were counted after four days under a microscope (x100 magnification). Similar procedures were used to study predation of *M. javanica* eggs by 203 specialized egg parasites such as *Paecilomyces* and *Pochonia* fungi. Dishes with nematodes 204 205 but without fungi were considered as control replicates.

206 2.7. *Statistical analyses*

207 Mean values were analyzed by one-way ANOVA and Kruskall-Wallis tests were used for all 208 pair-wise multiple comparisons. NF community patterns were explored through a Principal Component Analysis (PCA) of the diversity indices. Region grouping was tested using 209 Monte-Carlo tests on PCA eigenvalues (randtest, ade4). Calculations were performed and 210 211 graphics prepared using R language (readxl, base and ade4 packages) (R Development Core Team, 2011; Chessel et al., 2004; Dray and Dufour, 2007, Wickham, 2016), with a level of 212 significance = 0.05). A rarefaction regression was used to analyze the dependence between 213 root-knot nematodes (Meloidogyne spp.) and nematophagous fungi. 214

215

216 3. **Results**

217 *3.1. Nematofauna*

A non-significant gradient was present in plant-parasitic-nematodes (PPN), with a north-south increase of the population levels (Table 2). A parallel significant gradient was revealed in root-knot nematodes (RKN, *Meloidogyne* spp.). Free-living nematodes (FLN) were three times more abundant in the Souss olive nurseries than in the other regions. The ratios between FLN and PPN were different between all regions, the highest ratio being found in the Souss region.

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3.2. Nematophagous species and phylogenetic diversity

225 Several fungal isolates were recovered from the 305 soil samples examined. Observation of 226 characteristic conidia and traps around dead *M. javanica* juveniles revealed 149 soil samples 227 positive for NF. Morphological identification using microculture techniques revealed 73 NF 228 strains belonging to 11 genera.

229 In order to confirm the characterization of the fungal strains, the ITS regions of rDNA were 230 sequenced. Five species (Catenaria anguillulae, Nematoctonus leiosporus, Haptoglossa heterospora, Dactylaria sp. and Monacrosporium microphoides) were excluded from the 231 232 sequencing because it was not possible to purify them. The BLAST test showed that the ITS sequences of all sequenced strains were at least 99% similar to the corresponding GenBank 233 reference sequences (Table 3). The phylogenetic analysis including ITS sequences of the 234 Moroccan NF isolates and 16 reference sequences of identified close relatives revealed five 235 distinct clusters (Figs. 2 A-E): Purpureocillium lilacinum, Trichoderma, Fusarium 236 Arthrobotrys-Dreschslerella-Monacrosporium 237 oxysporum, **Talaromyces** and (family Orbiliaceae). 238

The combination of the morphological and the molecular analyses established that the isolated NF belonged to 19 species, eight families and six orders (Table 4). *Arthrobotrys* was the most diversified genus with five species, followed by *Trichoderma* (three species). The abundance of fungal species was low (less than five strains per species) except for *Paecilomyces lilacinus*

that represented 36 strains (50% of the strains). Half of the P. lilacinus strains detected were 243 244 isolated from the Souss region, and then gradually decreased northwards (12 in Haouz, four in Guerouane, two in Jbala). Arthrobotrys brochopaga, A. scaphoides, Monacrosporium 245 246 thaumasium, P. lilacinus, F. oxysporum, T. harzianum, T. asperellum and T. longibrachiatum were encountered in the four regions. A. oligospora, Dactylaria sp., Haptoglossa heterospora, 247 Monacrosporium microscaphoides, Nematoctunus leiosporus and Talaromyces assiutensis 248 were very rare (one isolate in one region). All the fungal species except A. oligospora were 249 250 detected in the Souss region. Only eight species were detected in the Jbala region. The Guerouane and Haouz regions hosted 11 and 12 species, respectively. 251

252 *3.3. Diversity patterns*

Because of their scarcity (only one nursery surveyed), the substrate samples from the Jbala 253 region were excluded from the dataset prior to running analyses. Richness (S) and local 254 255 diversity (H') were correlated to the PC1 axis, while the PC2 axis was related to numbers (N) and to evenness (E) of fungal isolates on its positive and negative sides, respectively (Fig. 256 257 3A). Region grouping was significant in the whole analysis and on the PC2 axis (Fig. 3B). Isolates were more numerous in the Souss region, whereas fungal communities were more 258 numerically alike in the Guerouane region. The Haouz region had lower NF richness and 259 diversity (non-significant PC1 coordinates). Indices were affected by the north-south 260 distribution of the nurseries (Figs. 3C-3E-3D). 261

The percentage of samples with NF was lower in the Haouz region than elsewhere (Table 5). Both trapping and endoparasitic fungi occurred more often in the Souss region than in the others regions, and no endoparasitic species were found in the Jbala region.

The rarefaction regression established between the occurrence of RKN (*Meloidogyne* spp.) and the occurrence of NF (Fig. 4) indicated a significant positive correlation, regardless of the region sampled.

268 *3.4.* In vitro *efficiency of the nematophagous strains*

NF were distinguished according to their ability to kill *M. javanica* J2s (Fig. 5). *Talaromyces assiutensis* killed all juveniles in all replicates. The Orbiliaceae species (*Arthrobotrys* spp., *Dreschlerella* spp., *Monacrosporium* spp.) were efficient against *M. javanica* since they trap
50 to 80% of the J2s using adhesives networks, buttons, constricting rings and hyphae
networks. *Paecilomyces* and *Trichoderma* strains killed 30 to 50% of the J2s. *Fusarium oxysporum* strains were less efficient (less than 20% of dead J2s). *P. lilacinus* and *P. chlamydosporia* strains infected all the *M. javanica* eggs.

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277 4. **Discussion**

Our first objective was to evaluate nematode populations in substrates from olive nurseries. In 278 all regions of Morocco, PPN abundance was greater than 1.4 nematodes/dm³ of soil and FLN 279 280 abundance greater than 1.9. As a comparison, Hamza et al. (2015) found 0.2 to 5.1 PPN/dm³ of soil and 0.3 to 4.3 FLN/dm³ of soil in 23 Souss and Haouz orchards. It can thus be 281 282 hypothesized that the multiplication of nematode populations may be boosted by acidity and by hydrophilic and non-degraded organic matter (Neher and Olson, 1999; Manlay et al., 2000; 283 Ou et al., 2005; McSorley, 2011). The usual dominance of FLN in olive soils could be due to 284 the origin of organic substrates (mountain soils, peat and manure) used in most nurseries 285 (Castillo et al., 2006). FLN are known to dominate in soil substrates not yet used for 286 agriculture (Hillocks, 1997). The FLN dominance in the Souss region, one of the most 287 intensively cultured areas in Morocco, was thus unexpected. 288

The FLN/PPN ratios were balanced in all regions except in the Souss where most of the soil substrates come from cropped areas (vegetables and citrus fruits). Because of the sandy texture of the soils in the Souss region, culture practices include high amounts of organic matter (especially cattle manure or tomato leaf compost), leading to an increase in FLN

populations and to a decrease in PPN populations because organic matter is unsuitable for PPN (Clark et al., 1998; Hominick, 1999; Hu and Qi, 2010). These mechanisms may explain the FLN/PPN ratio that is twice as high in the Souss region compared to the other regions The high percentages of RKN (*Meloidogyne* spp.) in Souss olive nurseries may be justified by the dominance throughout the area of vegetable crops that are highly susceptible to these nematodes (Sikora and Fernandez, 2005; Netscher, 1970).

Our second objective was to isolate and characterize nematophagous fungi able to control 299 300 RKN (Meloidogyne spp.). The success of fungal strain isolation from soils may be correlated with soil temperature, moisture and organic matter content (Akhtar and Malik, 2000; Cayrol 301 302 et al., 1992). The greatest difficulty encountered was in isolating pure NF strains because of the rapid growth of plant-pathogenic fungi and saprophytes such as Fusarium, Alternaria and 303 Aspergillus species. An increased pH of culture media may prevent the development of other 304 305 microorganisms (Gardner et al., 2000). Moreover, we found that direct cold soil powdering on hot media (37 to 40°C), like in Kelly et al., (2009), was preferable to aqueous soil suspensions 306 307 because it provided more concentrated fungal material (Davet and Rouxel, 2000; Hernández-308 Chavarría and Avendaño, 2001). Dispersing agents were avoided because they inhibited the growth of some NF strains (Davet and Rouxel, 2000). 309

This survey provided native NF from Morocco for the first time. The large number of soil 310 samples (305) allowed the detection of numerous NF strains, whereas former studies revealed 311 one strain at best (Bridge and Spooner, 2001). The strains detected as nematophagous 312 possessed different modes of action: adhesive networks, constricting rings, hyphal tips, 313 314 adhesive conidia and mycotoxins (Imerglik, 1981; Gaspard and Mankau, 1986). Despite this, ITS rDNA sequences could not be used to identify some NF strains due to their small size 315 316 (Pochonia chlamydosporia, for example), BLAST tests were useful to confirm the morphological characterization of other strains. The integrated taxonomical analysis 317

(morphological and molecular) of the NF provided a pool of 28 strains belonging to 19 species. The phylogenic analysis revealed that the ITS rDNA gene was able to distinguish species in a genus group (such as *Trichoderma*) but did not fully discriminate species belonging to the family Orbiliaceae, indicating the taxonomic proximity of *Dreschlerella*, *Arthrobotrys* and *Monacrosporium* species. Therefore, an integrative molecular analysis should be developed with other molecular markers (White et al., 1990) in order to improve NF identification.

Our third objective was to determine the occurrence and diversity of nematode-associated NF. 325 All NF species except A. oligospora were detected in the Souss region where they were more 326 327 dominant than in the other regions. This high diversity of NF might be due to the multiple habitat origins of the components (mountain, riverbank and field soils, cattle manure, plant 328 compost, etc.) used to make substrates for root olive plantlets. We hypothesize that the 329 330 microbial richness detected in the Souss region corresponds to the high endemic plant diversity characteristic of the Macaronesian region (Médail and Diadema, 2009; Msanda et 331 332 al., 2005). This could also be due to higher organic matter concentration in the soils (i.e., 333 saprophytic substrate for fungi that induce the formation and the activity of trapping structures) and to high PPN levels (i.e., parasitic substrates for fungi) (Den Belder and Jansen, 334 1994; Singh et al., 2012). 335

The number of NF isolates globally increased southwards, whereas evenness decreased (PCA2 axis). It is known that different thermal regimes affect soil microbial diversity (Bridge and Spooner, 2001). The absence of seasonality and the higher minimum temperatures in the Souss region may explain these developments where irrigation counteracts the arid to semiarid climate. The high occurrence of NF and the high evenness detected in the Guerouane region may be linked to a more continental climate. More than half of the 36 *P. lilacinus* strains came from the Souss region where the NF richness was the highest but the evenness the lowest. In a restricted soil area, the abundance of *P. lilacinus* may cause the rarity of the other species. For a constant number of species, maximal diversity is achieved when species have an even distribution. The Haouz, Jbala and Guerouane regions were characterized by less PPN and FLN than in the Souss region, explaining the lower NF richness in those regions.

Our fourth objective was to investigate the *in vitro* predation of the NF against *M. javanica* 348 juveniles and eggs. Talaromyces assiutensis (strain UIZFSA-31), whose nematode predation 349 350 was previously unknown, killed all the *M. javanica* juveniles in four days. The mode of action of T. assiutensis remains unknown but we hypothesize that the strain may produce specific 351 mycotoxins. In vitro trapping tests prove that the Orbiliaceae species (Arthrobotrys spp., 352 Dreschlerella spp., Monacrosporium spp.) were able to trap M. javanica juveniles. The 353 predatory capacity of A. oligospora was similar to data found in the literature (Singh et al., 354 2012). The mechanisms involved during predation are well known (Imerglik, 1981; Gaspard 355 and Mankau, 1986). Recognition and attachment of the mycelium to the cuticle of the RKN 356 357 juvenile is mainly due to compatible glycoproteins, e.g., lectins (Duponnois et al., 1996). Trichoderma species are recognizsed as control agents against nematodes, and various 358 mechanisms have been proposed to explain nematode killing, including antibiosis and 359 enzymatic hydrolysis (Sivan and Chet, 1992; Elad, 2000). Thomas (1996) demonstrated direct 360 interactions between T. harzianum and the cyst nematode Globodera rostochiensis: the 361 fungus penetrated eggs in the cysts, leading to the death of the juveniles (Sharon et al., 2001). 362 Precise information on the mechanisms involved is very limited and this misunderstanding 363 has hindered the selection of active strains and the development of improved biocontrol 364 methods. Paecilomyces lilacinus and Pochonia chlamydosporia are especially powerful 365 nematode egg parasites (Cayrol et al., 1982; Irving and Kerry, 1986), which could explain the 366 relatively low predation rate obtained on juveniles. These species can also act on the 367

movement of infested nematode juveniles via a paralytic toxin (Cayrol et al., 1989), purified
and identified as acetic acid by Djian et al. (1991). This molecule is abundantly produced
during fungal growth in liquid medium (*ibid*). *Fusarium oxysporum* strains, well-known plant
pathogens, exhibited lower predation rates, but some studies revealed that they are partly able
to kill nematodes by producing toxic sulfuric heterocycles (fusarenone and moniliformine)
(Ciancio et al., 1988; Cayrol et al., 1992).

Moreover, the significant co-occurrence of RKN and NF highlighted by the rarefaction curve 374 375 suggests a close interaction between prey (nematodes) and predators (fungi) in olive nurseries, as described by Lotka-Volterra models (Barbosa and Castellanos, 2005), probably 376 377 because of parasitism induction by nematodes (Jansson and Nordbring-Hertz, 1980). Even though the diversity of the NF detected in the four regions was variable at species and 378 population levels, we may expect symmetric dynamics (Marrow and Cannings, 1993) due to 379 380 possible co-evolutionary processes involved between nematodes and fungi. Such processes occur between competitive organisms in an ever-changing environment, as described by the 381 382 "Red Queen" hypothesis (Van Valen, 1973; Dawkins and Krebs, 1979).

383

384 5. Conclusion

Olive nursery solid substrates are infested with PPN, including *Meloidogyne* species. Various predatory fungi were able to kill RKN. Consequently, before selecting NF strains as candidates for biocontrol, studies must be extended to a wider range of *Meloidogyne* species and populations present on olive trees in Morocco in order to verify their specificity.

Indigenous NF strains were recovered from different substrates (different habitats), making it possible to undertake more research in order to understand the specificity of prey-predator interactions with more diverse PPN species. Predation efficiency in different cropping systems and in varied soil environments should also be explored in the future.

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400							
401	Conflict of interest statement						
402	We declare that we have no conflict of interest.						
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- 613

614 Legends for figures and tables

Figure 1 Distribution of the olive nurseries surveyed in Morocco. See Table 1 for moreinformation.

Figure 2 Neighbor-joining tree inferred from ITS rDNA sequences of the nematophagous fungi isolated (in bold) and their closest GenBank matches (with accession numbers). Bootstrap support values (1000 replicates) are provided as a percentage at the corresponding nodes when > 50. A to E = high similarity between phylogenetic groups

Figure 3 Diversity indices of the nematophagous fungus communities. (A) PCA loading plot for the diversity indices in soil samples (N = a total number of isolates; S = species richness; H' = Shannon-Wiener diversity; E = species evenness). (B) Score plot for the samples according to the region (J = Jbala; G = Guerouane; H = Haouz; S = Souss). (C, D, E) *N*, *H'* and *E* boxplots (*a-c* indicate significant groups, P < 0.05).

Figure 4 Rarefaction curve for root-knot nematodes (*Meloidogyne* spp.) and nematophagous
fungi detected in the olive nurseries according to the regions (J = Jbala; G = Guerouane; H =
Haouz; S = Souss).

Figure 5. *In vitro* efficiency of nematophagous fungal strains: percentage of dead *M. javanica* juveniles (*a-e* indicate significant groups, P < 0.05).

Table 1 Location and characteristics of the Moroccan olive nurseries surveyed. Number ofsamples for each cultivar in each geographic region.

Table 2 Average density (number of nematodes/dm³ of soil) of plant-parasitic nematodes (PPN) and free-living nematodes (FLN), percentages of samples infested with root-knot nematodes (RKN) and FLN/PPN ratios (*a-d* indicate significant groups, P < 0.05).

Table 3 BLAST results of ITS rDNA sequences of the nematophagous fungi isolated.

637 **Table 4** Nematophagous fungi associated with the Moroccan olive nurseries surveyed.

Table 5 Functional diversity of the nematophagous fungi (a-d indicate significant groups, P < d

639 0.05).

Table 1

641 Location and characteristics of the Moroccan olive nurseries. Number of samples for each cultivar in each geographic region.

Geographic region	Location	Climate	City	No. of nurseries	Main habitat origin of the substrates	Olive cultivar	No. of samples
Jbala	South-west face	Sub humid climate,	Ouazzane	1	Clay marls,	Picholine marocaine	5
	of the Rif Mountains	temperature from -6°			sand, forest soil	Haouzia	5
		to 32°C			and topsoil	Menara	5
Guerouane	Sais Plateau, between	More continental	Meknes	4	Yellow sand,	Picholine marocaine	15
	the Middle Atlas	climate, temperature			topsoil,	Haouzia	20
	to the south and the Rif	from -10° to 45°C			mature manure	Menara	20
	Mountains to the north				and local	Arbequina	5
					compost	Arbosana	5
						Picual	10
						Picholine Languedoc	5
Haouz	Northern slope	Semiarid climate,	Marrakech	5	Clay marls,	Picholine marocaine	25
	of the High Atlas	average temperatures			sand,	Arbequina	10
	Mountains	from -6° to 49.6°C			forest soil,	Haouzia	25
					mountain soil	Menara	25
					and topsoil	Picholine Languedoc	10
						Arbosana	5
			El Kelaa des Sraghna	3	Forest soil	Picholine marocaine	15
					and topsoil	Picholine Languedoc	15
						Menara	10
						Haouzia	10
			Sidi Abdellah Ghiat	1	Soil, clay and sand	Picholine marocaine	5
Souss	On the southern slope	Arid constant climate,	Agadir	8	Sand, topsoil	Picholine marocaine	15
	of the High Atlas	sunshine > 340 days a			and peat moss	Haouzia	20
	Mountains	year, average				Menara	5
		temperatures from 14° to 25°C	Khmiss Aït Amira	2	Topsoil, peat and manure	Picholine marocaine	10
			Biougra	1	Peat, soil and perlite	Menara	5
			~		· •		

642 **Table 2**

Average density (number of nematodes/dm³ of soil) of plant-parasitic nematodes (PPN) and
free-living nematodes (FLN), percentages of samples infested with root-knot nematodes

Regions	No. of PPN (/dm ³ of soil)	% of samples infested with RKN	No. of FLN (/dm ³ of soil)	FLN/PPN
Jbala	1,441	16.0 <i>d</i>	2,220 b	1.54 <i>b</i>
Guerouane	1,527	20.0 c	1,914 <i>b</i>	1.25 c
Haouz	2,003	58.1 b	2,081 b	1.04 <i>d</i>
Souss	2,395	76.4 <i>a</i>	6,194 <i>a</i>	2.59 a
P-value	0.153	0.000	0.000	0.000

645 (RKN) and FLN/PPN ratios (*a-d* indicate significant groups, P < 0.05).

646 **Table 3**

647 BLAST results of ITS rDNA sequences of the nematophagous fungi isolated.

Strains	GenBank reference strains				Maximum similarity (%)
	Species	Strain	GenBank	Number of	_
			accession no.	nucleotides (bp)	
UIZFSA-1	Paecilomyces lilacinus	MY683	GU980015	541	99
UIZFSA-2	Paecilomyce slilacinus	MY683	GU980015	541	99
UIZFSA-3	Purpureocillium lilacinum	NPF-4	KP308838	565	99
UIZFSA-5	Purpureocillium lilacinum	DF58	KT582081	713	99
UIZFSA-6	Purpureocillium lilacinum	MSEF24	KT310947	540	99
UIZFSA-12	Purpureocillium lilacinum	E303	KJ540087	540	99
UIZFSA-13	Arthrobotrys thaumasia	isolate 111	EU977532	561	100
UIZFSA-15	Purpureocillium lilacinum	SBTPI-001	KF766523	625	99
UIZFSA-18	Arthrobotrys scaphoides	CBS 226.52	KF494006	565	99
UIZFSA-20	Fusarium oxysporum	K9	JF807396	491	99
UIZFSA-21	Fusarium oxysporum	A2-3	KT876690	510	100
UIZFSA-22	Fusarium oxysporum	DSCF2	AB470914	509	100
UIZFSA-23	Fusarium oxysporum	Z11	EF611086	540	99
UIZFSA-24	Trichoderma asperellum	OTPT7	KC551811	581	100
UIZFSA-24	Trichoderma asperellum	SI14	KJ432865	548	100
UIZFSA-25	Trichoderma asperellum	OTPT7	KC551811	546	100
UIZFSA-26	Trichoderma asperellum	SVPP-3	KU215915	549	100
UIZFSA-27	Trichoderma harzianum	SZMC 20965	KP316410	567	100
UIZFSA-30	Trichoderma asperellum	CPCRI-TD-11	KT445769	594	99
UIZFSA-31	Talaromyces assiutensis	ph721	KF147920	537	99
UIZFSA-35	Trichoderma longibrachiatum	Y-14	KP281451	588	99
UIZFSA-55	Monacrosporium thaumasium	MTU51972	U51972	549	99
UIZFSA-97	Arthrobotrys brochopaga	ABU72609	U72609	525	100
UIZFSA-97	Arthrobotrys conoides	SBUG M12	AF106534	549	99
UIZFSA-98	Drechslerella brochopaga	2eA003	JF748753	493	100
UIZFSA-99	Drechslerella brochopaga	1eL002	JF748752	494	100
UIZFSA-100	Arthrobotrys oligospora	AOZ1	X94121	497	100
UIZFSA-101	Arthrobotrys thaumasia	CBS 322.94	AF106526	553	100

Table 4

Order	Family	Species	Number	Region			
		-	of strains	Jbala	Guerouane	Haouz	Souss
Orbiliales	Orbiliaceae	Arthrobotrys brochopaga	2	+	+	+	+
		A. conoides	1	-	+	-	+
		A. oligospora	1	-	+	-	-
		A. thaumasia	3	-	+	+	+
		A. scaphoides	1	+	+	+	+
		Dreschslerella brochopaga	2	-	+	-	+
		Dactylaria sp.	1	-	-	-	+
		Monacrosporium thaumasium	5	+	+	+	+
		M. microscaphoides	1	-	-	-	+
Eurotiales	Trichocomaceae	Paecilomyces lilacinus	36	+	+	+	+
		Talaromyces assiutensis	1	-	-	-	+
Agaricales	Pleurotaceae	Nematoctunus leiosporus	1	-	-	-	+
Blastocladiales	Catenariaceae	Catenaria anguillulae	1	-	-	+	+
Haptoglossales	Haptoglossaceae	Haptoglossa heterospora	1	-	-	-	+
Hypocreales	Clavicipitaceae	Pochonia chlamydosporia	4	-	-	+	+
	Hypocreaceae	Trichoderma asperellum	4	+	+	+	+
		T. harzianum	3	+	+	+	+
		T. longibrachiatum	1	+	+	+	+
	Nectriaceae	Fusarium oxysporum	4	+	+	+	+

649 Nematophagous fungi associated with the Moroccan olive nurseries surveyed.

650 +: detected; -: not detected.

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651
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Table 5

Functional diversity of the nematophagous fungi (*a-d* indicate significant groups, P < 0.05).

Region	Samples with NF (%)	Trapping species (%)	Endoparasitic species (%)
Jbala	66.66 <i>c</i>	7.13 <i>d</i>	0
Guerouane	$70.00 \ b$	25.12 <i>b</i>	12.13 c
Haouz	49.68 d	24.39 c	11.90 <i>b</i>
Souss	70.90 a	53.16 <i>a</i>	25.80 a
P-value	0.000	0.000	0.000



Figure 1. Distribution of the olive nurseries surveyed in Morocco. See Table 1 for more

⁶⁵⁸ information.



Figure 2. Neighbor-joining tree inferred from ITS rDNA sequences of the nematophagous fungi isolated (in bold) and their closest GenBank matches (with accession numbers). Bootstrap support values (1000 replicates) are provided as a percentage at the corresponding nodes when > 50. A to E = high similarity between phylogenetic groups.





Figure 3. Diversity indices of the nematophagous fungus communities. (A) PCA loading plot for the diversity indices in soil samples (N = a total number of fungal isolates; S = species richness; H' = Shannon-Wiener diversity; E = species evenness). (B) Score plot for the samples according to the region (J = Jbala; G = Guerouane; H = Haouz; S = Souss). (C, D, E) N, H' and E boxplots (*a-c* indicate significant groups, P < 0.05).

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Figure 4. Rarefaction curve for root-knot nematodes (*Meloidogyne* spp.) and nematophagous
fungi detected in the olive nurseries according to the regions (J = Jbala; G = Guerouane; H =
Haouz; S = Souss).

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Figure 5. *In vitro* efficiency of nematophagous fungal stains: percentage of dead *M. javanica* juveniles (*a-e* indicate significant groups, P < 0.05).