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

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1 Diversity of nematophagous fungi in Moroccan olive nurseries: Highlighting prey-predator  
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3

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19

20

21 **Abstract**

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

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

42

43        **1. Introduction**

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

51  
52        Several plant-parasitic nematode species (PPN) have been found to be associated with olive  
53        trees (Castillo et al., 2010; Ali et al., 2014; Hamza et al., 2015), including root-knot  
54        nematodes (RKN, *Meloidogyne* spp.) that were shown to impact olive growth (Afshar et al.,  
55        2014). RKN have been reported to be major pests on olive trees, mainly in nurseries where  
56        irrigation conditions are favorable to their multiplication (Nico et al., 2002; Sanei and  
57        Okhovvat, 2011). Moreover, most RKN infestations in olive orchards result from  
58        contaminated plant material produced in uncertified nurseries (Nico et al., 2002). To protect  
59        nurseries, vegetative propagation of olive is a crucial step to prevent plant pest dispersal.  
60        While some industrial olive-producing countries use synthetic substrates to cultivate olive  
61        trees, other countries use more traditional olive cultivation techniques, planting roots in soil  
62        substrates. In countries that have implemented large-scale planting programs (e.g., 1,220,000  
63        ha in the Morocco Green Plan, 2015), the “nursery risk” is a concern, with two main  
64        consequences: (i) the weakening of olive plants before their transplantation into orchards; and  
65        (ii) the introduction of pathogens into orchards. The nematode risk is now controlled in  
66        nurseries, either by multiplication of resistant varieties (Palomares-Rius et al., 2012) or by  
67        inoculating microbial antagonists in the substrates, such as mycorrhizae (Castillo et al., 2006).

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

77

78 Microbial antagonists of PPN include nematophagous fungi (NF), raising expectations for  
79 their use in integrated pest management (Waller, 2006; Larsen, 2006; Maingi et al., 2006).  
80 These fungi act by antibiosis, parasitism or predation (Imerglik, 1981; Gaspard and Mankau,  
81 1986). Fungi trap nematodes using adhesive networks or buttons. Some NF capture their prey  
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  
84 attachment of the mycelium to the nematode cuticle are mainly due to compatible  
85 glycoproteins such as lectins (Nordbring-Hertz et al., 2006).

86

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

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

97

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

107

## 108 2. Materials and methods

### 109 2.1. Site description and olive plantlet sampling

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

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

118 (Everris Company™).

119 In each nursery and for each variety, five olive plantlets (*Olea europaea* subsp. *europaea*)  
120 growing in plastic bags were sampled. Information about the origin and the preparation of the  
121 growth substrates and about the cultivars was recorded. A total of 305 olive plantlets were  
122 collected and maintained in the laboratory and kept under greenhouse conditions (12 h light at  
123 25 °C; 12 h dark at 20°C).

#### 124 2.2. *Nematode extraction and quantification*

125 A 250-cm<sup>3</sup> substrate subsample was removed from the rhizosphere of each olive plantlet and  
126 used for nematode extraction using the Oostenbrink (1960) elutriation procedure (ISO 23611-  
127 4). Free-living nematodes (FLN) and plant-parasitic nematodes (PPN), which specifically  
128 exhibit a stylet for plant-cell feeding, were enumerated in 5-cm<sup>3</sup> counting chambers (Merny  
129 and Luc, 1969) under a stereomicroscope (×60 magnification). Nematode population levels  
130 were expressed as the number of individuals per dm<sup>3</sup> of fresh substrate. Among PPN, root-  
131 knot nematodes belonging to the *Meloidogyne* genus (Mai and Mullin, 1996) were counted.

#### 132 2.3. *Isolation of nematophagous fungi*

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

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

#### 154 2.4. *Morphological and molecular characterization of the fungi*

155 The initial identification of the fungi was based on colony morphology and microscopic  
156 characteristics. Slide sub-cultures from pure NF cultures were observed under a dissecting  
157 microscope (up to x100 magnification). Genera and species were assigned according to  
158 specialized morpho-taxonomical keys (Cooke and Dickinson, 1965; Haard, 1968; Barron,  
159 1977; Yu et al., 2014; Philip, 2002).

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

168 mixture contained 2  $\mu$ l (10 ng) of template DNA, 1  $\mu$ l of each ITS1 and ITS4 primer (10  
169 mM), 4  $\mu$ l PCR buffer, 2.4  $\mu$ l MgCl<sub>2</sub> (25 mM), 0.6  $\mu$ l dNTPs (10 mM), 0.1  $\mu$ l BSA (0.1  
170 mg/ml) and 0.2 units of GoTaq<sup>®</sup> DNA polymerase.  
171 PCR cycling conditions consisted of an initial denaturation step at 94°C for 3 min, 35 cycles  
172 of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 10  
173 min. PCR products were checked for length, quality and quantity by agarose gel  
174 electrophoresis (1% (w/v) in 0.5x Tris-Acetate-EDTA (TAE).  
175 PCR products were sequenced from both ends by Eurofins MWG GmbH (Ebersberg,  
176 Germany), using the same ITS primers. CHROMAS LITE v2.1.1 (Technelysium Pty Ltd.)  
177 software was used to edit and assemble DNA sequences. BLAST similarity searches were  
178 performed in the non-redundant nucleotide database of GenBank (Altschul et al., 1997) to  
179 identify/verify species or genus affiliation of collected isolates. Sequences were aligned with  
180 ITS sequences of reference strains obtained from GenBank. Subsequently, the alignment was  
181 used to perform the phylogenetic tree with PHYLOGENY.FR (Dereeper et al., 2008) and  
182 Mega 6 (Tamura et al., 2007) using the neighbor-joining method (Saitou and Nei, 1987) and  
183 the Jukes-Cantor correct distance model (Jukes and Cantor, 1969). The phylogenetic tree was  
184 obtained from data using one of three equally parsimonious trees through 1,000 bootstrap  
185 replicates (Felsenstein, 1985) with a heuristic search consisting of 10 random-addition  
186 replicates for each bootstrap replicate.

#### 187 2.5. *Diversity of the fungal communities*

188 Four diversity indices were calculated to assess NF communities: total number of isolates  
189 found in each soil sample ( $N$ ); richness ( $S$  = number of species in the community); Shannon–  
190 Wiener local diversity index ( $H' = -\sum (p_i \cdot \ln p_i)$ , where  $p_i$  is the proportion of isolates with the  
191 species  $i$ ); and evenness ( $E = H' / \ln(S)$ ), which quantifies the numerical equality of  
192 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  
195    dominant in orchards) (Ali et al., 2016) was reared on RKN-susceptible tomato (cv. Roma) in  
196    a greenhouse (12 h light at 25°C; 12 h dark at 20°C). Fungal strains that exhibited trapping,  
197    adhesive or encysting organs were sub-cultured on WA in 9-cm-diameter Petri dishes. One  
198    week later, 100 second-stage juveniles (J2) of *M. javanica* (the only free form in the soil)  
199    were washed five times with 0.05% streptomycin-sulfate in sterilized distilled water,  
200    introduced into each fungal sub-culture and maintained at 25°C in darkness. Three Petri  
201    dishes per fungal strain were considered as replicates. Fungal predation structures were  
202    observed and predated/dead nematodes were counted after four days under a microscope  
203    (x100 magnification). Similar procedures were used to study predation of *M. javanica* eggs by  
204    specialized egg parasites such as *Paecilomyces* and *Pochonia* fungi. Dishes with nematodes  
205    but without fungi were considered as control replicates.

206        2.7.        *Statistical analyses*

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

215

216        3.    **Results**

217        3.1.        *Nematofauna*

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

### 224 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*  
231 *heterospora*, *Dactylaria* sp. and *Monacrosporium microphoides*) were excluded from the  
232 sequencing because it was not possible to purify them. The BLAST test showed that the ITS  
233 sequences of all sequenced strains were at least 99% similar to the corresponding GenBank  
234 reference sequences (Table 3). The phylogenetic analysis including ITS sequences of the  
235 Moroccan NF isolates and 16 reference sequences of identified close relatives revealed five  
236 distinct clusters (Figs. 2 A-E): *Purpureocillium lilacinum*, *Trichoderma*, *Fusarium*  
237 *oxysporum*, *Talaromyces* and *Arthrobotrys-Dreschlerella-Monacrosporium* (family  
238 Orbiliaceae).

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

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

### 252 3.3. Diversity patterns

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

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

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

268 3.4. In vitro efficiency of the nematophagous strains

269 NF were distinguished according to their ability to kill *M. javanica* J2s (Fig. 5). *Talaromyces*  
270 *assiutensis* killed all juveniles in all replicates. The Orbiliaceae species (*Arthrobotrys* spp.,  
271 *Dreschlerella* spp., *Monacrosporium* spp.) were efficient against *M. javanica* since they trap  
272 50 to 80% of the J2s using adhesives networks, buttons, constricting rings and hyphae  
273 networks. *Paecilomyces* and *Trichoderma* strains killed 30 to 50% of the J2s. *Fusarium*  
274 *oxysporum* strains were less efficient (less than 20% of dead J2s). *P. lilacinus* and *P.*  
275 *chlamydosporia* strains infected all the *M. javanica* eggs.

276

277 4. Discussion

278 Our first objective was to evaluate nematode populations in substrates from olive nurseries. In  
279 all regions of Morocco, PPN abundance was greater than 1.4 nematodes/dm<sup>3</sup> of soil and FLN  
280 abundance greater than 1.9. As a comparison, Hamza et al. (2015) found 0.2 to 5.1 PPN/dm<sup>3</sup>  
281 of soil and 0.3 to 4.3 FLN/dm<sup>3</sup> of soil in 23 Souss and Haouz orchards. It can thus be  
282 hypothesized that the multiplication of nematode populations may be boosted by acidity and  
283 by hydrophilic and non-degraded organic matter (Neher and Olson, 1999; Manlay et al., 2000;  
284 Ou et al., 2005; McSorley, 2011). The usual dominance of FLN in olive soils could be due to  
285 the origin of organic substrates (mountain soils, peat and manure) used in most nurseries  
286 (Castillo et al., 2006). FLN are known to dominate in soil substrates not yet used for  
287 agriculture (Hillocks, 1997). The FLN dominance in the Souss region, one of the most  
288 intensively cultured areas in Morocco, was thus unexpected.

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

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

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

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

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

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

336 The number of NF isolates globally increased southwards, whereas evenness decreased  
337 (PCA2 axis). It is known that different thermal regimes affect soil microbial diversity (Bridge  
338 and Spooner, 2001). The absence of seasonality and the higher minimum temperatures in the  
339 Souss region may explain these developments where irrigation counteracts the arid to semi-  
340 arid climate. The high occurrence of NF and the high evenness detected in the Guerouane  
341 region may be linked to a more continental climate. More than half of the 36 *P. lilacinus*  
342 strains came from the Souss region where the NF richness was the highest but the evenness

343 the lowest. In a restricted soil area, the abundance of *P. lilacinus* may cause the rarity of the  
344 other species. For a constant number of species, maximal diversity is achieved when species  
345 have an even distribution. The Haouz, Jbala and Guerouane regions were characterized by  
346 less PPN and FLN than in the Souss region, explaining the lower NF richness in those  
347 regions.

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

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

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

383

## 384 5. Conclusion

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

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

393

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400

#### 401 **Conflict of interest statement**

402 We declare that we have no conflict of interest.

403

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613

614 **Legends for figures and tables**

615 **Figure 1** Distribution of the olive nurseries surveyed in Morocco. See Table 1 for more  
616 information.

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

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

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

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

631 **Table 1** Location and characteristics of the Moroccan olive nurseries surveyed. Number of  
632 samples for each cultivar in each geographic region.

633 **Table 2** Average density (number of nematodes/dm<sup>3</sup> of soil) of plant-parasitic nematodes  
634 (PPN) and free-living nematodes (FLN), percentages of samples infested with root-knot  
635 nematodes (RKN) and FLN/PPN ratios (a-d indicate significant groups,  $P < 0.05$ ).

636 **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.

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

639 0.05).

640 **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 of the Rif Mountains	Sub humid climate, temperature from -6° to 32°C	Ouazzane	1	Clay marls, sand, forest soil and topsoil	Picholine marocaine	5
						Haouzia	5
						Menara	5
Guerouane	Sais Plateau, between the Middle Atlas to the south and the Rif Mountains to the north	More continental climate, temperature from -10° to 45°C	Meknes	4	Yellow sand, topsoil, mature manure and local compost	Picholine marocaine	15
						Haouzia	20
						Menara	20
						Arbequina	5
						Arbosana	5
						Picual	10
Haouz	Northern slope of the High Atlas Mountains	Semiarid climate, average temperatures from -6° to 49.6°C	Marrakech	5	Clay marls, sand, forest soil, mountain soil and topsoil	Picholine marocaine	25
						Arbequina	10
						Haouzia	25
						Menara	25
						Picholine Languedoc	10
			Arbosana	5			
			El Kelaa des Sraghna	3	Forest soil and topsoil	Picholine marocaine	15
						Picholine Languedoc	15
						Menara	10
						Haouzia	10
Sidi Abdellah Ghiat	1	Soil, clay and sand				Picholine marocaine	5
Souss	On the southern slope of the High Atlas Mountains	Arid constant climate, sunshine > 340 days a year, average temperatures from 14° to 25°C	Agadir	8	Sand, topsoil and peat moss	Picholine marocaine	15
						Haouzia	20
						Menara	5
			Khmiss Aït Amira	2	Topsoil, peat and manure	Picholine marocaine	10
			Biougra	1	Peat, soil and perlite	Menara	5

642 **Table 2**

643 Average density (number of nematodes/dm<sup>3</sup> of soil) of plant-parasitic nematodes (PPN) and  
 644 free-living nematodes (FLN), percentages of samples infested with root-knot nematodes  
 645 (RKN) and FLN/PPN ratios (*a-d* indicate significant groups, *P* < 0.05).

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

646 **Table 3**

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

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

648 **Table 4**

649 Nematophagous fungi associated with the Moroccan olive nurseries surveyed.

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

650 +: detected; -: not detected.

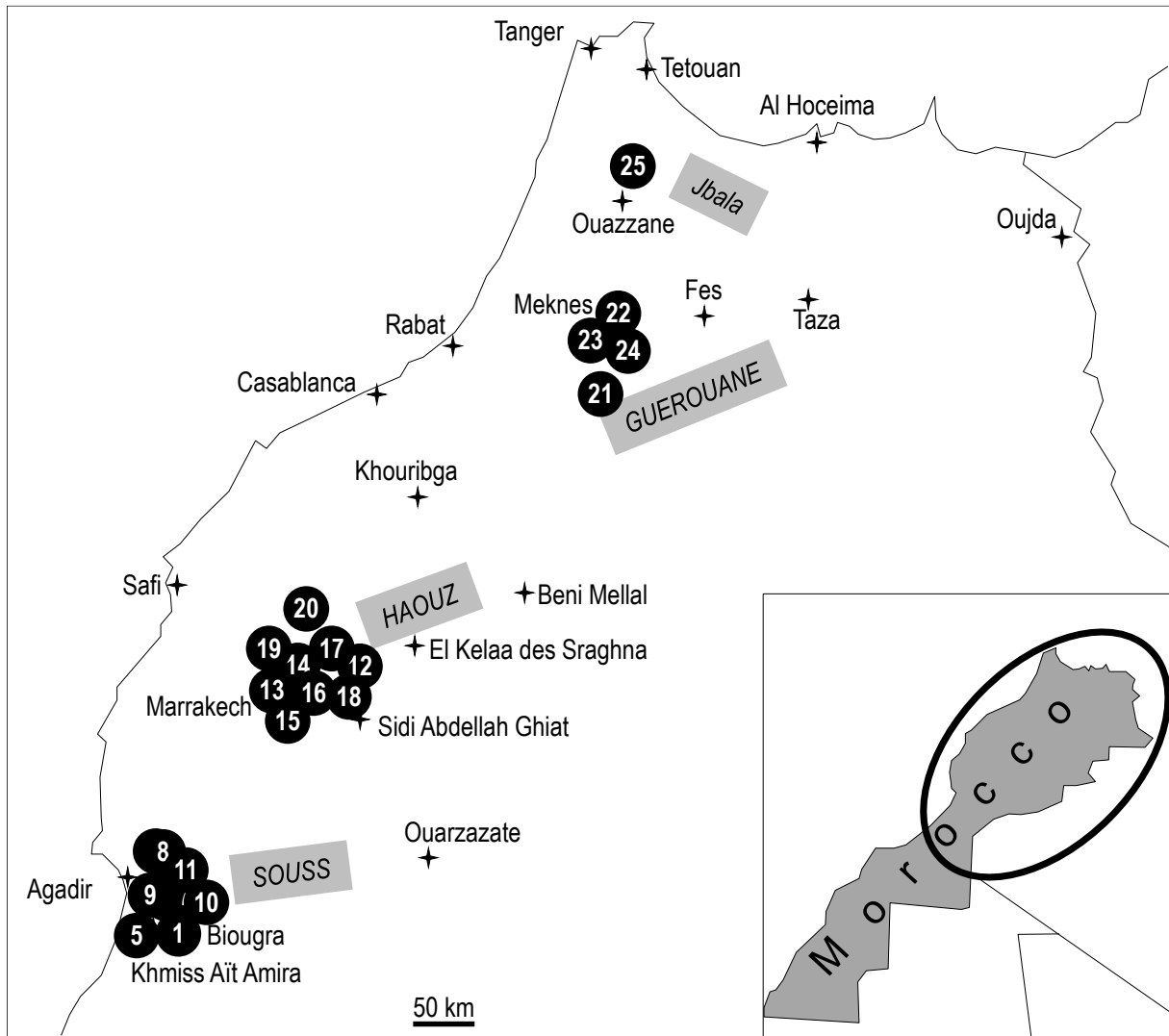
651

652 **Table 5**653 Functional diversity of the nematophagous fungi (*a-d* indicate significant groups,  $P < 0.05$ ).

654

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

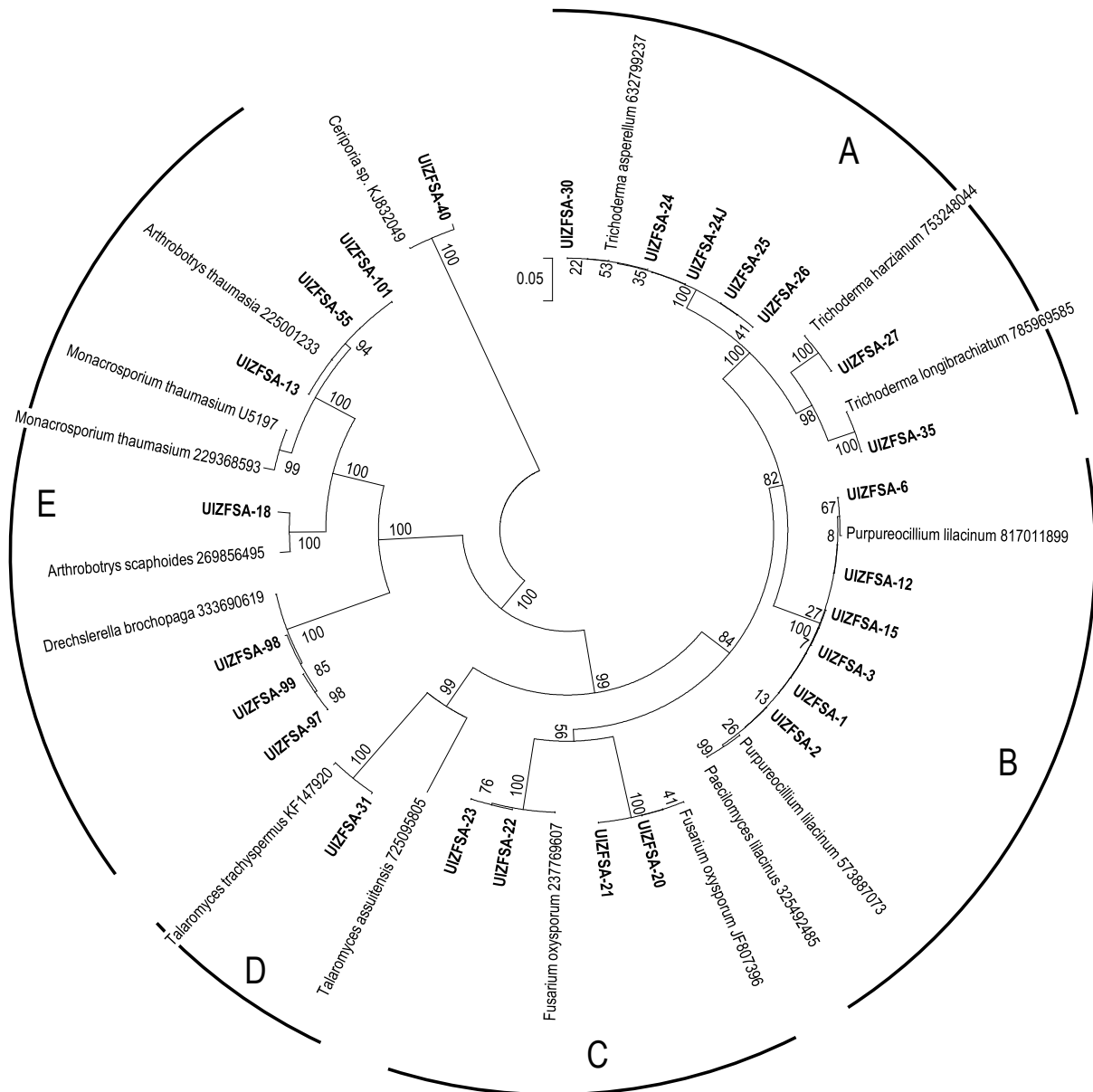
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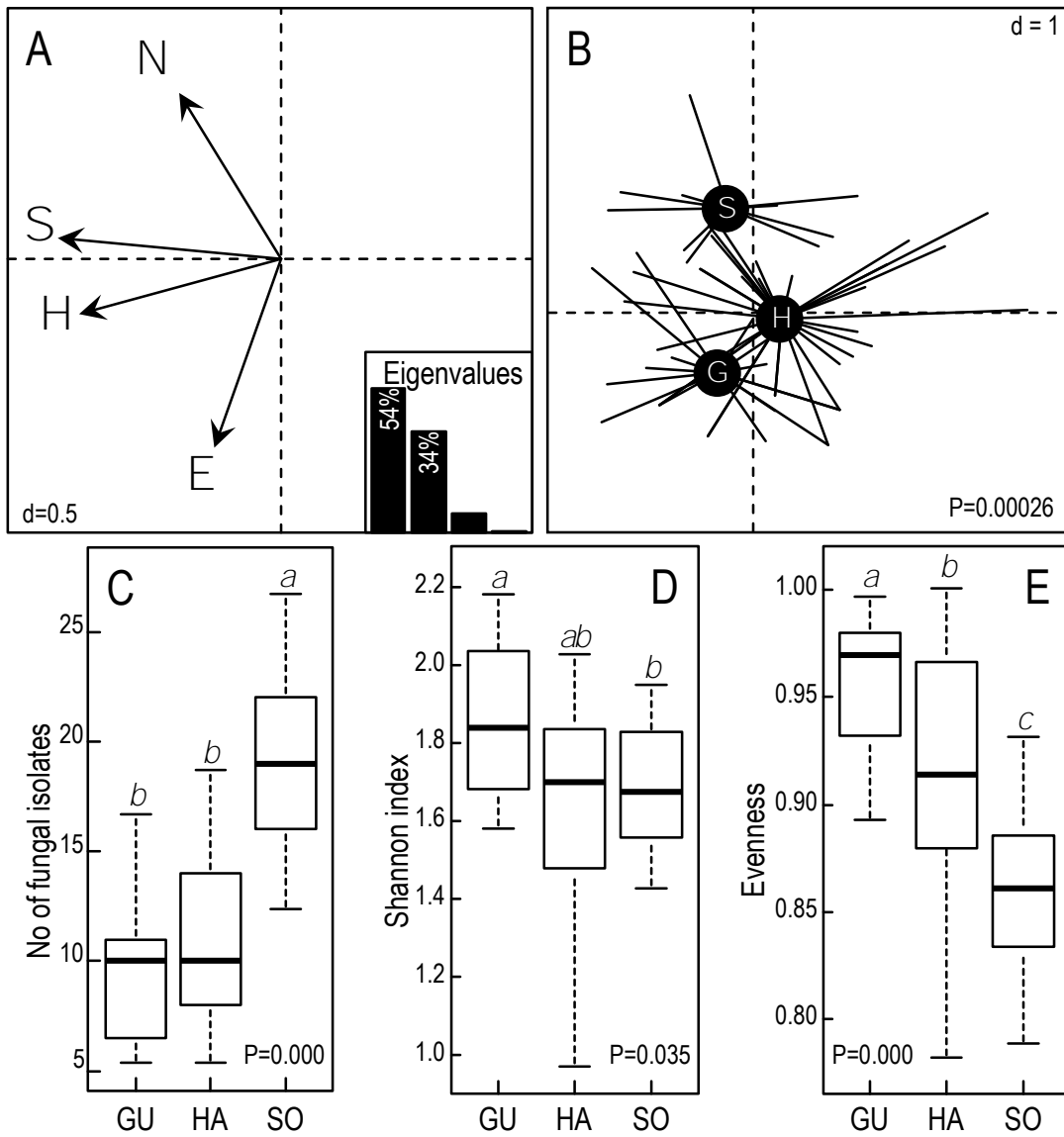
657 **Figure 1.** Distribution of the olive nurseries surveyed in Morocco. See Table 1 for more  
 658 information.

659



660

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

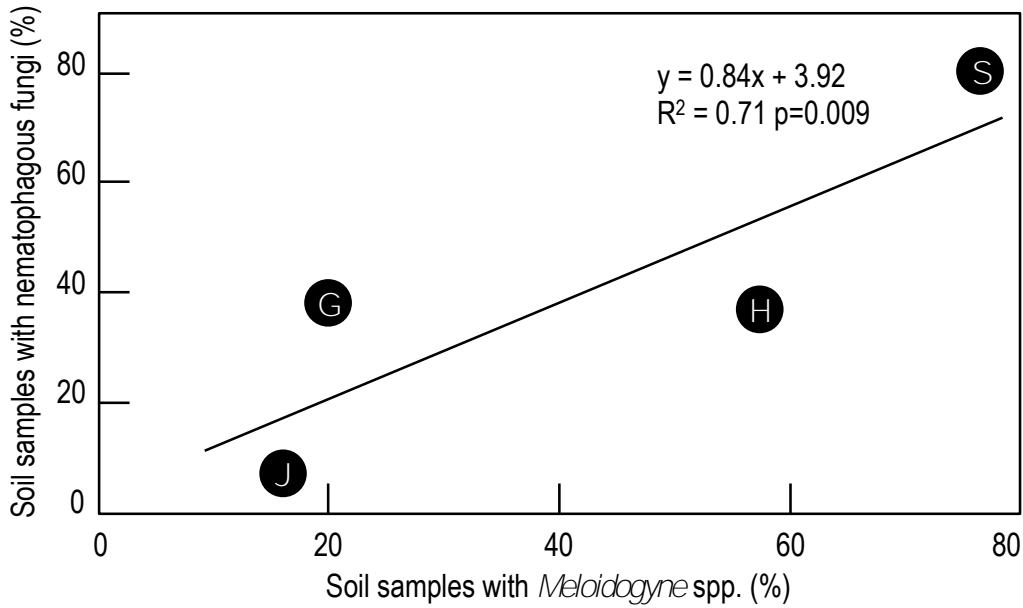


665

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

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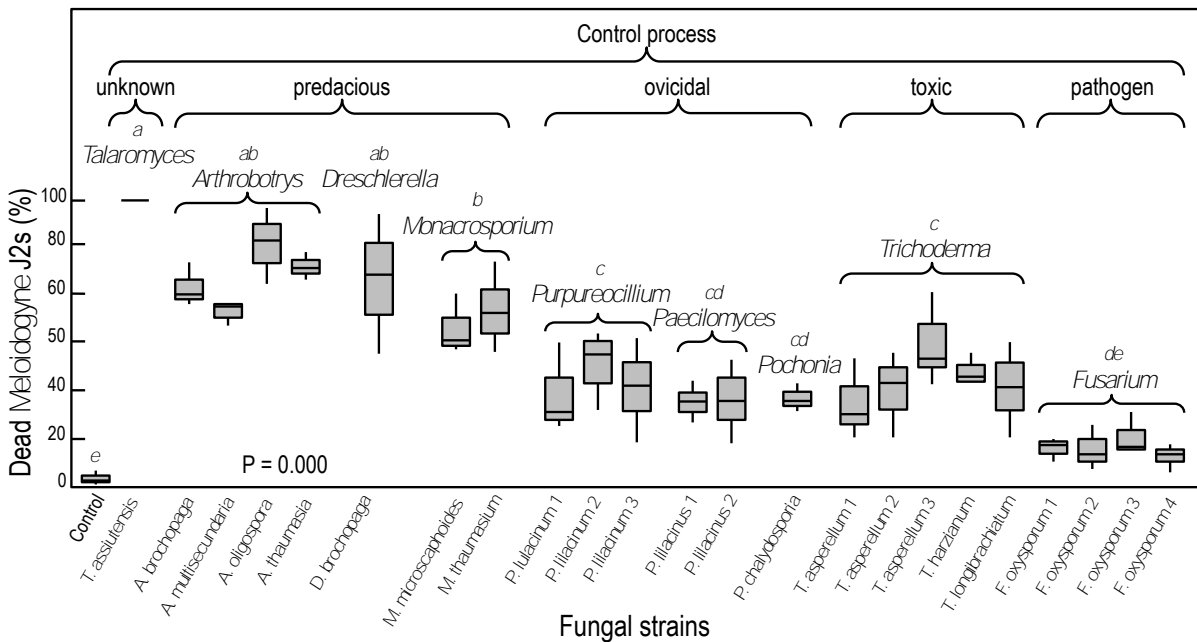
672



673

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

677



678

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