NEMATODES AND NEMATOPHAGOUS FUNGI ASSOCIATED WITH *CITRUS* FIELDS AND *PINUS HALEPENSIS-QUERCUS ROTUNDIFOLIA* FOREST SOILS

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Summary. Eight forest (*Quercus rotundifolia* and *Pinus halepensis*) and seven crop field (*Citrus* spp.) soils were surveyed in south-east Spain for nematode and nematophagous fungi. Nematodes were markedly more abundant in forest than in *Citrus* soils. The most commonly occurring group of nematodes were free-living nematodes (especially *Rhabditis* spp.) which occurred in both soil types. The mostly prevalent plant parasitic genera, which occurred in both soil types, were *Filenchus* sp., *Aphelenchus* sp. and *Pratylenchus* sp. Predatory nematodes (Mononchidae) were also found in the forest soil. The nematophagous fungi detected depended on the technique used. Only predatory fungi were found using the soil sprinkling technique and of these *Arthrobotrys oligospora* var. *oligospora* (adhesive network former) was the most frequent species (ca. 50% soil samples). *A. javanica, A. arthrobotryoides, A. oligospora* var. *microspora*, *A. oligospora* var. *sarmatica*, *A. musiformis* and *A. brochopaga* (constricting rings) were less frequently found. *Monacrosporium* sp. (bidimensional adhesive network) was found in both *Citrus* and forest soils. Predatory nematophagous fungi appeared earlier and in higher numbers in *Citrus* than in forest soils. The most common endoparasitic fungus was *Catenaria anguillulae* (ca. 80% soil samples) followed by *Myzocytium* sp. *Nematoctonus concurrens* was also found infecting nematodes. Our data show that the mediterranean soils studied acted as an important reservoir for a diverse range of nematophagus fungi which utilised different modes of action to attack nematodes and that some of these nematophagous fungi could have the potential to be developed as biocontrol agents of plant-parasitic nematodes.

Nematophagous fungi have been shown to have a worldwide distribution (Gray, 1982). The results of experiments into the biocontrol of plant parasitic nematodes in California (Jaffee *et al.*, 1998) are of particular interest to research workers in Spain since California has a similar Mediterranean climate and cultural conditions to those found in Spain. Gaspard and Mankau (1986) surveyed the nematophagus fungi in citrus fields in California and identified the species associated with the plant parasitc nematode *Tylenchulus semipenetrans*. It is important to study naturally occurring nematode populations in soil associated with nematophagous fungi, since these nematodes represent the source of food for many of the nematophagous fungi. Obligate parasites such as endoparasitic fungi are generally associated with high nematode densities since they are dependent on nematodes as their only food supply (Gray, 1985).

A recent study (Lopez-Llorca and Olivares-Bernabeu, 1997) indicated clear differences in the effect of organic matter (mainly derived from leaf-litter phenols) on nematophagous fungi. The aim of the present survey was to identify and compare the nematode and nematophagous fungi communities found in soils from the two Mediterannean environments: *Quercus rotundifolia -Pinus halepensis* forests and crop fields growing a citrus crop.

MATERIALS AND METHODS

The methodology and experimental approaches used in this survey were similar to those used by Persmark *et al.* (1996).

During October and November 1993 soil was collected from 15 sites in the Alacant, València and Castelló provinces (eastern Spain, Table I; see also Lopez-Llorca and Olivares-Bernabeu, 1997). Seven of these were agricultural soils from *Citrus* spp. fields, the rest were samples from forest soils dominated by either *Quercus spp.* (mainly evergreen oak *Q. rotundifolia* Lam.) and pines (*Pinus halepensis* Mill.). At each sampling site four points were marked at random and approximately 1.5-2 kg of soil was collected from them at a depth of 0-20 cm, after the uppermost leaf-litter layer (Ao horizon) had been removed. Samples were mixed in the laboratory, passed through a 2 mm sieve and stored at 4 °C in the dark prior to examination.

Nematodes were extracted from soil using the Baermann funnel technique (Barron, 1982). After each extraction, the numbers of nematodes were counted and expressed as nematodes per gram of dry soil. Three extractions were undertaken for each soil sample and three counts were undertaken per extraction to asses nematode numbers.

After the nematodes had been extracted from the soil, they were killed by immersion in a hot (60 °C) bath

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Table I. List of sampling sites for soils used in the experiments, together with their U.T.M coordinates.

Sample No.	Site location (U.T.M. Coordinates)	Site description
1 A	Alberic (Va); 30SYJ1532	*Citrus* Field
2 A	Alginet (Va); 30SYJ1748	*Citrus* Field
3 A-4A	Borriana (Ca), 30SYK4819	*Citrus* Field. Marro and Capote sites
5 A	Crevillent (Al), 30SXH9039	*Citrus* Field
6 A	El Puig (Va); 30SYJ3186	*Citrus* Field
7 A	Pego (Al); 30SYJ5207	*Citrus* Field
8F-11F	Alcoi (Al), Parc Natural de la Font Roja; 30SYH18	*Q. rotundifolia* forest, Sites: Santuari, Mas de Tetuan, Cava Coloma and Menejador
12F	Alcoi (A); 30SYH1684	*Pinus halepensis* forest
13 F	Bolulla (Al), Coll de Rates; 30SYH5185	Clear *Q. rotundifolia* forest plus *P. halepensis*
14 F	Xelva (Va), Sª del Negrete; 30SXJ68	Clear *Q. rotundifolia* forest
15 F	Xovar (Ca); 30SYK2916	*Q. rotundifolia* forest
16F-17F	Crevillent (Al), Sª de Crev.; 30SXH83	Clear *Q. rotundifolia* forest plus *P. halepensis* (sites 1-2)
18F-19F	Crevillent (Al), Sª de Crev.; 30SXH83	*Pinus halepensis* forest (sites 3-4)
20-21F	Eslida (Ca), Sª de Espadán; 30SYK2916	*Quercus suber* (sites 1-2)
22 F	Ibi (Al), Port del Biscoi; 30SYH0980	*Q. rotundifolia* forest
23 F-26 F	Tibi (Al), Sª del Maigmó; 30SYH06	*Q. rotundifolia* forest. (sites 1-4)

(All sample sites were located using Spanish Military Ordnance Survey Maps, scale 1: 50.000. Abbreviations: (Al) Alacant, (Va) València, (Ca) Castelló).

for 5 min to avoid contractions that may have altered their morphology and morphometrics. Nematodes were then fixed in TAF (Triethanolamine/Formaldehyde 40% aq./Water, 2:7:91, v/v/v) before being transferred to glycerin and mounted on microscope slides. Identification of the genera was carried out using the text of Southey (1978).

The original soil sprinkling technique (Drechsler, 1941), with slight modifications (Stirling, 1991), was used for the isolation of nematophagous fungi. Half to 1 g fresh (not dried) soil was aseptically sprinkled on 1% water-agar (WA) plates previously baited with approximately 5000 live nematodes. Axenic cultures of the free-living species *Panagrellus redivivus* (L.) Goodey (Jansson and Nordbring-Hertz, 1979) were used because this nematode is attacked by a large range of nematophagous fungi (Gray, 1983).

To increase the probability of isolating the nematophagous fungi to 90%, soil was sprinkled on six replicated Petri dishes with WA (Bailey and Gray, 1989). Plates were then kept at 25 °C and observed after 5-7 days. Soil sprinkling was carried out in the following two experiments.

In the first experiment (Experiment 1), predatory nematophagous fungi were checked for each week for one month to detect *Arthrobotrys*-like conidiophores (straight, hyaline and with denticles in the apex for conidium insertion in a terminal "rosette" or "umbrella"). Most of predatory nematophagous fungi form this type of conidiophores (De Hoog and Van Oorschot, 1985). Conidia from these structures were plated on corn meal agar (CMA) and the fungal species determined from pure cultures of the fungi.

In the second experiment (Experiment 2), the same conditions applied but all sprinkled plates were inspected weekly for six weeks. During that period, numbers of *Arthrobotrys*-like conidiophores/plate (n = 3 plates per soil studied) were scored per each soil sample and nematophagous fungus species were again recorded to detect other predatory fungi not observed in the first experiment. Furthermore, agar samples (n = 3) from soil sprinkled plates were also checked weekly under the microscope. This allowed the detection of predatory fungi with different structures from the conidiophore described above. Experiment 2 was also devised to detect nematodes infected with endoparasitic fungi. After the six week period, plates were renovated by adding (at 45 °C) a fresh 1% WA layer and further baiting with live nematodes (Duddington, 1955). After this, plates were further incubated (14 days) and then checked for

the presence of nematophagous fungi.

Due to their life history and small spores, endoparasitic nematophagous fungi are more difficult to isolate than predatory fungi. Thus they require particular techniques for their detection and study. Infected nematodes from soil sprinkled plates were added to healthy nematodes (in sterile water or on WA) to infect them (modified from Barron 1982). The nematode used for baiting soil sprinkled plates (*P. redivivus*) has also been used as a bait for the isolation of endoparasites (Jansson and Nordbring-Hertz, 1979). Nematodes used as bait must preferably be free of culture media particles. For this purpose a method based on discontinuous gradient centrifugation was devised (Lopez-Llorca and Olivares-Bernabeu, 1995). After centrifugation, axenically grown *P. redivivus* free from culture media were mixed with infected nematodes as bait for isolation of endoparasitic fungi.

Endoparasites were also isolated with the Baermann funnel (Barron, 1982). The method is based on the fact that a proportion of nematodes extracted with the funnel will be infected by fungal pathogens. Twenty g of soil were placed in the funnel and after 24 h nematodes were collected. One ml aliquots of nematode suspension were plated on 1% WA plates. These plates were baited with axenic and culture-medium-free *P. redivivus*. Plates were checked at 10 days intervals and infected nematodes were washed in sterile distilled water and inoculated on CMA plates to isolate nematophagous fungi present. WA was used instead of CMA to enhance endoparasites and inhibit saprophytes. To prevent bacterial contamination, 50 µg/ml penicillin and 50 streptomycin were added after sterilization and cooling of the medium at about 45 °C.

Endoparasitic fungi were also isolated using the differential centrifugation technique (Barron 1982). The bait nematode was *P. redivivus* from axenic cultures. WA plates were examined weekly to detect the presence of parasitized nematodes. These plates were poured a few days before the addition of nematodes and soil to prevent excess water and thus limiting development of bacteria and slime moulds.

RESULTS

The average numbers of nematodes per gram of dried soil of all soil samples studied are given in Table II. The main taxonomic groups of nematofauna found in forest and *Citrus* soils are also included in Table II. Nematodes were clearly more abundant in forest than in *Citrus* soils. Most nematodes found were free-living species specially belonging to the genus *Rhabditis* which was present both in forest and *Citrus* soils. Plant parasitic nematodes of genera *Filenchus*, *Aphelenchoides* and *Aphelenchus* were found in both types of soils studied. *Pratylenchus*, *Psilenchus*, *Lelenchus*, *Zygotylenchus*, *Tylenchus* and *Helicotylenchus* were only found in forest soils, but they were less abundant than free-living species. Predatory nematodes (Mononchidae) were also found in the forest soils.

Table III summarizes the nematophagous fungi found in both experiments. In the first experiment, only *Arthrobotrys spp.* were detected using soil sprinkling. The most frequent nematophagous fungus both in *Citrus* and forest soils was *Arthrobotrys oligospora* Fresenius var. *oligospora*, an adhesive tridimensional network former. Other species (less frequently found) were: *A. arthrobotryoides* (Berlese) Lindau, *A. oligospora* Fresenius *var. microspora* (Sopurnov) van Oorschot, *A. oligospora* Fresenius *var. sarmatica* (Jarowaja) van Oorschot, *A. javanica* (Rifai *et* R.C. Cooke) Jarowaja and *A. musiformis* Drechsler.

Table II. Abundance of nematodes present in the agricultural and forest soils sampled.

	Forest soils (N = 19)	Agricultural soils (N = 7)
Number of nem./g dry soil (Average; n = 3)	63.4 ± 16.7	22.0 ± 10.1
Percentage of free living nem.	91.8 ± 9.9	82.2 ± 20.0
Percentage of plant parasitic nem.	8.2 ± 9.9	17.7 ± 20.0
Predominant families and genera of free living nematodes	Fam. Rhabditidae (g. *Rhabditis*) Fam. Plectidae (g. *Wilsonema*) Fam. Cephalobidae (g. *Acrobeles*) Fam. Camacolaimidae (g. *Aphanoilaimus*) Fam. Dorylaimidae (g. *Aporcelaimellus*) Fam. Mononchidae	Fam. Rhabditidae (g. *Rhabditis*) Fam. Panagrolaimidae (g. *Panagrolaimus*) Fam. Cephalobidae (g. *Acrobeloides*)
Percentages of samples with plant parasitic nematodes	*Pratylenchus* (12.5%) *Psilenchus* (6.2%) *Filenchus* (75%) *Lelenchus* (12.5%) *Aphelenchoides* (18.7%) *Aphelenchus* (43.7%) *Zygotylenchus* (12.5%) *Tylenchus* (25%) *Helicotylenchus* (18.7%)	*Filenchus* (83.3%) *Aphelenchus* (66.7%) *Aphelenchoides* (16.7%)

Abbreviations: N (total number of soil samples); n (number of replicates /soil sample).

Soil sprinkling *Experiment 2* (which ran for six weeks) revealed, apart from the predatory species already described, other nematophagous fungi. *A. brochopaga* (Drechsler) Schenck, Kendrick *et* Pramer, a predatory nematophagous fungus forming constricting rings, was found in *Citrus* soils. *Monacrosporium* sp., a predatory fungus forming bidimensional adhesive networks, was found in both *Citrus* and forest soils.

The endoparasitic nematophagous fungi, *Catenaria anguillulae* Sorokin was the most commonly found species, appearing in virtually all soils studied. *Myzocytium sp.*, another endoparasite, was found in both forest and *Citrus* soils but less commonly than *C. anguillulae*. *Nematoctonus concurrens* Drechsler, a nematophagous basidiomycete with characteristic "clamp connections" in its hyphae, was found in agricultural soils. In spite of the variety of techniques used for isolation and detection (Table IV), only a few fungal endoparasites of nematodes were found and identified. It is noticeable that different isolation techniques isolated different species of nematophagous fungi.

Figure 1 shows the evolution of *Arthrobotrys* spp. in plates sprinkled with *Citrus* or forest soils, estimated by the average number of conidiophores per plate. An early development (1st week) of *Arthrobotrys spp.* in *Citrus* soils was in contrast with a later development of the fungus for forest soils (5th week). The most abundant and earliest nematophagous fungus in *Citrus* soils (Fig. 1a)

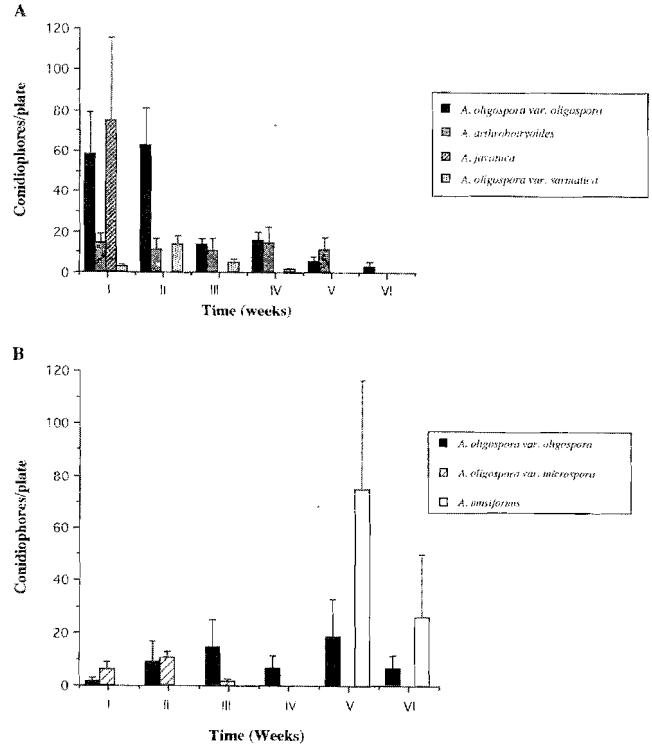

Fig. 1. Evolution of the abundance of *Arthrobotrys spp.* (average no. of conidiophores/plate) on 1% water agar plates sprinkled with soil and baited with the free-living nematode *Panagrellus redivivus*. A) Agricultural (*Citrus*) soils; B) forest (*Q. rotundifolia* and *P. halepensis*) soils.

Table III. Summary of the species of nematophagous fungi found in the soils sampled for the present study (see Table I for details).

	Species of nematophagous fungi	Presence in soils (sample No. see Table I)	Conidium size	
			Range	Average
(1)	*Arthrobotrys oligospora var. oligospora*	1A, 2A, 3A, 4A, 6A, 7A, 12F, 13F, 14F, 20F, 21F, 22F	19-24 x 10-12 µm	21.44 x 11.2 µm
(1)	*A. arthrobotryoides*	5A	13-22 x 10-13 µm	18.56 x 11.52 µm
(1)	*A. oligospora var. Microspora*	26F	13-22 x 6-10 µm	17.28 x 7.36 µm
(1)	*A. oligospora var. Sarmatica*	7A	32-38 x 10-13 µm	35.52 x 11.20 µm
(1)	*A. javanica*	3A, 4A	26-35 x 6-10 µm	28.50 x 9.50 µm
(1)	*A. musiformis*	20F, 21F	26-38 x 6-10 µm	31.68 x 8 µm
(1)	*A. brochopaga*	3A, 4A, 5A	38-45 x 6.45 µm	42.95 x 6.45 µm
(1)	*Monacrosporium* sp.	6A, 22F	(n.d.)	(n.d.)
(2)	*Catenaria anguillulae*	All soil samples	(n.d.)	(n.d.)
(2)	*Myzocytium* sp.	5A, 23F, 24F	(n.d.)	(n.d.)
(3)	*Nematoctonus concurrens*	5A	(n.d.)	(n.d.)

Data on the conidium size of the fungi where appropiate are given. Abbreviations: Numbers in brackets on the first column indicate the relative abundance of the fungi from (1) most abundant to (3) least abundant. n.d. = not determined.

Table IV. Endoparasitic nematophagous fungi present in soil samples (see Table I) and techniques used for their detection.

Technique	Forest soils	Agricultural soil
Soil Sprinkling on WA	*Catenaria anguillulae*	*Catenaria anguillulae*
Differential Centrifugation	*Myzocytium sp.*	*Myzocytium sp.* *Nematoctonus concurrens*
Baermann Funnel	Nematodes with sterile mycelia (no spores detected)	Nematodes with sterile mycelia (no spores detected)

was *A. javanica,* closely followed by *A. oligospora* var. *oligospora*. *A. musiformis* was the most abundant species in forest soils (Fig. 1b), but it appeared later than *A. oligospora* var. *oligospora*. It is also interesting to consider the prevalence of nematophagous fungi (% samples with a particular species) as well as abundance. The most prevalent species (nearly 50% of total soil samples analysed) was *A. oligospora* var. *oligospora* (Fig. 2). The rest of the fungi appeared in less than 10% of soil samples studied. *A. oligospora* var. *oligospora* was the most frequent in agricultural (Fig. 3a) (close to 90% of samples) and forest (Fig. 3b) soils (30% of samples) followed by *A. javanica* (30%) or *A. musiformis* (15%) for agricultural and forest soils respectively.

C. anguillulae was the most abundant endoparasitic species found in the soils tested. The remaining endoparasites appeared only irregularly and not in all soils. Figure 4 shows that the presence of the fungus decreased with time in soil sprinkling plates. One week after sprinkling, the fungus occurred in more agricultural than forest soils. After two weeks, the prevalence of the fungus was approximately the same for both soil types. The presence of the fungus decreased with time in both soil types.

Figure 5 provides a general view of the frequency of predatory and endoparasitic fungi in soil for the whole six week period of study. In agricultural soils (Fig. 5 solid bars) predatory fungi were found in all samples studied. *C. anguillulae* was found in 82% of the samples and other endoparasites were less frequent (less than 20%

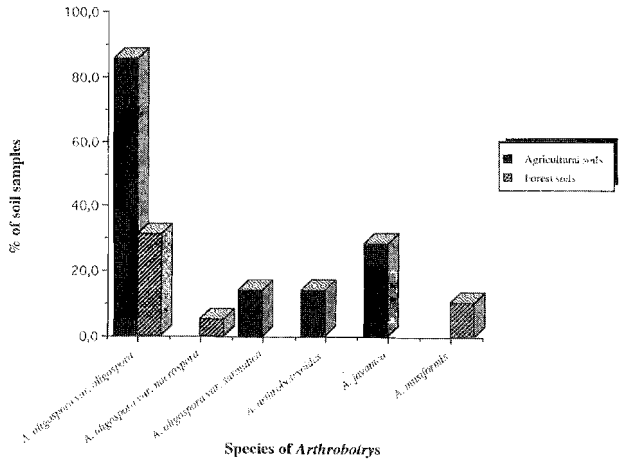

Fig. 2. Global prevalence of *Arthrobotrys* spp. (% of soils where the fungus was detected).

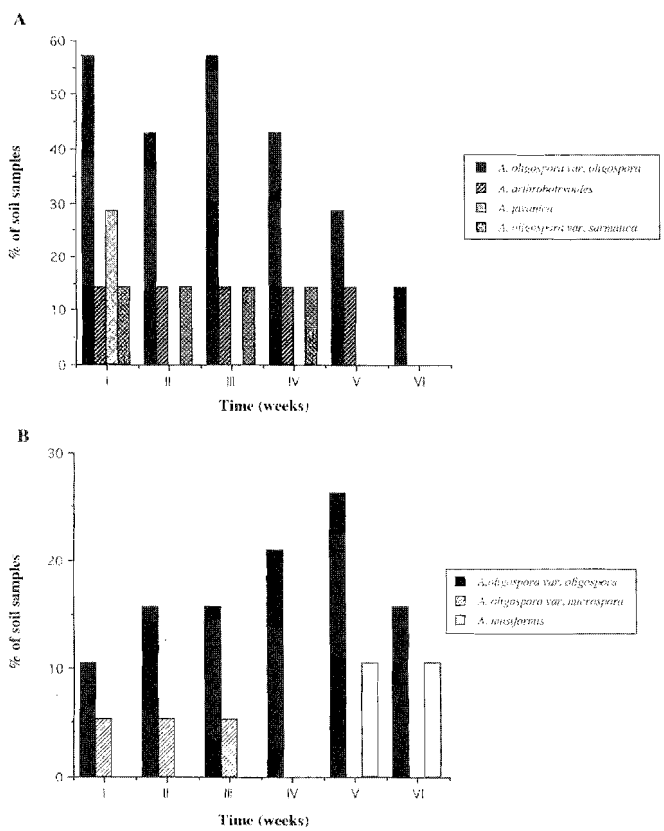

Fig. 3. Evolution of the frequency (as in Fig. 2) of *Arthrobotrys* spp. on 1% water agar plates sprinkled with soil (agricultural or forest) and baited with *P. redivivus* for the six week period of the experiment. A) agricultural soils; B) forest soils.

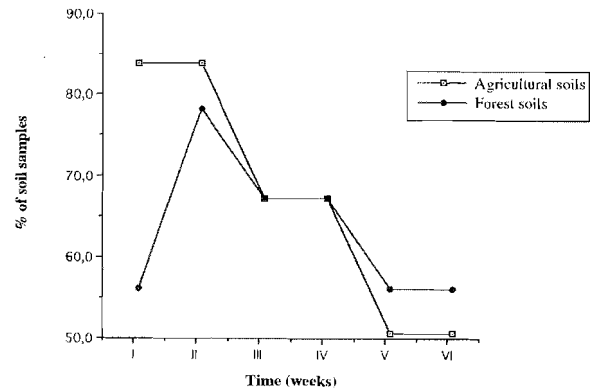

Fig. 4. Evolution of the frequency (as in Fig. 2) of the endoparasitic fungus *Catenaria anguillulae* on 1% water agar plates sprinkled with soil (agricultural or forest) and baited with *P. redivivus* for the six week period of the experiment.

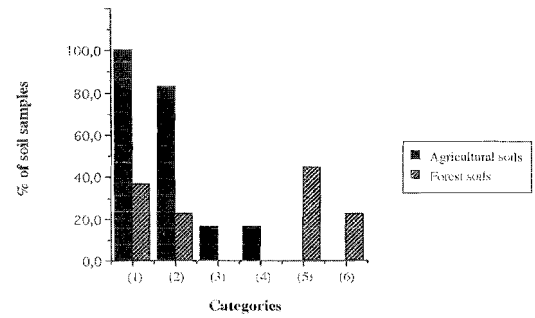

Fig. 5. Global frequency of predatory and endoparasitic nematophagous fungi in soil sprinkling plates. Values are percentage of soil samples (solid forest, hatched agricultural soils) where the fungi indicated were detected. Categories: (1) predatory nematophagous fungi; (2) predatory fungi + *C. anguillulae*; (3) predatory fungi + *C. anguillulae* + *Myzocytium sp.*; (4) predatory fungi + *C. anguillulae* + *Myzocytium sp.* + *Nematoctonus concurrens*; (5) *C. anguillulae* only; (6) *C. anguillulae* + *Myzocytium sp.*

of soils). In forest soils (Fig. 5, hatched bars) predatory fungi were less frequent than in agricultural soils. For 50% of samples studied, *C. anguillulae* was the only nematophagous fungus found.

DISCUSSION

Nematodes in this study were more abundant in forest than agricultural soils probably due to the higher organic matter content in the former that allows development of a more abundant microbiota (Gray, 1985). The most prevalent group of nematodes were free-living *Rhabditis* spp. which were present in both forest and agricultural soils. Predatory nematodes of Mononchidae family were also found in the forest soils. Plant-parasitic nematodes of the genera *Filenchus*, *Aphelenchoides* and *Aphelenchus* occurred in soils from both types of habitats. Nematodes of the genera *Pratylenchus*, *Psilenchus*, *Lelenchus*, *Zygotylenchus*, *Tylenchus* and *Helicotylenchus* were found only in forest soils. Their average numbers were lower than those of free-living nematodes. Populations of plant-parasitic nematodes may have been underestimated since they are associated with the rhizosphere and we did not sample soil around the roots as we set out to study global nematode populations.

Using the soil sprinkling technique the most common fungi isolated were predatory nematophagous fungi. Although soil sprinkling experiments are not strictly quantitative (Dackman *et al.*, 1987) we estimated the abundance (as no. conidiophores/plate) and prevalence (percentage of soil samples where a species appeared) of predatory species (*Arthrobotrys* spp.). The most prevalent nematophagous fungus was the adhesive net former *A. oligospora* var. *oligospora*, confirming the findings of Persmark *et al.*, (1996). The abundance of nematophagus fungi was influenced by soil type. In this respect phenolics in the forest soils of this study delayed the development of the fungus (Lopez-Llorca and Olivares-Bernabeu, 1997). The remaining *Arthrobotrys* species were less common but *A. javanica* and *A. musiformis* showed two peaks of abundance (in *Citrus* and forest soils respectively). Jansson (1985) concluded that adhesive net formers (i.e. *A. oligospora*) appear first on soil sprinkling plates and, after several weeks, fungi forming constricting rings (such as *A. brochopaga*) develop. Rings or conidial traps of *A. brochopaga* and *Monacrosporium* spp. networks were less common than structures of adhesive net forming *Arthrobotrys* spp. Differences between species sharing the same capture device (i.e. tridimensional adhesive networks) as found in this study, remain unexplained. Although predatory nematophagous fungi are nematode density independent (Stirling, 1991), nematodes in soil sprinkling plates may help them to overcome fungistasis by providing an alternative nutrient source (Mankau, 1962). Regarding endoparasitic nematophagous fungi, two zoosporic fungi *Catenaria anguillulae* and *Myzocytium* sp. were the most commonly found. The Mediterranean soils studied in this research are therefore an important reservoir of nematophagous fungi.

The present survey probably detected most of the predatory fungi in the soils tested, therefore further studies should concentrate on the facultative parasitic fungi i.e. those that usually parasitise nematode eggs. These latter antagonists have been used as biocontrol agents and studies on these fungi (Olivares-Bernabeu and Lopez-Llorca, 2002) are in progress.

ACKNOWLEDGEMENTS

This research was partly funded by Grants (PB 96-0413, AGL2001-0734) of the Spanish Ministry of Science and Technology.

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Accepted for publication on 7 October 2002.

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