

Departamento de Ciencias Ambientales y Recursos Naturales. Universidad de Alicante, Aptdo,  
Correos 99, 03080 Alicante, Spain\*

Zoology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, United Kingdom\*\*

## A CONTRIBUTION TO THE ECOLOGY OF FUNGI ASSOCIATED WITH FEMALES AND CYSTS OF *HETERODERA AVENAE* IN EASTERN SCOTLAND

by

L. V. LOPEZ-LLORCA\* and B. BOAG\*\*

**Summary.** Infected field soil was used to investigate the colonisation of cereal roots by fungi associated with *Heterodera avenae* in eastern Scotland. Results indicated that *Verticillium* invaded cereal roots before females of *H. avenae* and that the fungus colonised wheat roots more rapidly than oats while barley roots were invaded least rapidly. A greater number of *H. avenae* juveniles were found associated with roots infected by *Verticillium* compared with uninfected controls. After surface sterilization with sodium hypochlorite, white females of *H. avenae* developed only *Verticillium* colonies, suggesting that the fungus colonizes the female at an early stage of development. There was also evidence that *Verticillium* may produce metabolites that stimulate premature hatching of the juveniles contained in the eggs.

The fungal parasites affecting cyst nematodes have a dual action: they infect and destroy the female nematode (Kerry, 1980; Kerry and Crump, 1980) or penetrate and kill the developing egg (Kerry, 1980; Kerry and Crump, 1977; Morgan-Jones *et al.*, 1981). Cyst nematodes are also attacked by predaceous fungi, but fungal parasites of females and eggs are probably more effective in reducing nematode multiplication (Kerry, 1986).

Younger eggs are generally more susceptible to fungal attack than older ones (Cayrol *et al.*, 1982; Irving and Kerry, 1986). The physiological state of the eggs probably also influences their susceptibility to fungal colonization (Nigh *et al.*, 1980; Irving and Kerry, 1986). Although some aspects of the biology of fungi infecting the eggs of cyst nematodes are known, their mode of action is not yet fully understood. There is evidence that *Verticillium cblamydosporium* is able to colonise the cereal rhizosphere (Barron and Onions, 1966). Kerry *et al.* (1984) reported that *V. cblamydosporium* colonises the wheat rhizosphere, causing no damage to the plants.

In pot tests using soil containing the egg parasite *Dactylella oviparasitica* and inoculated with egg masses of *Meloidogyne incognita*, Stirling and Mankau (1979), observed that the percentage of eggs infected increased up to nearly 50% 50 days after inoculation. By means of field-soil sequential sampling Kerry *et al.* (1982) studied changes in egg-infection by naturally-occurring fungi in females and newly-formed cysts of *Heterodera avenae*. They found larger values of egg-infection in newly-formed cysts than

in females and an increase of infection with time. They also found large numbers of *H. avenae* females containing few eggs.

In a pot experiment (also with naturally infected soil) Ownley Gintis *et al.* (1983) found an increase with age in the extent of fungal colonisation of females and newly-formed cysts of *H. glycines*. They also observed a decrease in diversity, so that newly-formed cysts infected by fungi contained only few species (including *V. cblamydosporium*) compared with females. Godoy *et al.* (1983) studied fungal egg-infection in *M. arenaria* and also found a restricted fungal flora. Similar results were obtained by Tribe (1979) who studied fungal infection of cysts and females of *H. avenae* and *H. schachtii*.

Other important aspects of the biology of nematode egg-parasites such as their sensitivity to soil mycostasis are unclear but they have proved important in the ecology of soil fungi in general (Jackson, 1957; Ko and Lockwood, 1970) and nematophagous fungi in particular (Mankau, 1962; Lopez-Llorca and Boag, 1990). The mycostatic action of rhizosphere bacteria to fungal parasites of *H. avenae* was mainly restricted to young females (Lopez-Llorca and Boag, 1990) and it was concluded that fungistasis may have protected them from nematophagous fungi, but its effects decreased with the age of the nematodes.

In order to understand the fungal infection process it is relevant to study the sequence of events. Crump and Kerry (1977), using observation chambers, studied the maturation of *H. avenae* females and their infection by an

Entomophthora-like fungus (later identified by them as the new Oomycete *Nematophthora gynophila*). Observation chambers have been used in similar studies by other authors (La Mondia and Brodie, 1984).

Colonisation of cyst-nematode females is the crucial event in the destruction of nematode eggs by parasitic fungi. The main objectives of this paper were to ascertain the factors involved in the fungal ecology of the egg-parasites of the cereal cyst nematode *Heterodera avenae* Woll., relevant to their mode of action, such as: the role of the rhizosphere in the infection process, the presence of parasitic fungi in soil, the timing of the colonisation of nematode females by fungal parasites, and finally their effects on egg-production, egg-infection, juvenile hatching and egg-distortion.

Some of the problems in the estimation of the extent of parasitism found in previous studies on egg-parasites biology were overcome by the use of growth-restricting media (Lopez-Llorca and Duncan, 1986). In this paper, these media, and a micro-technique for plating eggs from a single female/cyst, were used in pot experiments to assess the various factors affecting egg-parasitism. The main approach used was sequential sampling of cereal roots containing nematodes (at different stages of development), which were infected naturally with fungi.

## Materials and methods

*Heterodera avenae* females and newly-formed cysts were obtained in pots with susceptible oats (*Avena sativa* L. cv. Peniarth) growing in naturally infested soil (Spittalfield, Blairgowrie, Scotland) known to contain endoparasitic fungi from a survey for fungal parasites of *H. avenae* in eastern Scotland (Boag and Lopez-Llorca, 1989).

The soil used for pot-experiments was collected (November 1986 and February 1987) from eight points of the same field, bulked and stored at 5 °C in the dark for at least 5 weeks before use. This helped to break the diapause and ensure that *H. avenae* would hatch (Fisher, 1981). To obtain a uniform invasion of the roots by *H. avenae* juveniles, the soil was left outdoors (from 20 April-10 May, 1987) before planting one-week-old oats seedlings. Five seedlings were planted per (13x13x11) cm pot, which were then placed in growth cabinets at 20 °C/16 hrs day-length. NPK fertilizer (15:8:8) was added to all the pots.

Six pots were sampled destructively at 4, 5, 6, 7, 9, 12 and 14 weeks after planting. On each sampling occasion the oat plants were lifted, together with the rhizosphere soil. The tops of the plants were removed and the roots were left for 15 min in tap water to loosen soil and debris. Then the root systems were washed gently in tap water and checked under the microscope for the presence of white females and/or newly-formed cysts.

Whole root-systems were plated to determine the extent of fungal colonisation by fungal egg-parasites. Oat root-systems were sampled at 1, 2, 3, 6, 7, 9 and 14 weeks after planting, from 2 to 5 pots (1 to 5 roots per pot). The roots were plated and incubated as for single cysts and females (described below) and the presence of *Verticillium* and other fungi scored. The number of *Verticillium* colonies per root-system was also scored. To test the effect of different cereal species on the root colonisation by egg parasites, barley (*Hordeum vulgare* L. cv. Esk) and wheat (*Triticum aestivum* L.) whole root systems were sampled, for barley at 1 and 2 weeks, for wheat at 1, 2 and 3 weeks after planting (from 2 pots, 4 to 5 roots per pot), then incubated and scored in the same way as for the oats.

To investigate the fungi associated with those fragments of root containing nematodes (females and newly-formed cysts appear frequently clumped together in the roots in necrotic areas), root pieces containing nematodes were sampled and plated on "Medium 1" (Lopez-Llorca and Duncan, 1986) modified as for females and cysts, together with the nematodes occurring in them, and the fungi developing from nematodes and root-pieces were scored. To count nematodes within the roots, after the roots had been scored for the presence of fungi, the roots were washed in water, stained in 0.05% (w/v) acid fuchsin in water/lactic acid/glycerol (1:1:1, v/v/v), destained in glycerol/water (1:1, v/v) and the nematodes counted (Hooper, 1970).

The soil from two pots was sampled 4 weeks after planting and used for soil dilution plating. The soil from one pot was mixed with 100 ml of water and shaken. A sub-sample was taken to include an equivalent of 10 g (dry weight) soil. The subsample was then diluted 1/10 in sterile distilled water (SDW), shaken for 20 min and then a dilution series (1/10, 1/100, 1/1000) was made, shaking each dilution for 5 min. One ml of each dilution was added to sterile empty 9 cm plastic Petri dishes. Cool (45 °C) malt extract agar (Oxoid), including the same additives as Medium 1 (modified as for females and cysts) was poured on to the plates. These were then shaken gently to mix the soil/water and the agar. The plates were incubated at 20 °C in the dark for 5-7 days before scoring fungal colonies, but after 8-10 days sporulation had occurred, thus enabling fungal identification.

Nematodes were picked-off the root systems, and the numbers of white females and newly-formed brown cysts per plant determined. White females were separated, according to their size and shape, into two age-categories viz. young females (small and elongated) and older females ('lemon-shaped' and bigger).

Females and cysts from roots, plus old cysts from soil in pots and field samples, were washed twice in sterile distilled water (SDW) and then plated on a growth-restrict-

ing medium ("Medium 1", Lopez-Llorca and Duncan, 1986) slightly modified (no rose bengal was included and penicillin was used instead of aureomycin) in 90 mm plastic Petri dishes. An average of 10 females or cysts per dish were plated. Plates were incubated at 20 °C in the dark for 6-7 days. Cysts and females were then inspected for the presence of fungi, which were identified.

To test the effect of surface sterilization on the incidence of fungi in white females, newly-formed cysts and the root pieces containing them, females, cysts and roots were treated for 1 min in 1% sodium hypochlorite and washed five times in SDW before plating and incubating them as for untreated specimens. To ascertain the percentage of eggs infected by fungi in each cyst or female, the following micro-technique was developed. Once plates containing females/cysts were scored, those which were colonised by fungi, were tested for egg infection as follows: females and/or cysts were picked up in sterile conditions and washed twice in SDW. They were then opened under a dissecting microscope in a droplet of SDW in sterile conditions, and the eggs released from the cyst. The cyst walls and, if present, the sub-crystalline layers from each cyst or female were then plated onto split (two-chambers) 90 mm diameter plastic Petri dishes (Sterilin Ltd., Feltham, England) containing Medium 1 modified as already explained. The eggs in the droplet were pipetted onto the plate (containing the cyst walls and the sub-crystalline layers) and spread using a capillary made by flaming the end of a 230 mm Pasteur pipette (John Poulten Ltd., Essex, England) and pulling it to the appropriate diameter size for collecting the eggs in the droplet and spreading them onto the agar. The eggs contained within each individual female, newly-formed cyst, or mature cyst were plated. The species of the fungus which was found colonizing the female/cyst whose eggs were being tested for fungal infection was recorded on the plates and the plates were incubated as before.

To determine the effects of egg-parasites (*Verticillium* in particular) upon eggs in females and newly-formed cysts, after incubation the following categories were scored per individual: INFECTED eggs (those developing a fungal colony), DISTORTED eggs (those whose contents were not identifiable as juveniles or embryos but in which no fungi were detected), egg-shells (empty), second-stage juveniles (hatched) and NORMAL eggs (containing embryos or second-stage juveniles). The numbers of egg-shells (empty) should equal those of second-stage juveniles (hatched) per individual female or cyst but in practice their numbers differ because some juveniles may have left the females before the count was made; or the numbers of egg-shells may be less than that of juveniles because, when counting large numbers, egg shells may be underestimated, as they are less conspicuous than juveniles. Consequently, the best estimate for the number of juveniles hatched per female or cyst is the greater of the

number of egg-shells and the number of juveniles, and this number was used in subsequent calculations. The proportions (%) of hatched, infected and distorted eggs were estimated. Fungi developing from cyst walls and sub-crystalline layers were also recorded.

## Results

*Verticillium* colonised roots before females of *H. avenae* were present and its incidence progressively increased (Table I). The effects of several species of cereals on root colonisation by the fungus were also tested at 1-3 weeks after planting (Table II). Wheat roots were colonised by *Verticillium* more rapidly than those of oats and those of barley were colonised least rapidly. The wheat roots, sampled one week after planting, from three pots, and plated to determine the presence of *Verticillium*, were separated into two groups according to the presence or absence of the fungus. Four roots of each group were stained and the numbers of *H. avenae* juveniles per root counted. Roots which developed *Verticillium* when plated contained on average  $12.25 \pm 6.3$  juveniles per root, whereas those which did not develop the fungus contained only  $1.75 \pm 0.8$  juveniles per root. Common soil fungi, most often *Cylindrocarpon* and less frequently *Acremonium*, *Paecilomyces* and *Penicillium*, were also detected on roots. As their role in egg parasitism was less important than that of *Verticillium* spp., their presence was not considered in this study.

In soil sampled four weeks after planting, only the dilution  $10^{-2}$  contained a number of colonies suitable for counting. Amongst these colonies, only a fraction were developed well enough to be identified. Several species of egg-parasites were detected (including *Verticillium*) but they were few compared with the total fungal flora of the soil (Table III).

TABLE I - Occurrence of *Verticillium* in oat root-systems grown in pots.

Weeks after planting	Percentage of root systems developing <i>Verticillium</i> (from 7 to 19 roots)	No. of <i>Verticillium</i> colonies developing per root
1	0	0
2	33	$0.3 \pm 0.2$
3	17	(not determined)
4	56	$0.68 \pm 0.17$
7	43	$0.71 \pm 0.35$
9	68	$1.22 \pm 0.28$
14	80	$0.9 \pm 0.18$

TABLE II - Colonisation of oats, barley and wheat root-systems by *Verticillium*.

Weeks after planting	Percentage of root systems developing <i>Verticillium</i>			No. of <i>Verticillium</i> colonies per root		
	Oats	Barley	Wheat	Oats	Barley	Wheat
1	0	0	40	0	0	1±0.3
2	33	0	70	0.3±0.2	0	2±0.5
3	16	n.d.	25	n.d.	n.d.	n.d.

Mean deviance ratio in analysis of deviance for binomial proportions for differences between plant species = 13.26 ( $P < 0.05$ ). n.d. = not determined.

TABLE III - Fungi in soil determined by dilution plating of soil from pots.

Total no. CFU	No. identifiable CFU	Fungal parasites (CFU)					
		B. fung.	A	T	C	V	P
POT1							
123000 (18300)	40500 (4550)	5680 (1740)	3900 (1570)	3200 (1210)	2480 (1320)	1060 (680)	2840 (1420)
POT2							
116000 (5560)	41500 (8920)	6700 (1450)	8690 (2280)	670 (670)	670 (670)	670 (386)	1000 (1000)

Abbreviations: CFU = colony forming units  $g^{-1}$  dried soil. B. fung. = 'Black fungus', A = *Acremonium* sp., T = *Trichoderma* sp., C = *Cylindrocarpon* sp., V = *Verticillium* sp., P = *Paecilomyces* sp. Average values of 4 estimations per pot. S.e.m. is given in brackets.

The numbers of females of *H. avenae* per plant reached a peak about 7 weeks after planting and then decreased (Fig. 1). Newly-formed cysts ('brown cysts') per plant were considerably fewer than white females and reached a maximum 12-16 weeks after planting, lower than that for white females. Where 'young' and 'old' females per plant were scored throughout the experiment, young females reached a maximum peak concurrent with and higher than that of 'old' ones.

The numbers of untreated specimens (rinsed in SDW only) which developed *Verticillium* only, increased with time as did the percentage of untreated females which developed *Verticillium* only (Fig. 2c-d). The percentage of newly-formed cysts, which developed *Verticillium* only was generally higher than that for females. In the later samplings, a decrease in newly formed cysts infected with *Verticillium* was observed.

With NaClO-treated females (Fig. 2a) only specimens developing *Verticillium* were found at weeks 6 and 9. The percentages of females from which fungal colonies developed were lower than for the corresponding untreated females (Fig. 2c). With newly-formed cysts (Fig. 2b) the pre-treatment with sodium hypochlorite did not decrease the occurrence of fungi as greatly as with white females. Also, fungi other than *Verticillium* were found in treated specimens, in approximately the same proportion (from week 9 until week 14) as for untreated. In newly-formed cysts no trends were evident in the percentages of nematodes developing *Verticillium* only, or those developing *Verticillium* and other fungi. The variation in the percentages was greater in untreated specimens (Fig. 2d).

In NaOCl-treated females (Fig. 2a) the numbers of specimens from which fungi other than *Verticillium* developed (including those containing other fungi) were greatly

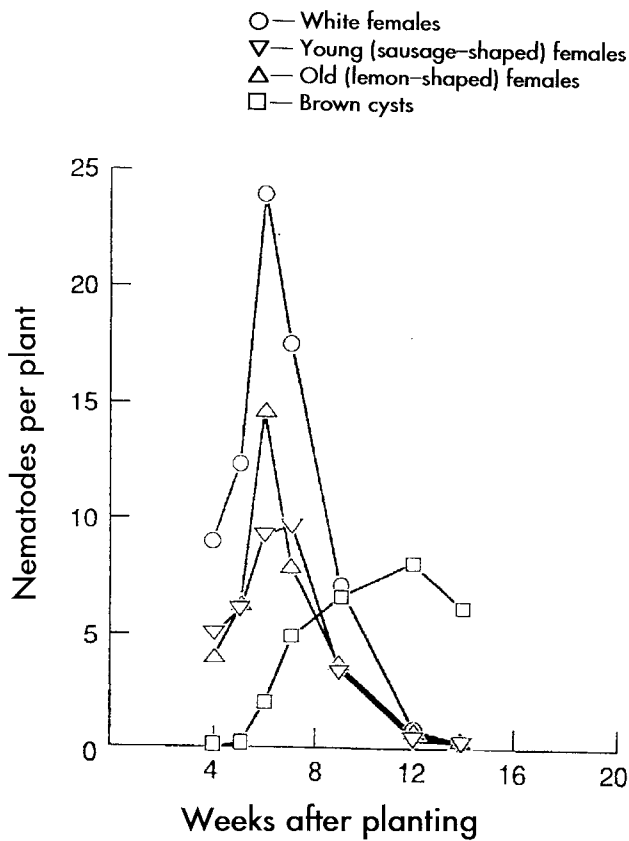


Fig. 1 - Numbers of *H. avenae* females and newly-formed cyst per plant in pots.

reduced in comparison with untreated specimens for the same sampling occasions (Fig. 2c). In newly-formed cysts, NaOCl-treated (Fig. 2b) and untreated (Fig. 2d), no such differences were observed.

Untreated females from the same root area showed extensive colonisation by *Verticillium* and other fungi (Fig. 3a). When females were treated with hypochlorite, very few developed fungi (Fig. 3b) and *Verticillium* was virtually the only fungus observed. In particular, Fig. 3b shows that one female out of more than 10 from the same 'cluster' was infected with *Verticillium*. Newly-formed cysts treated with hypochlorite (Fig. 3c) were extensively colonised by *Verticillium*. Root-pieces which had contained the nematodes developed, in untreated specimens (Fig. 3a), common soil fungi (mainly *Cylindrocarpum*) whereas in treated specimens (Fig. 3b, 3c) few or no fungi were found.

*Cylindrocarpum* and several sterile mycelia were the fungi, other than *Verticillium*, most commonly found in females and newly-formed cysts (Table IV). *Acremonium* spp., 'Black-fungus' (including probably several species of

melanic fungi), and other fungi in lower percentages such as *Paecilomyces*, *Trichoderma* and *Penicillium* were also found. Not all *Verticillium* isolates from infected females or cysts produced the dictyochlamydo spores typical of *V. chlamydosporium*. The percentage that did was recorded for the different sampling occasions and the results are

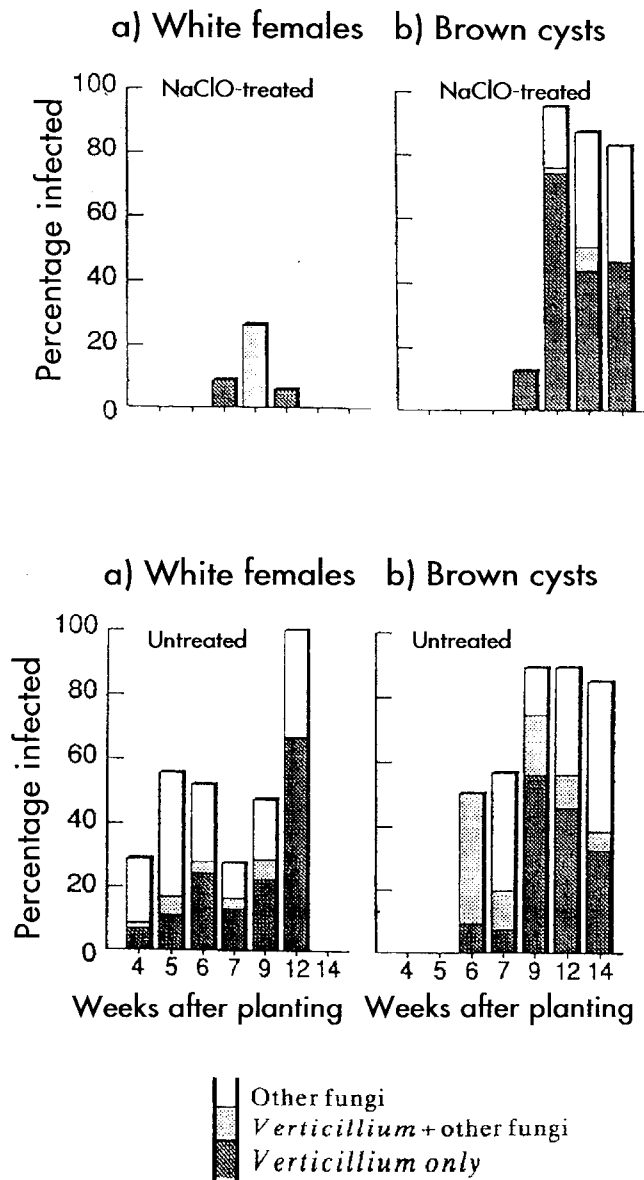


Fig. 2 - Fungal colonisation of females ('white') and newly-formed cysts ('brown') in pots. Specimens were either untreated (c-d) or treated (a-b) with 1% sodium hypochlorite. See key of shadowing in the histogram for fungi involved.

TABLE IV - Percentages of fungi, different from *Verticillium* ('other fungi') developing from females and newly-formed cysts of *Heterodera avenae* (numbers in brackets) in pots.

Week	Cyl.	st. myc.	Acre.	B. fung.	Other
4	38.4 (10)	19.2 (5)	15.4 (4)	7.7 (2)	19.2 (5)
5	41.6 (20)	16.6 (8)	8.3 (4)	6.2 (3)	27.1 (13)
6	29.7 (11)	29.7 (11)	8.1 (3)	13.5 (5)	18.9 (7)
7	31.8 (7)	45.4 (10)	4.5 (1)	0	18.2 (4)
9	26.6 (4)	33.3 (5)	0	0	40.0 (6)
(*)	28.6 (2)	57.1 (4)	0	14.2 (1)	0
12	15.8 (3)	36.8 (7)	36.8 (7)	5.2 (1)	5.2 (1)
(*)	54.5 (6)	18.2 (2)	9.1 (1)	18.2 (2)	0
14	13.3 (2)	46.6 (7)	26.6 (4)	6.6 (1)	6.6 (1)
(*)	11.1 (1)	44.4 (4)	0	11.1 (1)	33.3 (3)

(\*) Specimens treated with 1% sodium hypochlorite for 1 min. Abbreviations: Cyl. = *Cylindrocarpon*, st. myc. = sterile mycelia, Acre = *Acremonium*, B. fungus = 'Black fungus', 'Other' = 'other fungi', including *Paecilomyces*, *Trichoderma*, *Penicillium* and unidentified fungi.

TABLE V - Percentage of females and newly-formed cysts of *H. avenae* (numbers in brackets) from pots developing *Verticillium*, belonging to *V. chlamydosporium* (*V. c.*).

Week	Untreated		NaOCl-treated	
	<i>V. c.</i> (%)	Other isolates (%)	<i>V. c.</i> (%)	Other isolates (%)
4	15.4 (2)	84.6 (11)	n.t.	n.t.
5	3.8 (1)	96.1 (25)	n.t.	n.t.
6	2.0 (1)	98.0 (46)	n.t.	n.t.
7	11.1 (2)	88.9 (16)	0	100 (2)
9	23.7 (9)	76.3 (29)	18.9 (7)	81.1 (30)
12	16.7 (6)	83.3 (30)	30.0 (6)	70.0 (14)
14	0	100 (12)	0	100 (11)

n.t. = not tested.

shown in Table V. The percentage of *Verticillium* isolates producing chlamydospores was always lower than those which did not produce them.

Eggs from individual females/newly-formed cysts (276 in total) that had developed *Verticillium* when plated showed the highest percentages of infection, hatching and distortion, compared with those which had developed 'other fungi' (Figs 4-6). This was true for both untreated and hypochlorite-treated specimens. The total number of eggs in females and newly-formed cysts was similar (Fig. 7); however, the small sample size (especially of females in the last samplings) gave rise to variability.

The presence of fungi on the cyst walls and sub-crystalline layers of individual females or newly-formed cysts

was recorded. To estimate the difference between the fungi developed by the whole female/newly-formed cyst, and the fungi associated with its CW and SCL plated separately, the numbers were scored of CW and SCL respectively which developed fungi different from those developed by the original female/newly-formed cyst. In each case, the total number of CW or SCL developing fungi was divided by those figures to give an arbitrary index. The index assumes the value 1 if the flora of all the CW (or the SCL) was totally different from that of the original whole female or cyst. Therefore, values higher than 1 indicate increasing similarity between the flora of CW (or SCL) and that of females and newly-formed cysts. The results for the flora associated with the CW and the SCL and the index

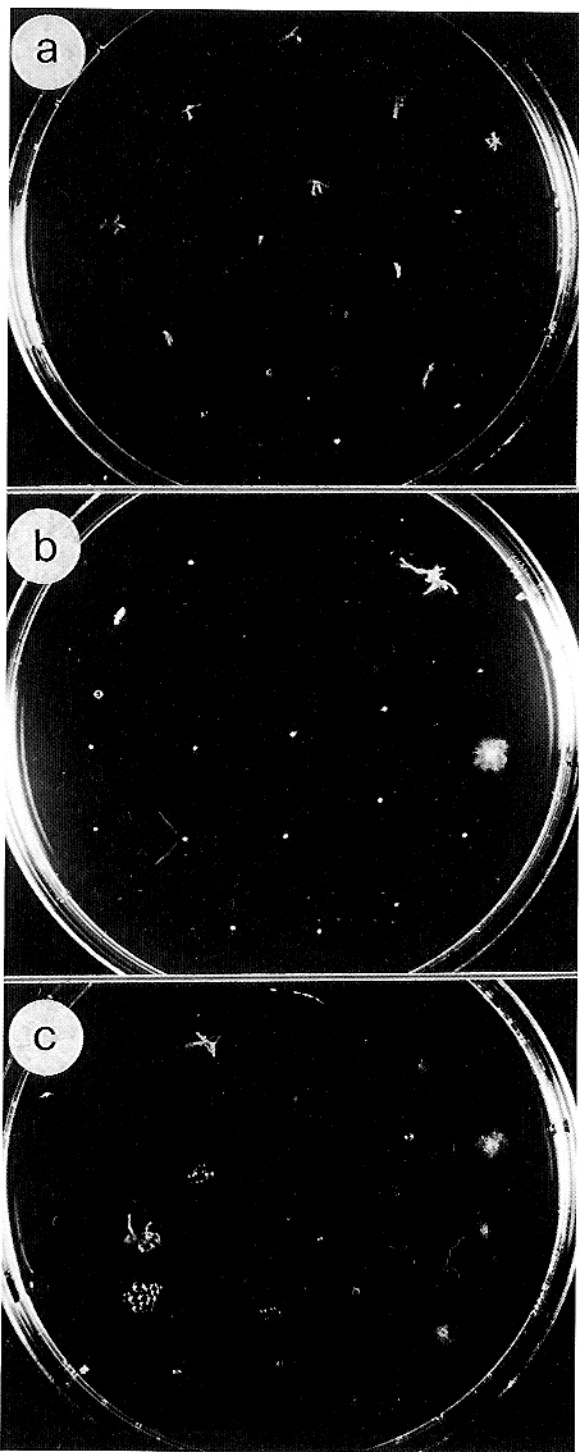


Fig. 3 - Fungal development from *H. avenae* females and newly-formed cysts from the same area of the root on growth restricting medium. (a) White females untreated; (b) white females treated with 1% sodium hypochlorite for 1 min; (c) newly-formed cysts treated with 1% sodium hypochlorite for 1 min.

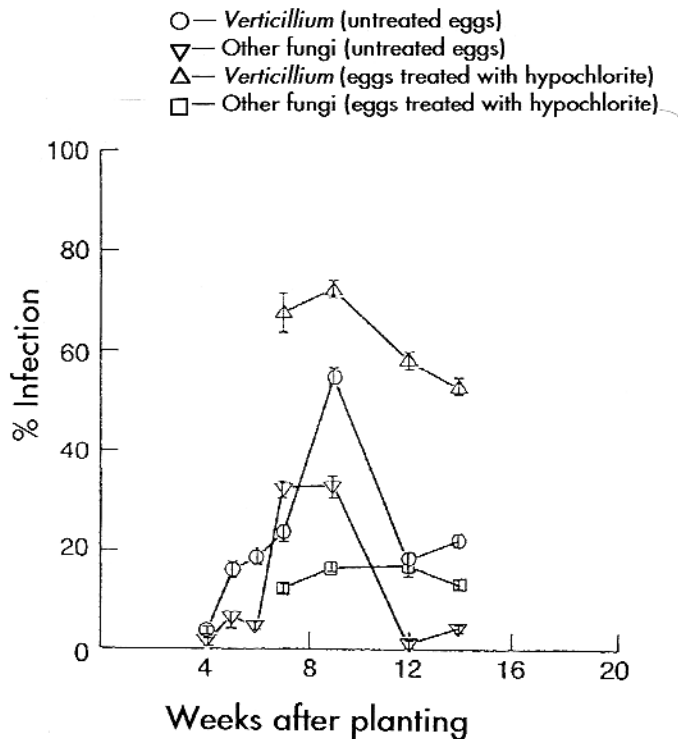


Fig. 4 - Percentage of egg-infection per single female and newly-formed cyst plotted against sampling occasions.

described above are shown in Table VI. The values of the index are low (but never 1, i.e. total dissimilarity) for the first sampling occasions and then increase to a relatively steady state. Treatment with hypochlorite reduces the differences in the flora developed by single CW/SCL and their original females/newly-formed cysts.

## Discussion

In pots and in field samples, *Verticillium* was found to colonise root-systems. The extent of colonisation, both in numbers of roots and numbers of colonies per root, increased with time. Wheat roots may be more susceptible to colonisation by *Verticillium* than those of oats, and oats more susceptible than barley. Parkinson *et al.* (1963) did not find *Verticillium* in young (1-10 day old) barley roots and the results described here agree with theirs.

*Verticillium* is known to colonise cereal roots (Jackson 1965a, 1965b; Mangan, 1967; Kerry *et al.*, 1984), while trapping-fungi such as *Arthrotrichy oligospora* have little association with living roots (Peterson and Katznelson, 1964, 1965). The sequence of events leading to colonisation of the root system may be as follows: sessile conidia

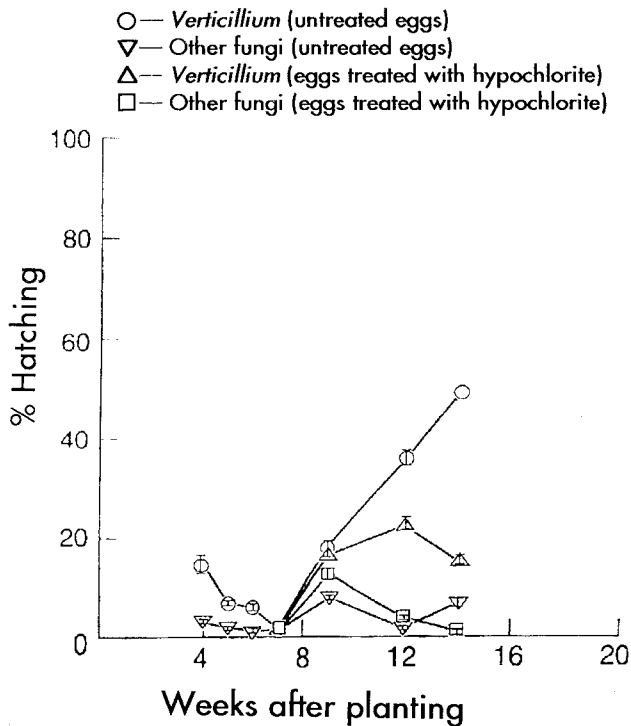


Fig. 5 - Percentage of hatching per single female and newly-formed cyst plotted against sampling occasions.

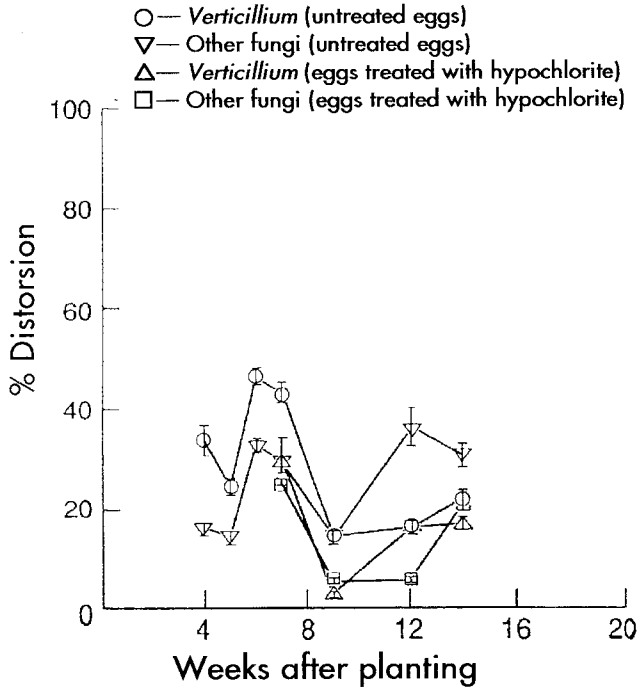


Fig. 6 - Percentage of egg-distortion per single female and newly-formed cyst plotted against sampling occasions.

and/or resting-spores of several isolates of *Verticillium* (including *V. chlamydosporium*) are induced to produce hyphae by the roots either directly, because of their movement in soil, or indirectly by their exudates (Olsson and Nordbring-Hertz, 1985; Elias and Safir, 1987).

Greater numbers of *H. avenae* juveniles per root were found in roots colonised by *Verticillium* than in uncolonised roots. It is possible that juveniles of *H. avenae* infecting roots provide a suitable environment for the colonisation by *Verticillium*. The fungus was frequently observed in the necrotic areas associated with juvenile penetration. Alternatively, nematodes could also be attracted to root areas colonised by the fungus. Jansson and Nordbring-Hertz (1979), studying the attraction of nematodes to nematophagous fungi, concluded that the mycelium of several nematophagous species was attractive to nematodes. Jansson (1982) found that conidia of endoparasitic fungi attracted nematodes. Conversely, nematodes respond to nematophagous fungi and produce substances which, in turn, affect these fungi. An example of this is the induction by nematodes of traps in predaceous fungi (Nordbring-Hertz, 1977; Burney and Estey, 1985).

Infection by female parasites such as *Nematophthora gynophila* whose resting spores were found in the soil used here (Boag and Lopez-Llorca, 1989) can explain, at least in part, the difference in numbers of females and newly-formed cysts, as females infected by those parasites are completely destroyed (Crump and Kerry, 1977). A progressive increase in fungal colonisation occurred as the females aged. Treating whole females with hypochlorite reduced the proportion from which *Verticillium* was subsequently recovered, especially in the very young females. Regarding the species involved, a reduction was found in their diversity as the nematodes aged; *Cylindrocarpon*, *Paecilomyces* and *Verticillium* (several isolates including *V. chlamydosporium*) were the fungi generally found in older cysts.

When female nematodes were treated with sodium hypochlorite only *Verticillium* were found as fungal colonisers. As fungi other than *Verticillium* were also found in untreated specimens, this suggests that they were only superficial saprophytes. The 'other fungi' found colonizing females and newly-formed cysts were mostly common soil fungi, and these were also found colonizing roots together with *Verticillium*. This supports the contention that females were colonised by *Verticillium* at an early stage before eggs were produced, the fungus having developed sufficiently within the females to withstand any adverse effect of the sterilant. Treatment of newly-formed cysts with sodium hypochlorite does not reduce the isolation of fungi to the same extent and probably indicates that *Verticillium* and 'other fungi' are well established within the nematode.

The percentage of nematodes colonised by *Verticillium* spp. (several isolates including *V. chlamydos-*



TABLE VI - Fungal colonisation of cyst walls (CW) and subcrystalline layers (SCL), as percentages of specimens developing fungi, and similarity with the flora of whole cysts or females as measured by an arbitrary index.

Weeks after planting	CW (% fungi)								SCL (% fungi)							
	V	C	S	A	B	O	-	Index*	V	C	S	A	B	O	-	Index*
5	7.1	14.3	7.1	7.1	0.0	7.1	57	2.00	0.0	0.0	20.0	20.0	0.0	0.0	60.0	1.00
6	16.6	13.9	16.6	2.8	5.5	8.3	36.1	2.30	33.3	16.7	8.3	16.7	8.3	8.3	8.3	2.75
7	24.1	15.5	20.7	6.9	5.2	6.9	20.7	3.06	25.0	15.0	10.0	10.0	15.0	15.0	10.0	2.57
9	23.9	19.7	25.3	15.5	0.0	9.8	5.6	1.97	20.7	41.4	13.8	13.8	6.9	3.4	0.0	1.93
(+)	52.8	3.8	15.1	0.0	1.9	1.9	24.5	4.00	76.2	14.3	9.5	0.0	0.0	0.0	0.0	4.20
12	42.1	15.8	15.8	15.8	5.3	5.3	0.0	3.80	25.0	12.5	25.0	37.5	0.0	0.0	0.0	2.70
(+)	33.3	29.2	8.3	4.2	8.3	8.3	8.3	3.14	22.2	44.4	0.0	11.1	11.1	11.0	0.0	3.00
14	13.0	4.3	52.2	0.0	0.0	21.7	8.7	1.75	16.7	16.7	33.3	0.0	0.0	33.3	0.0	1.50
(+)	46.7	6.7	33.3	0.0	6.7	6.7	0.0	3.75	66.7	16.7	16.7	0.0	0.0	0.0	0.0	3.00

V = *Verticillium* sp.; C = *Cylindrocarpon* sp.; S = Sterile mycelia; A = *Acremonium* sp.; B = 'Black fungus'; O = Other fungi; - = No fungi; (+) Specimens treated with 1% hypochlorite for 1 min.

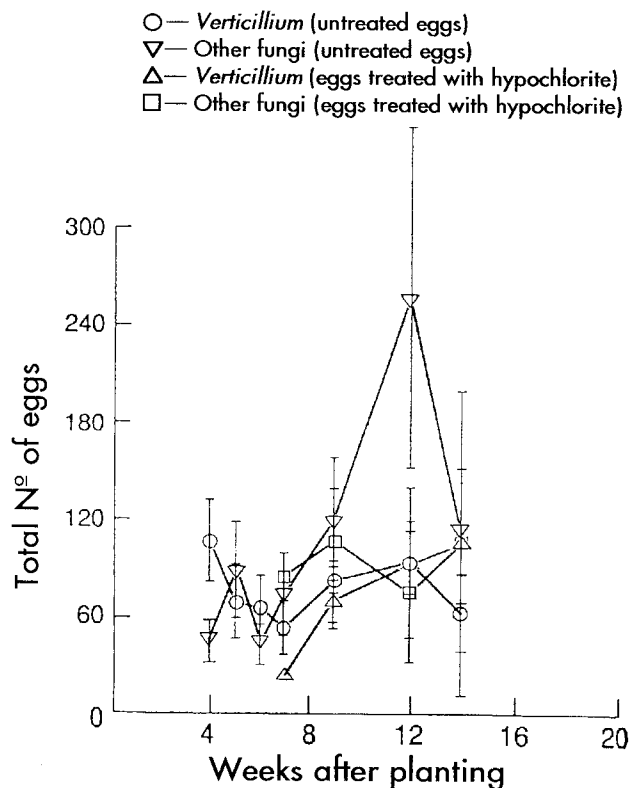


Fig. 7 - Total number of eggs per single female and newly-formed cyst plotted against sampling occasions.

*porium*) increases from females to newly-formed cysts, supporting the view that these fungi have a major role in the parasitism of *H. avenae* eggs in eastern Scotland, confirming other results (Lopez-Llorca and Duncan, 1986; Boag and Lopez-Llorca, 1989). The increase in occurrence of *Verticillium* may also indicate that the fungus competes with the 'other fungi' perhaps by producing inhibitory substances (Leinhos and Buchenauer, 1986), thus becoming the dominant egg parasite.

Kerry *et al.* (1982) calculated from field studies that the total percentage of eggs infected in nematode females increased from 3% to a maximum of 15% and then decreased to 7%. The evolution of egg-infection in pots from single females/newly-formed cysts showed a similar pattern. The maximum extent of infection also occurred at approximately the same time for all fungi, indicating that the different fungi involved in egg-parasitism operate on a similar time scale. The low incidence of egg infection during the first few weeks after planting (Fig. 4) could be due to a low proportion of females containing eggs, a low incidence of female infection or because young females infected with egg parasites contain few or no infected eggs. Although fungi develop inside the females and penetration of eggs may have started they are not destroyed until later (Lopez-Llorca and Duncan 1988, 1991).

The results obtained provide evidence that *Verticillium* enhances hatching from eggs, especially in newly-formed cysts, whether or not they are treated with sodium hypo-

chlorite. Fungi other than *Verticillium* may also, to a lesser extent, enhance hatching of *H. avenae*. Maximum percentage of infection did not coincide with maximum percentage of hatching; this suggests a double mode of action by *Verticillium* upon nematode eggs. When females are young and contain immature eggs, the eggs are readily penetrated and destroyed by the fungus (Lopez-Llorca and Duncan 1988, 1991). In older females/newly-formed cysts, which contain mostly fully-developed eggs, *Verticillium* may produce metabolites that stimulate premature hatching of the juveniles contained in the eggs. This effect has been observed by other authors (Jatala, 1986; Jatala *et al.* 1985).

The greatest proportion of distorted eggs was found 6-7 weeks after planting but before egg-infection had reached a maximum. It can be hypothesized that before being penetrated and destroyed by fungal parasites (mainly *Verticillium*), eggs can be killed and their contents 'distorted' by fungal metabolites; the effects of fungal metabolites of egg-parasites on nematode eggs have been discussed by several authors (Jatala, 1986; Morgan-Jones and Rodriguez-Kabana, 1985). Similarly, nematodes developing *Verticillium* (both untreated and treated with sodium hypochlorite) also contained the greatest proportion of distorted eggs.

Although the data are very variable, Fig. 7 shows that for weeks 7 and 9, nematodes developing fungi other than *Verticillium* had, on average, more eggs than those developing *Verticillium* both for untreated and hypochlorite-treated specimens. Kerry *et al.* (1982) suggested that in soils where parasitic fungi are present, female survival and fecundity are more important than female production in limiting *H. avenae* multiplication.

Regarding the fungal colonisation of cyst walls and sub-crystalline layers, the similarity index (Table VI) indicates that the flora associated with the outer parts (CW and SCL) of old females/newly-formed cysts are different from that developed by CW and SCL at early stages of colonisation (5 weeks after planting). In CW and SCL from hypochlorite-treated specimens, as opposed to untreated specimens, the associated flora are more similar to the flora developed by whole individuals. This can be explained if the (saprophytic) fungi, which were associated with external structures (CW and SCL) and were killed by the sterilant used, differed from the (parasitic) fungi within the female/cyst.

Dackman and Nordbring-Hertz (1985) found a *Verticillium* species (which they named *Verticillium* sp. 1) to be the most common fungal parasite infecting eggs in cysts of *H. avenae* in southern Sweden. They also found *V. chlamydosporium* parasitizing eggs and females but less commonly recovered from cysts. *Verticillium* sp. 1 differs from *V. chlamydosporium* because it grows at 6 °C and *V. chlamydosporium* does not and that *Verticillium* sp. 1 pro-

duces, on solid media, few submerged chlamydospores and none on the aerial mycelium. Both fungi were found in the experiments described in this paper, and also in females and newly-formed cysts containing infected eggs. Dackman and Nordbring-Hertz (1985) considered *V. chlamydosporium* as the root colonizer and *Verticillium* sp. 1 as the fungus that remains in the later stages overwintering in the cysts. This fungus was later described as *V. suchlasporium* (Gams, 1988). Dackman and Nordbring-Hertz (1985) isolated fungi from single, newly-formed cysts, and distinguished two categories of cysts: those infected with *Verticillium* sp. 1, other *Verticillium* sp., *V. chlamydosporium* and *P. lilacinus* which contained all eggs infected; and those developing *Cylindrocarpon* and several sterile mycelia which contained only a portion of eggs infected. They did not find empty egg-shells, and also reported that cysts with all the eggs infected contained few eggs (about 10 eggs each female/cyst). The results presented here were also on a 'per female/cyst' basis and are similar to theirs, although juveniles hatched and therefore empty egg-shells were observed.

## Literature cited

- BARRON G. L. and ONIONS A. H. S., 1966. *Verticillium chlamydosporium* and its relationships to *Dibeterospora*, *Stemphyliopsis* and *Paecilomyces*. *Can. J. Bot.*, 44: 861-869.
- BOAG B. and LOPEZ-LLORCA L. V., 1989. Nematodes and nematophagous fungi associated with cereal fields and permanent pasture in eastern Scotland. *Crop Research*, 29: 1-10.
- BURNEY K. and ESTEY H., 1985. A note on the non-diffusibility of a substance produced by nematodes that induced *Arthrobotrys oligospora* to form nematode traps. *Phytoprotection*, 66: 163-164.
- CAYROL J. C., VELASQUEZ-DOMINGUEZ M. and LEVAUX P., 1982. Etude préliminaire sur les possibilités d'utilisation des champignons parasites comme agents de lutte biologique. *EPPO Bull.*, 12: 497-503.
- CRUMP D. H. and KERRY B. R., 1977. Maturation of females of the cereal cyst nematode on oats roots and infection by an Entomophthora-like fungus in observation chambers. *Nematologica*, 23: 398-402.
- DACKMAN C. and NORDBRING-HERTZ B., 1985. Fungal parasites of the cereal cyst nematode *Heterodera avenae* in Southern Sweden. *J. Nematol.*, 17: 50-55.
- ELIAS K. S. and SAFIR G. R., 1987. Hyphal elongation of *Glomus fasciculatus* in response to root exudates. *App. Env. Microbiol.*, 53: 1928-1933.
- FISHER J. M., 1981. Further observations on the effect of temperature on *Heterodera avenae* Woll. *Nematologica*, 27: 228-234.
- GAMS W., 1988. A contribution to the knowledge of nematophagous species of *Verticillium*. *Neib. J. Pl. Path.*, 94: 123-148.
- GODOY G., RODRIGUEZ-KABANA R. and MORGAN-JONES G., 1983. Fungal parasites of *Meloidogyne arenaria* in an Alabama soil. A mycological survey and greenhouse studies. *Nematropica*, 13: 201-213.
- HOOPER D. J., 1970. Preserving and staining nematodes in plant tissues. In M.A.F.F. "Laboratory Methods for Work with Plant and Soil Nematodes". Southey, J. F. (ed.) H.M.S.O. 393-399.

- IRVING F. and KERRY B. R., 1986. Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. II. Factors affecting parasitism of cyst nematode eggs. *Nematologica*, **32**: 474-485.
- JACKSON R. M., 1957. Fungistasis as a factor in the rhizosphere phenomenon. *Nature*, **180**: 96-97.
- JACKSON R. M., 1965a. Studies of fungi in pasture soils. II. Fungi associated with plant debris and fungal hyphae in soil. *New Zealand J. Agric. Res.*, **8**: 865-877.
- JACKSON R. M., 1965b. Studies of fungi in pasture soils. III. Physiological studies on some fungal isolates from the root surface and from organic debris. *New Zealand J. Agric. Res.*, **8**: 878-888.
- JANSSON H. B., 1982. Attraction of nematodes to endoparasitic nematophagous fungi. *Trans. Br. mycol. Soc.*, **79**: 25-29.
- JANSSON H. B. and NORDBRING-HERTZ B., 1979. Attraction of nematodes to living mycelium of nematophagous fungi. *J. Gen. Microbiol.*, **112**: 89-93.
- JATALA P., 1986. Biocontrol of plant-parasitic nematodes. *Ann. Rev. Phytopathol.*, **24**: 453-489.
- JATALA P., FRANCO J., GONZALEZ A. and O'HARA C. M., 1985. Hatching stimulation and inhibition of *Globodera pallida* eggs by the enzymatic and exopathic compounds of some biocontrol fungi. *J. Nematol.*, **17**: 505 (Abs.).
- KERRY B. R., 1980. Biocontrol: fungal parasites of female cyst nematodes. *J. Nematol.*, **12**: 253-259.
- KERRY B. R., 1986. An assessment of the role of parasites and predators in the regulation of cyst nematode populations. In 'Cyst Nematodes' ed. by F. Lamberti and C. E. Taylor. NATO ASI Series Life Sciences Vol. 121: 433-450. Plenum Press.
- KERRY B. R. and CRUMP D. H., 1977. Observations on fungal parasites of females and eggs of the cereal cyst nematode, *Heterodera avenae*, and other cyst nematodes. *Nematologica*, **23**: 193-201.
- KERRY B. R., and CRUMP D. H., 1980. Two fungi parasitic on females of cyst-nematodes (*Heterodera* spp.). *Trans. Br. mycol. Soc.*, **74**: 119-125.
- KERRY B. R., CRUMP D. H. and MULLEN L. A., 1982. Studies of the cereal cyst nematode, *Heterodera avenae* under continuous cereals, 1974-1978. II. Fungal parasitism of nematode females and eggs. *Ann. appl. Biol.*, **100**: 489-499.
- KERRY B. R., SIMON A. and ROVIRA A. D., 1984. Observations on the introduction of *Verticillium chlamydosporium* and other parasitic fungi into soil for control of the cereal cyst nematode *Heterodera avenae*. *Ann. appl. Biol.*, **105**: 509-516.
- KO W. and LOCKWOOD J. L., 1970. Mechanism of lysis of fungal mycelia in soil. *Phytopathology*, **60**: 148-154.
- LA MONDIA J. A. and BRODIE B. B., 1984. An observation chamber technique for evaluating potential biocontrol agents of *Globodera rostochiensis*. *J. Nematol.*, **16**: 112-115.
- LEINHOS G. and BUCHENAUER H., 1986. Untersuchungen zur antagonistischen Wirkung des Bodenpilzes *Verticillium chlamydosporium* Goddard gegenüber Getreiderostpilzen. Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft p. 275. 45 Deutsche Pflanzenschutz-Tagung. Paul Parey. Berlin.
- LOPEZ-LORCA L. V. and BOAG B., 1990. Inhibition of *Verticillium suchlasporium* and other nematophagous fungi by bacteria colonizing *Heterodera avenae* females. *Nematol. mediterr.*, **18**: 233-237.
- LOPEZ-LORCA L. V. and DUNCAN G. H., 1988. A study of fungal endoparasitism of the cereal cyst nematode (*Heterodera avenae*) by scanning electron microscopy. *Can. J. Microbiol.*, **34**: 613-619.
- LOPEZ-LORCA L. V. and DUNCAN G. H., 1991. Effects of fungal parasites on naturally infected cereal cyst nematode (*Heterodera avenae* Woll.): a scanning electron microscopy study. *Can. J. Microbiol.*, **37**: 218-225.
- LOPEZ-LORCA L. V. and DUNCAN J. M., 1986. New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica*, **32**: 486-490.
- MANGAN A., 1967. Studies on wheat rhizosphere soil fungi. *Irish J. Agric. Res.*, **6**: 9-14.
- MANKAU R., 1962. Nematophagous fungi and soils fungistasis. *Phytopathology*, **52**: 611-615.
- MORGAN-JONES G., GODOY G. and RODRIGUEZ-KABANA R., 1981. *Verticillium chlamydosporium*, fungal parasite of *Meloidogyne arenaria* females. *Nematropica*, **11**: 115-120.
- MORGAN-JONES G. and RODRIGUEZ-KABANA R., 1985. Phytoneematode pathology: fungal modes of action. *Nematropica*, **15**: 107-114.
- NIGH E. A., THOMASON I. J. and VAN GUNDY S. D., 1980. Identification and distribution of fungal parasites of *Heterodera schachtii* eggs in California. *Phytopathology*, **70**: 884-889.
- NORDBRING-HERTZ B., 1977. Nematode-induced morphogenesis in the predacious fungus *Arthrobotrys oligospora*. *Nematologica*, **23**: 443-451.
- OLSSON S. and NORDBRING-HERTZ B., 1985. Microsclerotial germination of *Verticillium dahliae* as effected by rape rhizosphere. *FEMS Microb. Ecol.*, **31**: 293-299.
- OWNLEY GINTIS B., MORGAN-JONES G. and RODRIGUEZ-KABANA R., 1983. Fungi associated with several developmental stages of *Heterodera glycines* from an Alabama soybean field soil. *Nematropica*, **13**: 181-200.
- PARKINSON D., TAYLOR G. S. and PEARSON R., 1963. The development of fungi on young roots. *Plant Soil*, **19**: 332-349.
- PETERSON E. A. and KATZNELSON H., 1964. Occurrence of nematode-trapping fungi in the rhizosphere. *Nature*, **204**: 1111-1112.
- PETERSON E. A. and KATZNELSON H., 1965. Studies on the relationships between nematodes and other soil microorganism. IV. Incidence of nematode-trapping fungi in the vicinity of plant roots. *Can. J. Microbiol.*, **11**: 491-495.
- STIRLING G. R. and MANKAU R., 1979. Mode of parasitism of *Meloidogyne* and other nematode eggs by *Dactylella oviparasitica*. *J. Nematol.*, **11**: 282-288.
- TRIBE H. T., 1979. Extent of disease in populations of *Heterodera* with special reference to *H. schachtii*. *Ann. appl. Biol.*, **92**: 61-72.