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THE SIGNIFICANCE OF ECOLOGY IN THE DEVELOPMENT OF *VERTICILLIUM CHLAMYDOSPORIUM* AS A BIOLOGICAL CONTROL AGENT AGAINST ROOT-KNOT NEMATODES (*MELOIDOGYNE* SPP.)

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STELLINGEN

1 Bij de selectie van een biologisch bestrijdingsmiddel moet de nadruk niet zozeer liggen op de screening van zoveel mogelijk isolaten, maar meer op het ontwikkelen van een zo volledig mogelijk begrip van de ecologie van een beperkt aantal veelbelovende isolaten.

2 Effectieve isolaten van *Verticillium chlamydosporium* tegen *Meloidogyne* spp zijn rhizospheer competent. Dit betekent echter niet dat rhizospheer competente isolaten altijd effectief zijn tegen *Meloidogyne*. Dit proefschrift.

3 De conidia van *V. chlamydosporium* dragen wezelijk bij tot de verspreiding van deze schimmel door de bodem. Dit proefschrift.

4 Het hydrofiele karakter van *V. chlamydosporium* conidia, wijst erop dat verspreiding van deze conidia door bodeminsecten van ondergeschikt belang is. Dit proefschrift.

5 Het is onwaarschijnlijk dat een zaadbehandeling met *V. chlamydosporium* effectieve bescherming biedt tegen *Meloidogyne*.

6 De effectiviteit van *V. chlamydosporium* voor de bestrijding van *Meloidogyne* wordt in grote mate bepaald door de waardplant. Dit proefschrift.

7 In het algemeen kan *V. chlamydosporium* alleen gebruikt worden samen met andere bestrijdingsmethoden. Een juiste timing van de toediening is echter een voorwaarde. Dit proefschrift.

8 Het opleggen van economische criteria in een vroeg stadium van het screeningsproces voor biologische bestrijdingsmiddelen vermindert de kans om isolaten te vinden die biologische en economische waarde hebben. Dit proefschrift.

9 Het doen van onderzoek zonder goede methodieken is als de beklimming van "Mount Everest" in een rolstoel.

10 Het doel van onderzoek is het blootleggen van de interacties die optreden tussen de verschillende elementen. Dit geldt zeker voor onderzoek gericht op biologische bestrijding. E. Mach (1910) Popular Scientific lectures.

11 Het besef dat het economisch is om ecologisch te denken is waarschijnlijk de beste garantie voor het behoud van het milieu. Dit betekent echter dat economen ecologisch onderlegd moeten worden en vice versa.

12 Geloof in reïncarnatie zou een aanzet kunnen zijn om de rijkdommen die deze planeet nog steeds bezit op een verantwoorde wijze te exploiteren.

13 De braakregeling laat de mogelijkheid voor een minder intensieve, niet vervuilende landbouw, op een jammerlijke manier braak liggen.

14 Het ideaal van een verenigd Europa is alleen mogelijk wanneer de individualiteit van de verschillende volkeren gerespecteerd wordt.

15 Bij politieke verkiezingen zouden kiezers de mogelijkheid moeten hebben om hun stem te verdelen over verschillende politieke partijen.

THEOREMS

1 In selecting microbial and fungal biological control agents, emphasis should not be placed on screening as many isolates as possible, but on gaining understanding of the ecology of a limited number of promising isolates.

2 Effective isolates of *Verticillium chlamydosporium* against *Meloidogyne* are rhizosphere competent. However, this does not mean that all rhizosphere competent isolates are effective against *Meloidogyne*. This thesis.

3 The conidia of *V. chlamydosporium* aid the distribution of this fungus through soil significantly. This thesis.

4 The hydrophilic character of the conidia of *V. chlamydosporium* indicates that dispersal of these conidia by soil insects is of limited importance. This thesis.

5 It is unlikely that seed treatments with *V. chlamydosporium* will provide effective protection against *Meloidogyne*.

6 The host plant has a large effect on the effectiveness of *V.chlamydosporium* to control *Meloidogyne*. This thesis.

7 As a rule *V. chlamydosporium* can only be used, properly timed, in integrated control programmes. This thesis.

8 Imposing economic criteria on the development of biological control agents in an early stage of the research programme reduces the chance of finding isolates which are effective in a biological and economical sense.

9 Doing research without good methods is like climbing "Mount Everest" in a wheel chair.

10 "The aim of research is the discovery of the equations which subsist between the elements of phenomena". This is certainly true for research in biological control. E. Mach (1910) Popular Scientific Lectures.

11 The realisation that it is economic to think ecologically is probably the best guarantee for the preservation of the environment. This, however, means that economists must become ecologically educated and vice versa.

12 Belief in reincarnation could be an incentive to exploit the richness which this planet still possesses in a responsible way.

13 "Set aside" sets aside the possibility of non-intensive, non- polluting agriculture.

14 The ideal of a united Europe is only possible if the individuality of the different peoples in the EEC is respected.

15 A voting system which aims at proportional representation should give individuals the possibility of giving partial votes to different political parties.

Frans de Leij.
The significance of ecology in the development of *Verticillium chlamydosporium* as a biological control agent against root-knot nematodes (*Meloidogyne* spp.).
Wageningen, 28 February 1992.

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FOREWORD

"A thousand mile journey starts with a single step ....", is a saying which when kept in mind would make any journey an enjoyable experience. Like any journey, the area covered and the quality of the journey, is not only dependent on the skills of the explorer, but also on maps which describe the area, roads which can be used and guides which help to overcome obstacles on the way. First of all I would like to thank my co-promoter and supervisor Dr Brian Kerry for the way he helped me to find my way into the subject, the useful discussion about the work, and the critical revision of the papers written. Then there is Jackie Dennehy who accompanied me for one and a half years as my assistant. Without her far less ground would have been covered. Special thanks go to Dr Keith Davies and Dr David Crump for their willingness to discuss specific problems and help to get used to new techniques. For that matter I have to compliment all those members of staff Rothamsted who helped wherever possible.

I also wish to thank the Agricultural Genetics Company, which provided the funds to undertake this project. Without them I would not have started.

Research has the tendency to become more and more high tech. However, computer facilities, high power microscopes and clever analytical techniques are meaningless when experiments are badly managed. This is especially true for ecological research in glasshouse situations, where plants have to grow for months, before they can be harvested. The care taken by the glasshouse staff, especially by Albert Callewaert is therefore very much appreciated.

I mentioned that journeys are difficult to undertake when there are no proper roads through the area. Much of this thesis is built on the work of Ian Kirkwood and Jane Barba who were largely responsible for the development of a semi-selective medium for *Verticillium chlamydosporium*. This medium made it possible to quantify many of the interactions which occur between *V. chlamydosporium*, the soil, the rhizoplane and the nematode.

The account of my work would not be in this final form where it not for Dr Ken Evans who did some excellent editing of the manuscripts, and the effort made by Jackie Fountain, who did most of the typing.

I wish to thank my promotor Prof. dr. ir. A.F. van der Wal for the useful

suggestions on the work, and the critical reading of this thesis. His support, interest, and help were essential for the completion of this work and is very much appreciated.

Last but not least I want to thank my wife Fiona for her moral support and my father who has always stimulated me to develop my career in whatever way I liked.

CHAPTER I

GENERAL INTRODUCTION

Root knot nematodes: damage, economical importance and control

Root-knot nematodes (*Meloidogyne* spp.) are worldwide in their distribution and are known to attack a wide variety of crops (Goodey, *et al.*, 1965). Of a total of 70 *Meloidogyne* species identified so far (Luc *et al.*, 1988) only the species *M. incognita* (Kofoid & White) Chitwood, *M. javanica*(Treub) Chitwood, *M. arenaria* (Neal) Chitwood and *M. hapla* Chitwood, are of major economic importance. From 400 populations of *Meloidogyne* identified world wide, 97% were identified as belonging to these four species (Sasser, 1979). The economic damage inflicted by this group of nematodes is enormous; estimates of worldwide crop losses is in the region of 13% (Sasser, 1979) with the subtropics and tropics being the most badly affected areas.

The life cycle of most *Meloidogyne* species can be described as follows: second-stage juveniles hatch from eggs which are deposited in a gelatinous matrix. After hatching, the juveniles migrate through the soil and invade a suitable host plant near the root tip. After penetration, the juveniles migrate through the root cortex, until suitable feeding sites are found. There they become established, with the anterior end in contact with the vascular cylinder, where, in susceptible hosts, induction of giant cells takes place upon which the nematode feeds. At this stage a gall generally forms in which the nematode stays embedded during its further development. Juveniles gradually assume a flask shape and undergo three further moults. The last moult is a true metamorphosis for the male, which appears as a long filiform nematode folded inside the cuticle of the fourth juvenile stage; the adult female at first retains the same shape of the last juvenile stage, but as it matures it enlarges and becomes pyriform. The females secrete a gelatinous matrix into which they extrude a large number of eggs, usually 500-1,000, but sometimes more (Tyler, 1933). Although the female stays embedded in the gall, cellulytic enzymatic activity of the gelatinous matrix might induce a channel to the gall surface, so that the egg-mass usually can be formed on the outside of the gall (Orion, *et al.*, 1987).

Many species of *Meloidogyne* are parthenogenic and development of males only occurs when feeding sites are poor (Triantaphyllou, 1960) or when temperatures

are supra-optimal for development (Laughlin *et al.*, 1969; Webber & Fox, 1971). Depending on temperature, the complete life cycle can be finished in less than a month (Guiran & Riter, 1979) and new generations will follow continuously as long as conditions are suitable.

Because of the enormous variety of suitable hosts (Goodey, 1965, lists over 700 host species and varieties for *M. incognita*), the life cycle which is almost completely confined to the inside of the host plant, and their enormous reproductive capacity, root- knot nematodes are very difficult to control.

Although chemical control is still a common method for reducing nematode populations, there is considerable public pressure, particularly in Europe and the United States, to limit or even ban the use of nematicides which were a few years ago still widely used (Thomason, 1987). Many nematicides are highly toxic and sometimes very mobile in soil because of their solubility in water. In the USA and elsewhere, ground water has been contaminated with many organic chemicals including the nematicides DBCP, EDB, the 1,2-dichloropropane component of DD, aldicarb, carbofuran and ethoprop (Peoples *et al.*, 1980; Zaki *et al.*, 1982; Wixted *et al.*, 1987). Concern over these chemicals has led to an increased interest in biological control in its widest sense, in order to achieve more environmentally benign methods of reducing nematode damage.

Most research on biological control agents for nematodes has concentrated on the identification and biology of nematophagous micro-organisms (Barron, 1977). Quantitative research on the interactions between agent, soil, host plant, and nematode pest are frequently lacking (Kerry, 1990). This lack of ecological understanding has often led to failures when attempts have been made to use microbial agents against plant parasitic nematodes. Also, to demonstrate the effectiveness of biological control agents, experiments must include adequate control treatments, estimates of the survival and proliferation of the agent in soil, and re-isolation of the agent from the host (Stirling, 1988). Such standard procedures are frequently not followed. In 25 experiments purporting to demonstrate biological control using the egg-parasite *Paecilomyces lilacinus* (Thom) Samson, only 15% reached the necessary experimental standards to prove that the agent applied had caused the observed reduction in the nematode population (Kerry, 1990). It is often difficult to

satisfy such standards. In addition, the use of microorganisms to control nematode pests faces much scepticism. This might be due to a lack of consistent results, caused by a lack of understanding of the ecology of the system under study.

Verticillium chlamydosporium

There are a few well documented examples of nematode suppression by biological control agents in arable crops. Probably the best documented one, is the decline of the cereal cyst nematode, *Heterodera avenae* Woll., in England. When cereals were grown continuously over a period of 13 years, nematode populations peaked two years after the start of the experiment and then fell rapidly to a level below the economic threshold. This low level was maintained for the rest of the experiment (Gair *et al.*, 1969). Applications of a drench of formalin equivalent of 3000 l/ha to a nematode suppressive soil, restored the multiplication of *H. avenae*, suggesting that biological agents were responsible for the suppression of this nematode (Williams, 1969; Kerry *et al.*, 1980; Crump & Kerry, 1981). Four main species of nematode parasitic fungi were found in cereal fields infested with *H. avenae*, with *Nematophthora gynophila* Kerry & Crump and *Verticillium chlamydosporium* Goddard being the most widespread and abundant ones (Kerry *et al.*, 1982).

In contrast with *N. gynophila, V. chlamydosporium* is a facultative parasite, which means that the fungus can survive and proliferate in soil and on the rhizosphere of plants as well as destroy nematodes when it contacts them. All stages of the fungus (hyphae, conidia and chlamydospores) occur in soil, but infection of nematode eggs and females is thought to result from penetrative hyphae that develop from actively growing mycelium (Morgan-Jones *et al.*, 1983). Because *V. chlamydosporium* attacks nematodes only when fully developed females or egg-masses appear on the root surface, initial damage on young plants cannot be prevented using this fungus. However, population control of the first generation will result in subsequent generations of nematodes doing less damage. In this way *V. chlamydosporium* has the same effect on nematode populations as a partially resistant crop cultivar.

Because of its role in the decline of *H. avenae*, its common association with all the major cyst and root knot nematodes (Willcox & Tribe, 1974; Morgan-Jones *et al.*, 1981; Freire & Bridge, 1985) and the ease with which it can be cultured on all sorts

of substrates, makes *V. chlamydosporium* an ideal organism to be used for augmentative purposes. Tribe (1980) considered it a likely candidate for direct addition to soil, but progress towards this end has been slow and only a few experiments have been conducted so far (Kerry *et al.*, 1984; Godoy *et al.*, 1983; Rodriguez-Kábana *et al.*, 1984).

The thesis

From the above it is clear that there is a need for alternative methods of controlling nematodes, especially root-knot nematodes. However work on biological control of nematode pests is often empirical and lacks the necessary backbone of ecological understanding of the organisms involved, either because adequate experimental techniques are lacking, or resources have not been applied to the multidisciplinary approach that is generally recognized as essential for the development of a biological control agent (Kerry, 1990).

This thesis is about the development of a particular isolate of *V. chlamydosporium* as a biological control agent for root knot nematodes. The work is an attempt to gain insight into the key factors which govern the efficacy of the fungus as a biological control agent.

Chapter two describes the selection of an effective isolate of *V. chlamydosporium*; this includes rhizosphere compatibility, survival in soil, ability to kill nematodes, introduction into soil and non plant pathogenic properties.

In chapter three the effectiveness of *V. chlamydosporium* alone and in combination with the obligate parasite *Pasteuria penetrans* (Thorne) Sayre & Starr is tested against *M. incognita*. The treatments are compared with an aldicarb treatment of 3.75 kg a.i./ha. This chapter also describes how gall size might influence the effectiveness of the two organisms.

Chapter four expands on how nematode density affects nematode damage and how this affects the effectiveness of *V. chlamydosporium* at different inoculation rates.

Chapter five focuses on how temperature affects the interactions which occur between *V. chlamydosporium* and three nematode species (*M. incognita, M. javanica* and *M. arenaria*) with tomato plants as their host.

Chapter six describes how watering might influence soil conditions and spread

of *V. chlamydosporium* and what the consequences are in terms of ability of the fungus to infect eggs of *M. incognita*.

Chapter seven reports on how the soil itself affects establishment of *V. chlamydosporium* and the consequences this has on nematode control. This chapter also describes the effectiveness of *V. chlamydosporium* in a micro-plot experiment in field soil against *M. hapla*.

Finally, in chapter eight the previous chapters are brought together and some extra data are presented to fill in some of the gaps. The results are discussed mainly in terms of the potential of *V.chlamydosporium* as a biological control agent for root-knot nematodes.

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CHAPTER 2

THE NEMATOPHAGOUS FUNGUS, *VERTICILLIUM CHLAMYDOSPORIUM* AS A POTENTIAL BIOLOGICAL CONTROL AGENT FOR *MELOIDOGYNE ARENARIA* (NEAL) CHITWOOD

F.A.A.M. de Leij and B.R. Kerry

Summary

The potential of three *Verticillium chlamydosporium* isolates as biological control agents against *Meloidogyne arenaria* on tomato plants was investigated under glasshouse conditions. All three isolates survived well in soil but showed marked differences in their ability to colonise uninfected roots, nematode galls and nematode eggs. Significant population reductions of >80% after the first nematode generation, were achieved with one isolate, which resulted in significant damage control, but not in population control, in subsequent generations. Establishment of *V. chlamydosporium* in soil was significantly greater if the fungus was introduced without a foodbase, i.e. as hyphal fragments and chlamydospores rather than colonised sand-bran. The fungus did not invade the root cortex and there were no adverse effects of the fungus on plant growth.

Introduction

Verticillium chlamydosporium Goddard was first recognised as a parasite of cyst nematodes after it had been isolated from the eggs of *Heterodera schachtii* Schmidt (Willcox & Tribe, 1974) and *H. avenae* Woll. (Kerry, 1975). The fungus was considered a major parasite which caused the decline of cereal-cyst nematode populations in monocultures of susceptible crops (Kerry, Crump and Mullen, 1982). *Verticillium chlamydosporium* has a wide host range amongst cyst and root-knot nematodes but it is very variable and only some isolates may have potential as commercial biological control agents. When *V. chlamydosporium* has been applied to soil, significant levels of control of *H. avenae* (Kerry, Simon and Rovira, 1984) and *Meloidogyne arenaria* (Neal) Chitwood (Godoy, Rodriguez-Kábana and Morgan-Jones, 1983) have been achieved in pots. The fungus was as effective as *Paecilomyces lilacinus* (Thom) Samson against *M. arenaria* but did not appear to survive as well in soil (Godoy, Rodriguez-Kabana and Morgan-Jones, 1983). *Verticillium chlamydosporium* colonised the rhizosphere but did not invade the root cortex, cause lesions or affect the growth of wheat (Kerry, Simon and Rovira, 1984). Such colonisation prolonged the survival of the fungus in soil (Kerry, 1988) and enabled it to increase close to developing female nematodes.

Cyst nematodes are most effectively controlled by *V. chlamydosporium* when females are parasitised early in their development. However, early infection is also important with root-knot nematodes because immature eggs are more susceptible to parasitism by *V. chlamydosporium* than those containing second-stage juveniles (Irving and Kerry, 1986). Root-knot nematodes may be less easily controlled because immature females remain embedded within galls and only the egg-masses are exposed to fungal parasitism on the root surface.

Stirling (1988) was critical of many experiments that purport to demonstrate the biological control of nematodes because too often there have been inadequate controls for comparison with the treatments applied and few attempts have been made to monitor the rates of infection and survival of the fungus in soil. The development of a semi-selective medium for the isolation of *V. chlamydosporium* has enabled its growth and survival in non-sterile soils to be monitored (Kerry *et al.*,

1990). This paper describes how *V. chlamydosporium* is added to soil for the control of *M. arenaria* and its effects on nematode multiplication, the numbers of eggs parasitised, plant damage, and its survival on roots and in soil are reported.

Materials and methods

Three isolates of *V. chlamydosporium* were selected for these studies from a collection of nematophagous fungi maintained on silica gel stored at 5oC (Smith & Onions, 1983). Isolates 10 and 43 were originally collected from eggs of *M. incognita* (Kofoid & White) Chitwood and *H. schachtii* respectively, whereas isolate 35 was established from a chlamydospore extracted from soil suppressive to *H. avenae*. In preliminary studies, isolates 10 and 43 were more effective than isolate 35 in their ability to infect eggs of *M. incognita* in a standard *in vitro* test (Irving & Kerry, 1986). Also, all three grew well *in vitro* but only isolate 10 produced chlamydospores freely on corn meal agar (Oxoid) and 35 produced none. It was not known whether such differences affected the ability of the isolates to infect root-knot nematodes and to survive in soil and so they were compared in the first experiment.

Production of fungi and their application to soil. Isolates were grown in Czapek Dox broth in shaken liquid cultures for 1 wk at 18oC (Kerry *et al.*, 1986). A 10ml sample of the hyphal and conidial suspension was transferred to each 100ml aliquot of a 1:1 (v/v) sand/wheat bran medium in 250ml conical flasks and incubated for 3 wks at 18oC. Unless otherwise stated, the colonised sand/bran was added to a non-sterile peat/sand soil at a rate 1% w/w soil. The inoculum was thoroughly mixed with the soil before dispensing 500g of the mixture into each plastic pot (diam. 12.5cm). Uncolonised sand/bran was mixed at the same rate (1% w/w soil) and added to pots and unamended soil was added to others to act as control treatments. The pots were then planted with 1 month old tomato plants cv. Roter Gnom and placed in randomised blocks in the glasshouse at 23oC for two weeks.

Addition of second-stage juveniles and estimation of nematode population densities in roots and soil. Second-stage juveniles (<1wk old) were hatched from egg-masses picked from the roots of infected tomatoes. The juveniles were counted and a suspension made up to give the required inoculum in 10ml water. This was added

2 wks after transplanting around the roots of the tomato plants. To determine nematode multiplication, juveniles and eggs were extracted from the root systems using the methods of Coolen & d'Herde (1972). The proportion of eggs infected with *V. chlamydosporium* was estimated by taking 10 egg-masses at random, dispersing the eggs in 3ml water using an homogeniser (Jencons), and plating them out on water agar (0.8%) in a Petri dish (Kerry & Crump, 1977). After 2 days at 22oC infected eggs were readily identified and the proportion (%) infected was determined by examination of 100 eggs on each dish. After two days the fungus had produced few conidia and so the plates were re-examined after 4 days when sporulation was more widespread and the fungus could be identified as *V. chlamydosporium*. Plates were examined after 2 days because after longer periods of incubation the growth of contaminants in some samples made assessments of parasitism difficult.

Estimation of fungal densities in soil and on roots. The isolates of *V. chlamydosporium* used in these experiments were known to grow readily on the semi-selective medium developed by Kerry *et al.* (1990). The medium contains: 37.5mg carbendazim, 37.5mg thiabendazole, 75mg rose bengal, 17.5g NaCl, 50 mg each of streptomycin sulphate, aureomycin and chloramphenicol, 3ml Triton X 100, and 17g corn meal agar (Oxoid) in a litre of distilled water. Soil samples from pots of each treatment were removed using a cork borer. At each sampling, two cores (diam 7mm) were taken from each pot. Soil dilution plates using the semi-selective medium were prepared using standard techniques (Kerry *et al.*, 1990). To assess root colonisation, root systems were carefully washed to remove soil, then blotted dry, weighed and cut into small segments (>1cm); 1g samples of each were taken at random and crushed with a sterilised pestle and mortar. The root fragments were suspended in 10ml of agar solution (0.05%) and a dilution range prepared and plated onto the selective medium as before. The length of a root sample (1g) was estimated using the intercept method (Tennant, 1975) and the average diameter of ungalled and galled (>25 galls/sample) calculated. This enabled the numbers of colony forming units (cfu) developing from root samples to be presented in terms of surface area. Colonies were counted at x50 magnification after 1-2 wks incubation at 18oC.

Experiment 1: To determine the potential of three isolates of *V. chlamydosporium* for the control of *M. arenaria* on tomato plants in pots. Isolates 10, 35 and 43 were

cultured and introduced into soil on the sand/bran mixture as described. In this test, only uninoculated sand/bran was used as a control treatment. As described above, second-stage juveniles were collected and 1,000 added to soil around the roots of tomato plants which had been growing, for 16 days, in soil amended with uncolonised sand/bran or with sand/bran colonised by each of the fungal isolates. Each treatment was replicated 6 times and the pots arranged in randomised blocks in the glasshouse at 23oC. The numbers of cfu/g soil of each isolate were estimated immediately after adding the fungus to soil and in pairs of pots sampled 20, 30 and 50 days after the nematodes were added. At the same time, the number of cfu/cm^2 of each fungal isolate was determined on galled and ungalled roots as described above. Nematode populations were estimated at the end of the experiment (50 days after nematode inoculation) and the proportion of infected eggs determined. The fresh tops and roots of each tomato plant were weighed at each harvest.

Experiment 2: <u>To determine the effect of inoculum form and rate of isolate 10 on the control of *M. arenaria* on tomato plants in pots</u>. Isolate 10 was cultured as before and 0.5g and 5g of the colonised sand/bran added to soil (0.1% or 1% w/w soil, respectively) in eight replicate pots. The same rates of uncolonised medium were added to similar numbers of pots as controls. Chlamydospores of the fungus were separated from the sand/bran by washing aliquots equivalent to the two application rates on a 58μm sieve and collecting the sediment on a 10μm sieve. The sediment from each aliquot containing chlamydospores and some hyphal fragments was mixed with 25g sand (20-100 mesh) and added to soil as before. A 'sand only' control was also used, hence, two rates of chlamydospores and unamended sand were compared with the sand/bran treatments. The numbers of cfu/g soil were estimated for each soil treatment immediately after addition of the fungus. To estimate the number of chlamydospores, aliquots (1g) of colonised sand/bran were added to 9ml 0.05% agar, and vigorously agitated on a mixer for 5 mins. A dilution series of the suspension was prepared and the numbers of chlamydospores were counted in a haemocytometer. The mean number of colony forming units and chlamydospores were 7×10^7 and 8×10^6/g sand/bran respectively. As before, nematodes (600 second-stage juveniles) were added around the roots of each tomato plant 2 wks after application of the fungal inoculum. The pots (56) were arranged in the

glasshouse at 23oC, in randomised positions in two blocks so that each treatment was replicated 4 times in each block.

The numbers of cfu/g soil were also estimated 21, 33, 61, 82, 98 and 110 days after the addition of the fungus in each of 4 pots for each treatment. The amount of fungus on roots was estimated 61 and 110 days after the soil was inoculated, when 4 pots were sampled to estimate the nematode population densities. At 23°C it was anticipated that after 47 days the nematode would have completed one generation and after 96 days 2 or 3 generations. The extent of root galling was assessed (Bridge & Page, 1980), the roots weighed, and in the final sampling the total length/g root was calculated for each root system. The numbers of nematodes and the extent of fungal infection was estimated as before.

Experiment 3: The effect of isolate 10 on the growth of tomato plants. The growth of tomato plants and the colonisation of their roots were examined in soils inoculated with *V. chlamydosporium* isolate 10 and the known plant pathogen, *V. albo-atrum* Reinke & Berthold. The latter fungus had been isolated from tomato roots and maintained on malt agar. Both fungi were grown on sand/bran and added to soil as described above for *V. chlamydosporium* before the tomatoes were planted. Four pots were established for each fungus and uninoculated sand/bran controls and placed in randomised positions in the glasshouse at 23oC. After 23 days the plants were harvested, and the fresh tops and roots weighed. A root sample (c 1g) was taken from each plant and divided into two equal sub-samples; one was washed in sterile distilled water while the other was surface sterilised in 0.2% sodium hypochlorite solution for 3 mins. After treatment the sterilised roots were washed five times in sterile distilled water. Each sample was crushed in a sterilised pestle and mortar as described before and a dilution series prepared. The suspension of root fragments from soil inoculated with *V. chlamydosporium* or *V. albo-atrum* were spread onto the semi-selective medium developed by Kerry *et al.* (1990) or on to a modified Jordan's (1971) medium respectively. The latter contained: 2g sorbose, 0.05g aureomycin, 0.05g streptomycin sulphate, 0.05g chloramphenicol and 10g agar in a litre of water. The colonies of each fungus developing from surface sterilised and untreated roots were counted between 1 and 2 wks incubation at 22oC.

Results

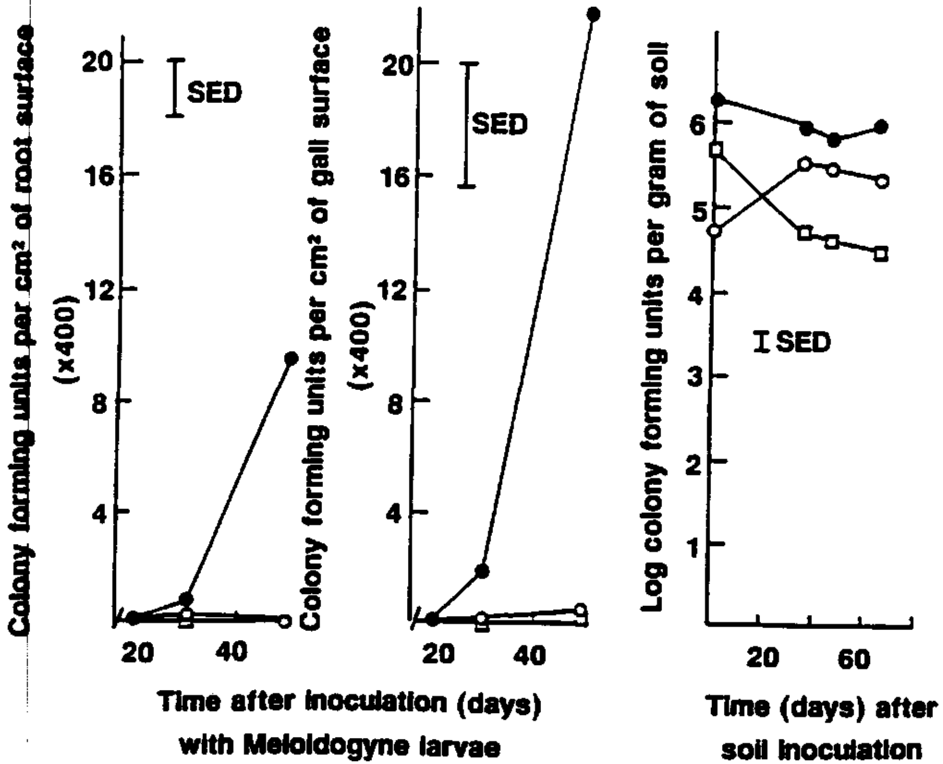

Fig. 1. Changes in numbers of colony forming units of three *V. chlamydosporium* isolates 10(●), 35(O) and 43 (□) on the surface of tomato roots and *M. arenaria* galls and in soil. Means of duplicate samples.

Experiment 1: <u>Effects of three isolates on multiplication of *M. arenaria*</u>. All three isolates of the fungus survived well in soil (Fig 1.) and remained numerous for the duration (66 days) of the experiment; isolate 10 was present in greatest numbers (approximately 10^6 cfu/g soil) on all sampling occasions. Although isolates 35 and 43 both established in soil, they were not rhizosphere competent and only isolate 10 increased significantly around the roots. The growth of isolate 10, but not the others, was greater on galled root tissue than on unaffected roots (Fig 1). There were no

significant effects of the fungus on the growth of the tomato plants.

Only applications of isolate 10 resulted in a significant (P<0.001) reduction in the numbers of nematode eggs and juveniles, which were approximately 80% fewer in soil treated with the fungus than in untreated soil (Table 1). Also, this isolate was the only one which could be re-isolated from nematode eggs at the end of the experiment, when approximately 30% were infected. Hence, isolate 10 was selected for further tests.

TABLE I

The effect of three isolates of *V. chlamydosporium* on post-cropping populations of *M. arenaria* 50 days after nematode inoculation

Treatment	*Total eggs and juveniles/g soil*	*Egg infection (%)*
Control	137	0
Isolate 10	29	32
Isolate 35	124	0
Isolate 43	164	0
S.E.D.	9.4	

Experiment 2: Effect of inoculum form and application rate of isolate 10 on the control of *M. arenaria*. Both types of application of isolate 10, either as colonised sand/bran containing hyphae, conidia and chlamydospores or the washings from sand/ bran

containing predominantly chlamydospores and some hyphal fragments became established in soil and survived throughout the experiment (110 days). At both application rates the fungus without the food base established itself within 33 days and was more numerous ($P<0.05$) on all subsequent sampling occasions than when it was applied in sand bran (Fig. 2). At the 0.1% and 1% application rates approximately 10^5 cfu/g soil and 10^6 cfu/g soil respectively were established and these differences persisted throughout the experiment.

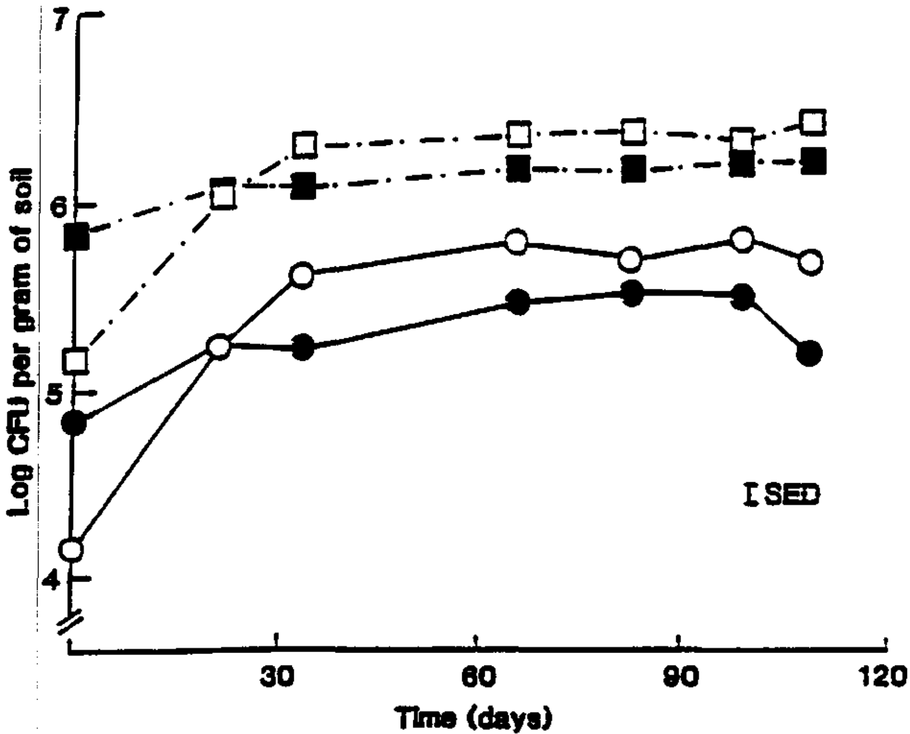

Fig. 2. Establishment and survival of *V. chlamydosporium* isolate 10 added to soil in colonised sand/bran at a rate (w/w) of 0.1% (●) and 1% (■) or as propagules (mainly chlamydospores) washed from equivalent weights of sand bran (0.1%; 0; 1%: □). Means of four replicates.

TABLE II

Effect of *V. chlamydosporium* isolate 10 on a population of *M. arenaria* on tomato plants on two sampling occasions 47 and 96 days after inoculation with *M. arenaria* larvae (means of four replicates)

Treatment	*Amount of sand bran (% w/w)*	*Sampling I*				*Sampling II*			
		Juveniles (Nos/g soil)	*Viable eggs (Nos/g soil)*	*Total population (log_{10} nos)*	*Egg infection (%)*	*Juveniles (Nos/g soil)*	*Viable eggs (Nos/g soil)*	*Total population (log_{10} nos)*	*Egg infection (%)*
No fungus	0	75	241	2.49	0	195	1260	3.13	0
	0.1	50	163	2.31	0	105	924	2.87	0
	1.0	35	169	2.29	0	426	4552	3.50	0
Fungus	0.1	16	50	1.79	35	202	1744	3.16	58
	1.0	6	41	1.65	38	187	1820	3.26	59
Fungus washed	0.1	21	63	1.90	36	332	2451	3.43	43
from :	1.0	9	43	1.68	48	174	1762	3.19	51
			S.E.D.	0.11				N.S.	

The higher application rate also resulted in greater numbers ($P<0.05$) of propagules of the fungus on the root surface (Fig.3), with greatest densities developing where the fungus had been added to soil at the 1% rate in sand/bran. The extent of root colonisation was similar in soils to which the fungus had been added with or without an energy source.

After 47 days populations of *M. arenaria* were significantly ($P<0.001$) smaller in all soils to which the fungus had been introduced (Table 2). The larger application rate resulted in smaller ($P<0.05$) populations of eggs and juveniles than the 0.1% rate

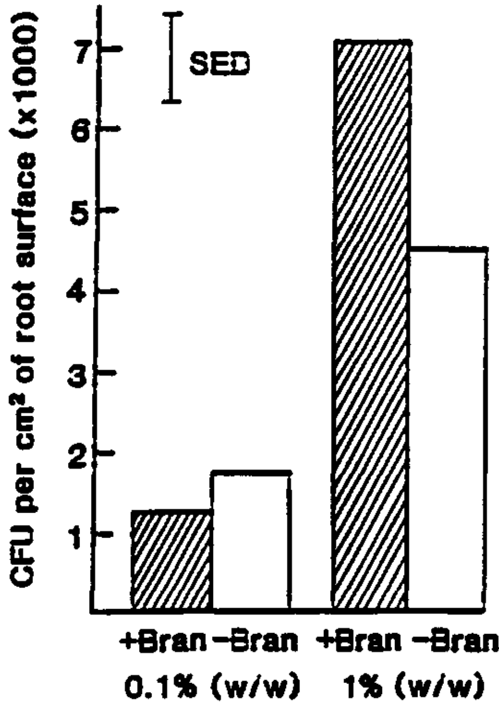

Fig. 3. Propagule densities (cfu/cm^2 root) of *V. chlamydosporium* isolate 10 on the root surface of tomato plants growing in soil treated with the fungus at two rates with and without a sand bran food source. Means of four replicates sampled after 61 days.

but at each rate there was no significant difference in numbers of nematodes in soils in which the fungus had been applied with bran compared to those where bran had not been applied. After 96 days the numbers of eggs and juveniles were similar in all treatments. *Verticillium chlamydosporium* was re-isolated from nematode eggs on both sampling occasions in soils treated with fungus (Table 2) but neither the form nor the rate of application affected the proportion of eggs infected after 47 or 96 days. Substantial proportions (up to 59%) of eggs were infected on the latter sampling occasion and galling was significantly ($P<0.001$) reduced and root length/g root significantly ($P<0.001$) increased where isolate 10 had been added to soil (Table

TABLE III

Effect of *V. chlamydosporium* isolate 10 on damage caused by *M. arenaria* to tomato plants 96 days after inoculation with juveniles (Means of four replicates)

Treatment	*Amount of sand/bran (% w/w)*	*Root gall index (1-10)*	*Root length (cm/g root)*
No fungus	0	7.5	177
	0.1	7.5	234
	1.0	6.9	221
Fungus	0.1	5.3	364
	1.0	4.5	430
Fungus washed from :	0.1	5.0	353
	1.0	4.4	488
	S.E.D.	0.45	46.4

3). Such damage to plants was less ($P < 0.05$) at the higher than lower application rate but again, at each rate, damage control was similar whether or not the fungus had been applied with wheat bran. None of the treatments significantly affected the fresh weight of roots.

Experiment 3: Effect of *V. chlamydosporium* on plant growth. The plant pathogen *V. albo-atrum* applied to soil significantly reduced the top weight of tomato plants ($P < 0.001$) but applications of *V. chlamydosporium* had no effect (Fig. 4). Surface sterilisation of roots had little effect on the numbers of *V. albo-atrum* cfu/g root but significantly ($P < 0.05$) reduced those of *V. chlamydosporium* (Fig. 5). This indicates that the plant pathogen grew inside the root and was physically protected from the sterilant whereas *V. chlamydosporium* grew on the root surface without penetrating the epidermis and cortex and so was killed by the treatment. No lesions were observed on roots growing in soil treated with *V. chlamydosporium* whereas those from soil inoculated with *V. albo-atrum* showed necrosis.

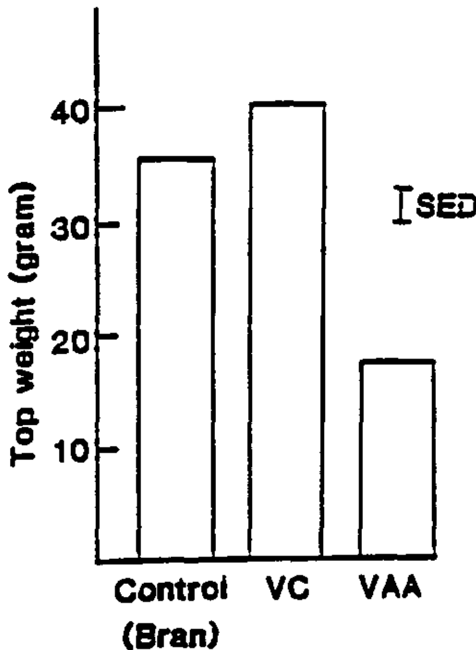

Fig. 4. Fresh weight (g) of the tops of tomatoes growing in soil inoculated with *V. chlamydosporium*, *V. albo-atrum* or uncolonised sand bran. Means of four replicates.

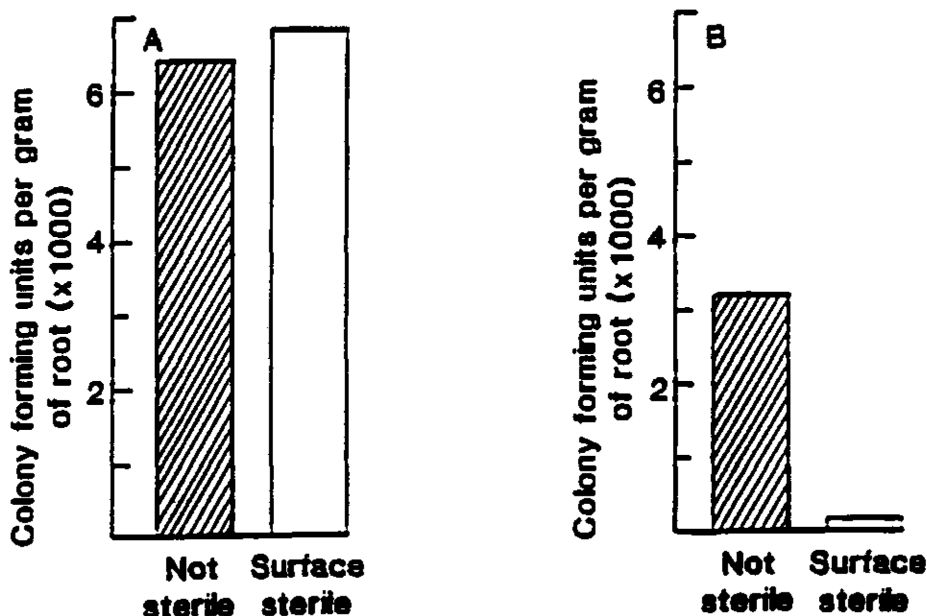

Fig. 5. Propagule densities (cfu/g root) of *V. albo-atrum* (A) and *V. chlamydosporium* (B) from surface sterilised and untreated roots of tomatoes. Means of four replicates.

Discussion

There is a need for careful selection of isolates in the development of a biological control agent. Variation between isolates of several species of nematophagous fungi has been widely reported (Stirling & Mankau, 1978; Nigh *et al.*, 1980; Kerry *et al.*, 1986). Although all three isolates of *V. chlamydosporium* used in our experiments parasitised eggs of root-knot nematodes *in vitro*, only one was effective in soil. The fungi survived well throughout the experiment in numbers considerably in excess of those found in soils that naturally suppress cyst-nematode multiplication (Kerry *et al.*, 1990). Only the isolate that extensively colonised the root surface caused significant reductions in nematode populations but this isolate was also the only one that was originally isolated from root-knot nematodes. Although isolate 10 was effective in three pot experiments, more extensive testing is necessary to determine its effectiveness in a range of conditions.

Significant reductions in the multiplication of *M. arenaria* were achieved after the first generation of the nematode in soil treated with isolate 10. However, the control

was insufficient to prevent the development of large nematode infestations in subsequent generations. The roots of plants growing in untreated soil were severely damaged and unable to support large numbers of egg–laying females in subsequent generations. Hence, differences in nematode populations on treated plants which had healthier roots and were able to support more females were less marked after more than one generation. In pot tests in the glasshouse *Meloidogyne* spp. multiply rapidly and soon exceed the carrying capacity of plant roots in untreated soil. Hence, it is more appropriate to determine the efficacy of biological control agents, such as *V. chlamydosporium*, by comparing the extent of plant damage, after more than one generation, with that of plants growing in untreated soil, and the level of nematode population control with that resulting from the application of an efficient nematicide. Also, in the experiments described, observations ceased after only one or two generations of the nematode. As yet nothing is known of the effect of the fungus on the survival of nematodes between crops. Some nematophagous fungi readily parasitise eggs in egg-masses added to soil (Stirling, 1979; Cabanillas *et al.*, 1989).

In contrast to colonisation of roots by *V. albo–atrum* our methods demonstrated that *V. chlamydosporium* was largely, and probably exclusively, confined to the rhizoplane; extensive colonisation of the root cortex and lesion development did not occur. These characteristics would probably prevent the development of *V. chlamydosporium*, which is related to some species of plant pathogens, as a biological control agent. Because *V. chlamydosporium* cannot colonise the root cortex, egg-masses developing inside large galls are protected from fungal infection. The severity of galling is dependent upon the susceptibility of the host plant and the nematode density. More prolonged control might be achieved at smaller pre–cropping nematode densities or on less susceptible crops than tomato; in both cases the size of galls would be less, and so a larger proportion of the egg-masses would be produced on the root surface. The susceptibility of the crop was considered important in determining the biological control efficacy of the fungus, *Dactylella oviparasitica* Stirling & Mankau, which effectively controlled root–knot nematodes on peach but not on vines, on which the egg-masses were approximately seven times the size (Stirling, McKenry and Mankau, 1979). The fungus appeared to be more prevalent on galled than on non–galled roots. This may be due to the

leaching of more nutrients from nematode damaged than undamaged tissue. *Paecilomyces lilacinus* was also found more frequently on galls (Hewlett *et al.*, 1988) and these authors stressed the importance of the root system in determining the spread of the fungus and its efficacy as a biological control agent.

Monitoring the survival of a nematophagous fungus applied to soil and measuring the numbers of nematodes colonised are important in determining its efficacy and potential as a biological control agent (Stirling, 1988). However, such data can be misleading without a proper understanding of the epidemiology of the fungus. The control of beet cyst nematodes was not related to the numbers of cysts colonised by different isolates of *V. chlamydosporium* at the end of the experiment but was dependent upon the proportion of young females infected within 2 wks of their emergence on the root surface (Kerry, 1988). Hence, an understanding of the time of infection and the spread can be important in the selection of potential biological control agents. Also, the ability to survive in soil may not be related to the levels of nematode infection unless the fungus is also able to colonise the root surface.

Chlamydospores and some hyphal fragments of *V. chlamydosporium* isolate 10 rapidly proliferated and survived for up to 110 days in non-sterile soil. Other isolates have been successfully established from similar inocula but proliferation of the fungus depended on soil texture and the isolate (Kerry *et al.*, 1990). An energy source colonised by the fungus was considered essential for the establishment of inocula of hyphae and conidia applied to mineral soils. The numbers of colony forming units established in soil used in these experiments was significantly greater when the fungus was applied as chlamydospores washed from sand bran than from an equivalent application of the same colonised growth medium. Presumably, application of the sand bran growth medium supported the growth of other soil micro organisms that successfully competed with *V. chlamydosporium* and limited its proliferation in soil. Chlamydospores tend to leak only small amounts of nutrients and have a negligible effect on the residual soil microflora (Bruehl, 1976). Hence, the fungus is able to establish more easily in soil from chlamydospores than from an external food source. The successful establishment of some isolates of *V. chlamydosporium* in soil from applications of chlamydospores without an energy source greatly simplifies experimentation, as controls to measure the amendment

effect of the food source itself are not required. In many experiments the food source applied with the fungus limited nematode multiplication when applied alone. Several authors have recommended the use of the uncolonised or autoclaved colonised growth medium as controls so that the effects of the fungus can be separated from those of its food source (Baker *et al.*, 1984; Kerry *et al.*, 1984; Stirling, 1988) but interpretation of results is often difficult.

Biological control agents are unlikely to have the widespread effectiveness of some chemical treatments. The efficacy of biological agents is likely to be affected by the level of nematode infestation, host plant, and other biotic and abiotic factors. The significance of these factors needs to be elucidated so that application rates and methods can be developed to deliver sufficient inoculum to give effective nematode control in a range of conditions. Selected isolates of *V. chlamydosporium* have considerable activity in non-sterile soils and survive in large numbers throughout the growing season. *V. chlamydosporium* is a promising nematophagous fungus for control of some cyst and root-knot nematodes and selected isolates are being developed as commercial biological control agents.

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CHAPTER 3

THE USE OF *VERTICILLIUM CHLAMYDOSPORIUM* AND *PASTEURIA PENETRANS* ALONE AND IN COMBINATION TO CONTROL *MELOIDOGYNE INCOGNITA* ON TOMATO PLANTS

F.A.A.M. de Leij, K.G. Davies and B.R. Kerry.

Summary

The potential control of *M. incognita* using an isolate of *Verticillium chlamydosporium* and *Pasteuria penetrans*, alone or in combination, was tested in a pot experiment at rates of 10^3 and 10^4 spores/g soil for each organism at two nematode population densities of 250 or 1500 second-stage juveniles per tomato plant. The treatments were compared with the effects of an aldicarb drench equivalent to 3.75 kg/ha. Root galling, invasion, numbers of females, egg production and infection were monitored 7 and 14 weeks after nematode inoculation. Aldicarb was the most effective treatment at reducing galling, as measured at the first harvest; by the second harvest, all treatments were giving similar root galling indices. Significantly fewer eggs and juveniles were produced after some of the treatments with biological control organisms than after treatment with aldicarb at the second harvest which gave *c* 55% population control. The biological control agents tended to complement each other, giving up to 92% population control at the second harvest, although neither reduced the initial invasion significantly. *Verticillium chlamydosporium* was most effective at infecting eggs at low nematode population densities when the galls were small and the majority of the egg-masses, produced on the root surface, were colonised by fungus resulting in population control levels of around 80%. *Pasteuria penetrans* infected up to 65 % of first generation females, but also infected subsequent generations of nematodes produced within large galls.

Introduction

Pasteuria penetrans (Thorne) Sayre & Starr is an obligate bacterial parasite of root-knot nematodes (Sayre & Starr, 1988) and has considerable potential as a biological control agent (Mankau & Imbriani, 1975; Sayre, 1980; Stirling, 1984). The natural suppression of root-knot nematodes in West Africa (Mankau, 1980), and on vines in South Australia (Stirling & White, 1982), have been associated with a large proportion of spore-encumbered juveniles in soil. *Pasteuria penetrans* reduces nematode populations when spores on the cuticle germinate and form micro-colonies, which proliferate in the pseudocoelom of the developing female and eventually mature into spores (Sayre & Wergin,1977); this prevents infected root-knot nematodes from producing eggs (Mankau, 1980; Sayre, 1980). *Pasteuria penetrans* may also reduce the numbers of second-stage juveniles that invade roots (Stirling, 1984; Brown & Smart, 1985; Davies, Kerry & Flynn, 1988). Although populations of *P. penetrans* exhibit varying affinities for different populations of nematodes (Stirling, 1985; Davies, Kerry & Flynn, 1988), virulent populations, with a high affinity of the spores for nematode cuticle at spore densities of between 10^4 and 10^6 spores per gram of soil, have provided very effective control of *Meloidogyne javanica* (Treub) Chitwood from soil (Stirling, 1984). Large numbers of second-stage juveniles may be encumbered with spores as they migrate towards the roots. However, those that hatch from eggs on, or in the root can reinvade without coming into contact with the soil and being exposed to spores (Stirling, 1984). Hence, control of root-knot nematodes is dependent on the infection of the first generation of juveniles (Kerry, 1987).

Verticillium chlamydosporium Goddard is a facultative parasite of cyst and root-knot nematodes (Willcox & Tribe, 1974; Kerry, 1975; Godoy, Rodriguez-Kabana & Morgan-Jones; 1983; Freire & Bridge, 1985). Experiments monitoring the growth of a *V. chlamydosporium* isolate in soil have shown the fungus to proliferate from 10^4 to 5×10^5 cfu g^{-1} soil over a period of 30 days; increases in the rhizoplane, especially at those sites where gall-formation caused by the nematode took place, were even greater (de Leij & Kerry, 1991). When the egg-masses emerged on the root surface, one isolate of the fungus colonised the gelatinous matrix and parasitised the eggs. In a pot test, where approximately 10^4 chlamydospores and their associated hyphal

fragments were introduced into soil, the numbers of *M. arenaria*(Neal) Chitwood were reduced by around 80%; this significantly decreased the amount of galling during subsequent generations of nematodes (de Leij & Kerry, 1991). Because the fungus survives and continues to proliferate saprophytically on the root surface during successive nematode generations, nematode control is not limited to the first generation.

The introduction of *P. penetrans* into soil to reduce root invasion and egg production, together with *V. chlamydosporium* as a rhizosphere coloniser and facultative egg parasite, may improve nematode control compared with either of the organisms alone. In this paper the use of *V. chlamydosporium* and *P. penetrans* at different inoculum densities, singly and in combination against different densities of *M. incognita*, is described together with a report of infection of subsequent generations of nematodes. This is discussed in relation to the epidemiology of both organisms, and their use and potential in the biological control of root-knot nematodes.

Materials and methods

Fungal and bacterial inocula. An isolate of *V. chlamydosporium* originally from *M. incognita* was chosen which had previously been shown to be effective against *M. arenaria* in pot tests (de Leij & Kerry, 1991). The fungus was cultured on a mixture of sand and bran (1 : 1 v/v) inoculated with two 5 mm agar plugs containing fungus and incubated at 18°C. After three weeks incubation the cultures were washed through a 50 μm aperture sieve with a fine water spray to remove the sand and bran and the fungal propagules were collected on a 10 μm sieve. The deposit was further washed to remove conidia and small hyphal fragments leaving mainly chlamydospores and some hyphal fragments. The chlamydospores were counted using a haemocytometer. The inoculum was prepared by mixing the fungus with fine sand which acted as an inert carrier.

Spores of *P. penetrans* were obtained from infected females of *M. incognita* containing mature spores following the method of Stirling and Wachtel (1980); the females were homogenized with a 1 ml uniform homogeniser (Jencons) to release the endospores, which were counted using a haemocytometer. The bacterial inoculum

was also mixed with fine sand as described above.

Each pot, containing 500 g unsterilised peat/sand compost (1 : 1 v/v) was inoculated with the fungus and/or bacterium inoculum in 10 g fine sand to produce the final concentrations of inoculum in the soil as shown in Table 1.

TABLE I

The initial inoculum rates of *Pasteuria penetrans* and *Verticillium chlamydosporium* used in the thirteen treatments of the experiment (spores x 10^3 per gram soil).

Treatments	1	2	3	4	5	6	7	8	9	10
V. chlamydosporium	1	10	0	0	1	1	10	10	0	0
P. penetrans	0	0	1	10	1	10	1	10	0	0
Aldicarb ($\mu g\ g^{-1}$)	0	0	0	0	0	0	0	0	0	1.5

Controls consisted of fine sand not containing the agents and an aldicarb drench of 1.5 μg active ingredient per gram compost added the day prior to nematode inoculation.

Experimental design. Ten treatments were each applied to sixteen pots. Each pot was planted with a one month old tomato seedling (cv. Roter Gnom) which was allowed to establish for two weeks before half of the pots were inoculated with 250 *M. incognita* juveniles and half with 1500 juveniles. Juveniles of less than 1 week old were obtained from infected tomato roots using a modified Baermann funnel technique (Whitehead & Hemming, 1965). The juveniles were counted and the required inoculum was introduced in 10 ml water, by pipetting the juveniles into three holes around the roots of the transplanted tomatoes. Four pots of each treatment were harvested after 7 weeks (1st harvest), while the other four were harvested after a further 7 weeks (2nd

harvest). The experiment was set out as four randomised blocks, each block representing one of four replicates. The results were analyzed using multivariate analysis of variance; data were transformed when necessary.

Fungal establishment. The amount of *V. chlamydosporium* present in the compost was measured immediately after inoculation of the experiment and at both harvests, using a semi-selective medium (de Leij & Kerry 1991). Dilution plates using the semi-selective medium were prepared using standard techniques. Before the root systems were processed to assess the extent of parasitism of the nematodes by both the fungus and the bacterium, root galling was determined using a gall-rating of 0-10 (Bridge & Page, 1980). Colonisation of the root surface was assessed at each harvest: root systems were washed carefully, then blotted dry, weighed and cut into small segments (*c* 1 cm length) and mixed thoroughly. From each root system a 1 g sample was taken, and crushed with a sterilised pestle and mortar in 9 ml of agar solution (0.05 %). A dilution range of this suspension was plated onto the semi-selective medium as previously described (de Leij & Kerry, 1991).

Infection of the first and subsequent nematode generations and the effect of root galling. At both harvests, half of the chopped root segments were used for estimating the number of females and their infection by *P. penetrans*. The roots were digested in Pectinex (Novo Enzyme Products Ltd., Farnham, U.K.) for 24 h at room temperature, homogenised (Atomix, Measuring and Scientific Equipment Ltd., Crawley, U.K.), for 20 seconds, and the females extracted by washing the slurry through a 0.8 mm aperture sieve and collecting them on a 53 μm aperture sieve (Davies, Kerry & Flynn, 1988). A sample of the residue was obtained and the females counted in a counting dish (Doncaster, 1962). A further ten females were individually squashed on a microscope slide and examined at x 400 for *P. penetrans* infection.

Half of the remaining root sample was used to estimate the number of eggs and juveniles using the methods described by Coolen & d'Herde (1973). The proportion of eggs in egg-masses infected with *V. chlamydosporium* was estimated by taking egg-masses from the remaining roots, dispersing the eggs in 3 ml water using a homogenizer (Jencons), and plating *c* 0.5 ml of this egg suspension onto water agar (0.8%) in a Petri-dish (Kerry & Crump, 1977). After 2 days infected eggs could be recognised by fungal hyphae growing from the eggs; 100 eggs on each dish were

examined and the proportion parasitised estimated. After 2 days the fungus had produced few conidia and so the plates were re-examined after 4 days when sporulation was more frequent, so the fungus could be identified as *V. chlamydosporium*. Plates were examined after two days because after longer periods of incubation the growth of contaminants not associated with the nematode eggs in some samples made assessment of parasitism difficult.

At the first harvest, egg-masses were picked off the surface of galled roots and examined for infection; at the second harvest, when a proportion of the egg-masses were produced within the root gall, infection of eggs on the root surface was compared with the infection of eggs and juveniles within galls. Each sample consisted of twelve randomly chosen egg-masses picked of from the root surface or picked out of dissected galls. The number of juveniles and the numbers of healthy and infected eggs were counted, and the presence or absence of chlamydospores in each individual egg-mass was recorded; this was done using a high power microscope (X 200). *Pasteuria* infection was studied by removing ten large galls from the *P. penetrans* treated pots and carefully dissecting each gall separately to release the second-stage juveniles from inside the gall. The juveniles were then examined microscopically (X 400) and the numbers of spores adhering to their cuticles counted.

Results

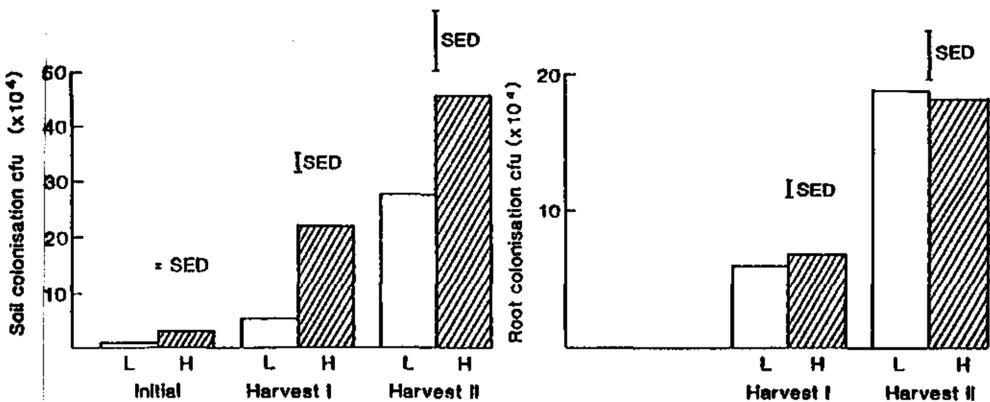

Fig 1 Colonisation of soil and rhizosphere by *Verticillium chlamydosporium* inoculated at rates of 1000 (L) and 10,000 (H) chlamydospores per gram soil.(n=24)

Fungal establishment. During the experiment there was a rapid increase in *V. chlamydosporium* both in the compost and on the root surface in all the pots to which it was added (Fig 1). Throughout the experiment there was a large difference in the establishment of *V. chlamydosporium* in the compost between the high and low rates of inoculum; however, differences in soil populations of the fungus in the compost did not result in significantly different population densities on the root surface (Fig. 1).

Effects of V. chlamydosporium and P. penetrans on nematode populations and root galling.

TABLE II

Galling index (1-10; Bridge & Page, 1980) of tomato roots grown in compost inoculated with 250 or 1500 juveniles of *Meloidogyne incognita* per plant and treated either with *Verticillium chlamydosporium* at rates of 10^3 (VL) or 10^4 (VH) chlamydospores/g soil, or with *Pasteuria penetrans* at rates of 10^3 (PL) or 10^4 (PH) spores/g soil, or the combinations of the two (VL+PL; VL+PH; VH+PL; VH+PH), or with aldicarb at a rate of 1.5 ppm, or left untreated. (n=4)

Harvest time	7 weeks		14 weeks	
Treatment/Pi	250	1500	250	1500
Control	1.3	3.3	7.3	8.0
Aldicarb	0.4	0.6	4.3	5.8
VL	1.0	2.5	4.5	6.3
VH	0.9	2.5	4.0	5.0
PL	1.3	2.8	5.8	7.0
PH	1.1	2.0	5.3	7.0
VL+PL	0.6	2.5	5.3	5.8
VL+PH	1.0	1.8	6.0	5.3
VH+PL	0.8	3.3	4.3	4.8
VH+PH	0.6	2.3	2.5	4.0
SED	0.3^{*}	0.3^{***}	0.6^{***}	0.5^{***}

TABLE III

Number of *Meloidogyne incognita* females (FEM)/root system, and their % infection extracted from tomato roots inoculated with 250 or 1500 juveniles per pot, and treated with: *Verticillium chlamydosporium* at rates of 10^3 (VL) or 10^4 (VH) chlamydospores/g soil, or *Pasteuria penetrans* at rates of 10^3 (PL) or 10^4 (PH) spores/g soil, or the combinations of the two (VL+PL; VL+PH; VH+PL; VH+PH) or aldicarb at a rate of 1.5 ppm, or left untreated. (n=4)

Harvest time	7 weeks				14 weeks			
Treatments/Pi	250		1500		250		1500	
	FEM	% infection	FEM	% infection	FEM	% infection	FEM	% infection
Control	88	0 -	374	0 -	1697	0 -	6161	0 -
Aldicarb	36	0 -	142	0 -	1468	0 -	2703	0 -
VL	99	0 -	364	0 -	1021	0 -	3287	0 -
VH	89	0 -	308	0 -	1037	0 -	3944	0 -
PL	106	19 (-1.10)	280	25 (-0.77)	1275	6 (-2.00)	4622	8 (-1.75)
PH	64	50 (0.00)	214	65 (0.68)	1210	52 (0.15)	5655	33 (-1.79)
VL-PL	41	17 (-1.30)	396	19 (-1.55)	1416	8 (-2.14)	2854	2 (-2.39)
VL-PH	72	38 (-0.53)	324	31 (-0.85)	1670	50 (0.01)	4552	52 (0.13)
VH-PL	53	13 (-2.07)	199	17 (-1.77)	1205	8 (-2.14)	2590	8 (-1.75)
VH-PH	58	59 (0.34)	360	58 (0.34)	1046	67 (0.92)	2910	50 (0.00)
SED	21	0.327	91	0.49	530	0.62	1287	0.69
Significance level p	0.05	<0.001	NS	<0.001	NS	<0.001	NS	<0.001

* parenthesis logit $x = \log_e(x/1-x)$

After the first harvest, galling was reduced by almost all treatments compared with the untreated controls (Table 2). Aldicarb was the most effective treatment, producing a significant reduction in galling in both the low and high rates of nematode inoculation. At the second harvest, all treatments reduced galling rates significantly irrespective of the initial nematode level. *Verticillium chlamydosporium* applied at 10^4 chlamydospores/g soil was significantly better than *P. penetrans* and comparable to aldicarb in reducing galling. The combined high rates of *V. chlamydosporium* and *P. penetrans* caused the greatest reduction in root galling index, at the second harvest.

Aldicarb was the only treatment which produced a significant reduction in the number of females on the root. However this was only true at the first harvest, by the second harvest there were no significant differences between any of the treatments (Table 3). The effectiveness of the biological control agents could best be evaluated by examining the numbers of juveniles and eggs produced (Tables 4, 5). Numbers of juveniles were reduced by all treatments at the first harvest (Table 4). Greatest reductions in numbers of juveniles were achieved with those treatments where *V. chlamydosporium* was added either alone or in combination with *Pasteuria penetrans*, resulting in population control comparable with the control achieved with aldicarb (Table 4). Greatest reductions in numbers of juveniles were achieved with the combination of a high level of *P. penetrans* with a high level of *V. chlamydosporium*, resulting in 92% control; a high dose of *V. chlamydosporium* on its own resulted in 85% control. Both treatments gave significantly greater reductions in the number of juveniles than aldicarb (52% population control). The data for eggs showed similar trends to the results for juveniles (Table 5).

Infection of *M. incognita* by *V. chlamydosporium* and *P. penetrans*. When *P. penetrans* was applied alone there were highly significant differences (Table 5) in the percentage infection of females between the low rate of inoculation (6 to 25%) and the high inoculum rate (33 to 65%). The addition of *V. chlamydosporium*, in combination with *P. penetrans*, did not significantly affect female infection; nematode density did not affect female infection by *P. penetrans* either (Table 5). After the first harvest none of the second-stage juveniles extracted from gall tissue had any *Pasteuria* spores attached to their cuticles. After the second harvest 50% of the juveniles extracted from gall tissue treated with the high dose of *P. penetrans* had spores attached to their

TABLE IV

Number of juveniles per root system extracted from tomato roots inoculated with 250 or 1500 *Meloidogyne incognita* juveniles per pot and treated with *Verticillium chlamydosporium* at rates of 10^3 (VL) or 10^4 (VH) chlamydospores/g soil, or *Pasteuria penetrans* at rates of 10^3 (PL) or 10^4 (PH) spores/g soil, or the combination of the two (VL+PL; VL+PH; VH+PL; VH+PH), or aldicarb at a rate of 1.5 ppm, or left untreated. (n=4)

Juveniles (x 100) per root system

Harvest	7 weeks		14 weeks	
treatment/Pi	250	1500	250	1500
Control	78.0 (1.88)*	592 (2.72)	2086 (3.30)	1728 (3.20)
Aldicarb	14.0 (1.01)	73 (1.77)	894 (2.89)	814 (2.84)
VL	18.1 (1.24)	95 (1.81)	784 (2.80)	1564 (2.87)
VH	13.1 (0.98)	74 (1.74)	307 (2.46)	782 (2.01)
PL	77.5 (1.77)	219 (2.33)	768 (2.83)	541 (2.69)
PH	48.9 (1.56)	176 (2.20)	1596 (3.02)	883 (2.89)
VL+PL	17.0 (1.18)	107 (1.98)	1226 (3.05)	1493 (3.12)
VL+PH	49.2 (1.57)	180 (2.06)	955 (2.97)	401 (2.39)
VH+PL	8.6 (0.92)	153 (2.16)	682 (2.80)	893 (2.84)
VH+PH	12.4 (0.94)	89 (1.93)	177 (2.22)	293 (2.36)
SED	0.230	0.230	0.168	0.168
Significance level (p)	<0.001	<0.01	<0.001	<0.001

* data not normally distributed Y = Log (X + 1)

TABLE V

Number of eggs/root system extracted from tomato roots inoculated with 250 or 1500 *Meloidogyne incognita* juveniles per pot and treated with *Verticillium chlamydosporium* at a rate of 10^3 (VL) or 10^4 (VH) chlamydospores/g soil, or *Pasteuria penetrans* at rates of 10^3 (PL) or 10^4 (PH) spores/g soil, or the combination of the two (VL+PL; VL+PH; VH+PL; VH+PH) or aldicarb at a rate of 1.5 ppm, or left untreated.(n=4)

Eggs (x 100 per root system)

Harvest	7 weeks				14 weeks	
	Total eggs		Healthy eggs		Total eggs	
Nematode inoculum	250	1500	250	1500	250	1500
Control	364	2083	364 (2.54)*	2083 (3.31)	9336 (3.88)	9703 (3.97)
Aldicarb	85	292	85 (1.92)	292 (2.41)	3759 (3.50)	4425 (3.61)
VL	189	1047	76 (0.18)	392 (2.57)	4171 (3.50)	7896 (3.77)
VH	305	1114	98 (1.93)	412 (2.60)	3562 (3.48)	5940 (3.75)
PL	209	1201	209 (2.22)	1201 (3.06)	6523 (3.74)	4040 (3.59)
PH	160	712	160 (2.15)	712 (2.83)	8734 (3.78)	7220 (3.77)
VL+PL	166	989	86 (1.93)	468 (2.62)	7643 (3.87)	7255 (3.83)
VL+PH	172	906	93 (1.93)	489 (2.55)	6704 (3.81)	4353 (3.62)
VH+PL	120	925	59 (1.70)	446 (2.64)	5218 (3.68)	3359 (3.45)
VH+PH	124	889	62 (1.79)	467 (2.64)	1043 (2.10)	2097 (3.32)
SED	64	281	0.15	0.16	0.20	0.16
significance level (p)	<0.001	<0.001	<0.001	<0.001	<0.01	<0.05

* data not normally distributed Y = Log (X + 1)

cuticles, with an average of more than four spores per juvenile. Approximately 15% of the juveniles from the treatments with *P.penetrans* at 10^3 spores/g soil had spores attached at an average of little more than one spore per juvenile. Egg-masses which did not break through the gall surface were physically protected from *V. chlamydosporium* by root tissue and contained. Therefore no infected eggs. Egg-masses produced on the outside of the gall were all colonised by the fungus; this resulted in more than 90 % of the eggs being infected (Fig. 2).

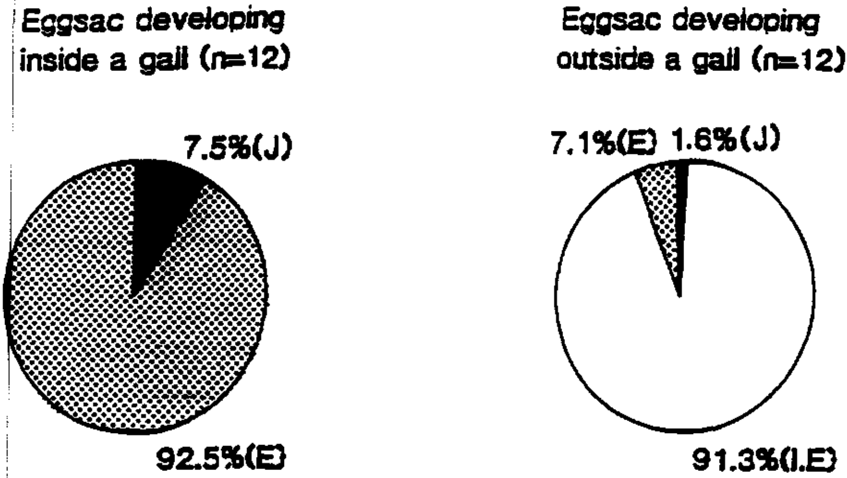

Fig 2 Availability of *Meloidogyne incognita* eggs developing inside and outside the root gall to *Verticillium chlamydosporium* (E = healthy eggs; IE = infected eggs; J = juveniles).

Discussion

Aldicarb was the most effective treatment at reducing galling, as measured at the first harvest. However, by the second harvest a similar degree of control of root galling was being obtained by the biological control organisms. Reductions in the numbers of eggs and juveniles after addition of biological control agents were

significantly greater than after aldicarb treatment at the second harvest. Aldicarb acts by contact with the motile second-stage juvenile and reduces their ability to locate host roots and feed (Wright, 1981); its ability to control second-stage juveniles is limited to about six weeks (Hague & Gowen, 1987) and, therefore, any subsequent generation of juveniles coming from nematodes which escaped its initial effect will not be controlled without further applications. Both of the biological organisms continued to act on subsequent generations of nematodes, although the amount of infection varied between the two and was dependent on the extent to which large galls had formed.

Meloidogyne spp. have a great reproductive potential (de Guiran & Ritter, 1979). Size and health of the root systems are therefore very important factors which determine to a large extent the numbers of nematodes and their reproduction in the roots at high nematode densities (Triantaphyllou, 1960). At the second harvest where restricted root growth and large numbers of nematodes prevailed, there were some treatments with more nematodes than suitable feeding sites on the roots to support the development of females. This has led to increased variation which made the interpretation of results after the second harvest difficult. Because of excessive damage caused by the nematodes in the untreated controls after the second harvest, nematode populations were in general smaller in pots receiving the large nematode inoculum than in those receiving the small inoculum. It may, therefore, be more appropriate to compare nematode control by the biological treatments at harvest two with the degree of control after aldicarb treatment.

Colonization of egg-masses by *V. chlamydosporium* was confined to those exposed to the soil on the root surface; no egg-masses embedded in the root were colonised. At the first harvest, most egg-masses had developed on the root surface as there were few large galls, resulting in more than an 80 % reduction in nematode populations; this level of control was similar to that obtained with aldicarb. Colonisation of egg-masses by *V. chlamydosporium* after the first harvest was not dependent on nematode density, making the fungus very useful in situations where nematode densities were low. As the amount of fungus detectable on the root surface more than doubled during the 7 weeks between the first and second harvest it seems likely that the effectiveness of the fungus should increase with time. However, in subsequent generations more and more nematodes developed inside the root, in large

galls, making the fungus less effective as it does not penetrate the root cortex (de Leij & Kerry, 1991).

Neither *V. chlamydosporium* nor *P. penetrans* had any significant effect on the numbers of females. Although *P. penetrans* has been observed to reduce invasion (Stirling, 1984; Davies, Kerry & Flynn, 1988), in this experiment, where juveniles were inoculated into pots with well established root systems, the distance the nematodes had to migrate through the soil before finding a suitable invasion site was presumably too short for them to become encumbered with sufficient spores. Therefore, for *P. penetrans* to have an effect on invasion, either the number of spores, or the length of time the second-stage juveniles are active in the soil, needs to be increased. It has been suggested that 10^5 spores per gram soil are required to reduce invasion (Stirling, 1988). However, fewer spores would be necessary if the distance juveniles have to migrate was lengthened.

No parasitism by *P. penetrans* could be observed on second-stage juveniles dissected from galls at the time of the first harvest. This observation alone, would confirm the view that *P. penetrans* has to be sufficiently active to control the initial invasion if it is to be a useful biological control agent. At the second harvest it was expected that the percentage of females infected with *P. penetrans* would decline, because juveniles would reinvade without coming into contact with the soil and would therefore remain uninfected. However, our results showed that after the second harvest, at the high *P. penetrans* inoculation rate a relatively high percentage of the females were infected with *P. penetrans*. As spores were also observed on second-stage juveniles dissected from large galls, this suggests that either they had left the root and become encumbered with spores in the soil before reinvading, or they had picked up spores from inside the root from disintegrating females. As spores were only observed on second-stage juveniles dissected from galls at the second harvest and not at the first, the latter explanation is the most likely.

In this pot-test *V. chlamydosporium* and *P. penetrans* parasitised different stages of the nematode life-cycle and were able to produce strong complementary effects when both organisms were present in high concentrations. Both organisms reduced the reproductive potential of the female nematode. *V. chlamydosporium* was very effective after the first harvest when most egg-masses were produced in the

rhizosphere and the fungus could colonise the egg-masses. Later, when egg masses were produced within galls and were protected from the fungus, *P. penetrans* liberated from disintegrating females may have produced a degree of secondary infection, hitherto unobserved.

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CHAPTER 4

THE EFFECT OF FUNGAL APPLICATION RATE AND NEMATODE DENSITY ON THE EFFECTIVENESS OF *VERTICILLIUM CHLAMYDOSPORIUM* AS A BIOLOGICAL CONTROL AGENT FOR *MELOIDOGYNE INCOGNITA*

F.A.A.M. de Leij, B.R. Kerry & J.A. Dennehy

Summary

Five application rates of *Verticillium chlamydosporium* (0, 50, 500, 5000 and 50,000 chlamydospores/g soil) and five inoculum densities of *Meloidogyne incognita* (0, 50, 500, 5,000 and 25,000 juveniles/plant) were tested in a factorial experiment on tomato plants grown in pots. The effects of nematode and fungal inoculum density on fungal colonisation of soil and tomato roots, as well as nematode control and invasion of the roots were studied. Even when roots were extensively colonised by *V. chlamydosporium*, invasion of juveniles of the roots was not affected by the fungus. Establishment of *V. chlamydosporium* in soil was dependent on the initial fungal inoculum rate used. Increases in numbers of colony forming units in soil were greater from a small initial inoculum than from a large one. This suggests that *V. chlamydosporium* depends partly on external nutrients in soil for its establishment. Colonisation of the rhizoplane depended on initial fungal inoculum and on galling caused by *M. incognita*; higher fungal and nematode inoculum levels resulted in greater colonisation of the roots. Although galled roots were most extensively colonised by *V. chlamydosporium*, the fungus was least effective in controlling *M. incognita* at high nematode densities, presumably, because many egg-masses stayed embedded in the gall tissue, and were therefore protected from fungal attack.

Introduction

Verticillium chlamydosporium Goddard is a facultative parasite of cyst- and root-knot nematodes (Willcox & Tribe, 1974; Kerry, 1975; Morgan-Jones, Godoy & Rodriguez-kábana, 1981; Freire & Bridge, 1985). An isolate of the fungus which was able to colonise the rhizoplane of tomato plants and especially sites where galls formed, was an effective biological control agent for *Meloidogyne incognita* Kofoid & White, *M. javanica* Treub, *M. arenaria* Neal and *M. hapla* Chitwood (de Leij & Kerry, 1990, 1991).

Besides abiotic and biotic constraints such as temperature, aeration and competition from other organisms, establishment of *V. chlamydosporium* on the root surface seems to be governed by two main factors: (a) fungal inoculum density in soil, which determines the initial propagule density on the root surface, and (b) the amount of stimulus the roots provide for the fungus to grow. De Leij & Kerry (1991) found that more fungus established on galled tissue than on healthy roots. The extent of colonisation of the rhizoplane may therefore be related to the density of nematodes in the roots. Presumably infected root tissue leaks more nutrients into the rhizosphere than healthy root tissue.

However, large nematode infestations may reduce the effectiveness of *V. chlamydosporium*. On susceptible plants like tomato, gall size induced by *Meloidogyne* spp. is proportional to the number of nematodes developing at a certain site in the root (Dropkin, 1954). At high nematode densities large galls are induced in which a significant proportion of the egg-masses may stay embedded in the gall tissue. These egg-masses are physically protected from fungal attack (de Leij, Davies & Kerry, 1992). The higher the proportion of embedded egg-masses, the less the control by *V. chlamydosporium*.

Colonisation of roots by rhizosphere bacteria have been reported to reduce nematode invasion (Schroth & Hancock, 1981; Oostendorp & Sikora, 1989). Because *V. chlamydosporium* is rhizosphere competent, roots that are extensively colonised by *V. chlamydosporium* may inhibit nematode invasion. In this paper the effect of fungal application rate and nematode density on fungal establishment in soil, fungal establishment on the root surface, invasion of roots by juveniles, and nematode control by *V. chlamydosporium* are reported.

Materials and methods

Fungal inoculum. An isolate of *V. chlamydosporium* (CMI cc 334168) originally isolated from *M.incognita* eggs was chosen because it had previously been shown to be effective against *M. arenaria* , *M. javanica*, *M. hapla* and *M. incognita* in pot tests (De Leij & Kerry, 1990). The isolate was stored on silica gel at 5°C (Smith & Onions, 1983).

For experimental use, a few silica gel crystals were sprinkled onto a water agar plate and incubated at 25°C for two weeks, allowing the fungus to grow away from the crystals onto the agar. The fungus was mass-produced by inoculating a sterile mixture of moist milled barley grain and sand (1 : 1 $^v/_v$) with two 7 mm agar plugs colonised by *V. chlamydosporium*. After two weeks at 25°C, the cultures were washed through 50 and 10μm aperture sieves with a fine spray of water to remove the culture medium. The residue on the 10 μm sieve was further washed to remove conidia and small hyphal fragments, leaving mainly chlamydospores. The chlamydospores were counted in a haemocytometer. Fungal inoculum was prepared by mixing the residue on the 10 μm sieve with fine sand to a concentration of 10^7 chlamydospores/g sand. This inoculum was further mixed with an unsterilised mixture of peat and sand (3 : 5 $^v/_v$) to provide a standard, infested potting compost (hereafter referred to as soil).

Treatments. Five fungal application rates were used: 0, 50, 500, 5,000 and 50,000 chlamydospores/g soil. For each rate, 25 pots (diam. 12.5 cm) were filled with soil (700 g per pot) and each pot was planted with one tomato seed (cv. Pixie). The pots were placed in the glasshouse (25 to 30°C) in five randomized blocks. One month after planting of the seed, nematodes were inoculated into the soil through a cut of pipette-tip (1 ml), which was fixed into a hole made halfway each pot. *Meloidogyne incognita* juveniles (less than one week old) were added to the soil via the pipette tip in 10 ml water to give concentrations of 0, 100, 500, 5,000 or 25,000 juveniles/plant.

Effect of fungal inoculum level and nematode density on invasion. Three weeks after inoculation with nematodes, one block was harvested. Roots were carefully washed to remove the soil and blotted dry. After weighing the roots, damage was estimated using the gall-rating chart described by Bridge & Page (1980). The roots from each pot were cut into small segments (c 2 cm) and mixed thoroughly. The ratio of root

weight : surface area, is greater in heavily galled roots than in those less infected by the nematode. Because *V. chlamydosporium* is confined to the root surface (de Leij & Kerry, 1991), data presented in terms of cfu/g root can be misleading if roots with different galling indices are compared. Hence, the density of the fungus on the roots is expressed as the number of cfu/cm^2 root, which is estimated as follows: The root length of a 1 g root sample was measured using the intercept method (Tennant, 1975). From this and the total root weight, total root-length and surface area/g root could be calculated by assuming that fresh roots consist mainly of water and so have a specific gravity of unity and that roots are round cylinders of uniform diameter. Then, $r^2 l\pi = 1$ (l = length of a 1 g root sample; r = root radius). It follows that $r = \sqrt{(1/\pi l)}$. The surface area of 1 g root is $2\pi rl$. Combining the two equations results in: Surface area = $2\sqrt{(\pi l)}$. Because there was a strong negative correlation (r= -0.90) between root length and galling in this experiment (Fig. 1), root length can also be expressed in terms of galling (Fig. 1). Because the length of 1 g of healthy roots varies considerably depending on host plant and soil type, while galling is also dependent on host plant and nematode species used (Nickle, 1984), the last equation is only of use in this particular experiment.

A sample (3-6 g) of the remaining roots was used to estimate numbers of females and males in the roots. The roots were digested in Pectinex (Novo Enzyme Products Ltd, Farnham, U.K.) for 24 h at room temperature. The slurry was washed through a 0.8 mm screen with a strong water jet onto a 10 μm aperture sieve. The residue was suspended in 100 ml water, and the nematodes in two samples of 10 ml were counted using a low power microscope (x 6).

Effect of fungal inoculation rate and nematode density on control. Seven weeks after nematode inoculation the remaining four blocks were harvested. Root weight and galling were measured as described for the first block. Numbers of nematodes in the roots were estimated using the methods described by Coolen and d'Herde (1973). About ten egg-masses from *V. chlamydosporium* treated plants were taken to confirm egg infection and presence of chlamydospores. Each egg-mass was crushed in a drop of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix and examined at 100X magnification.

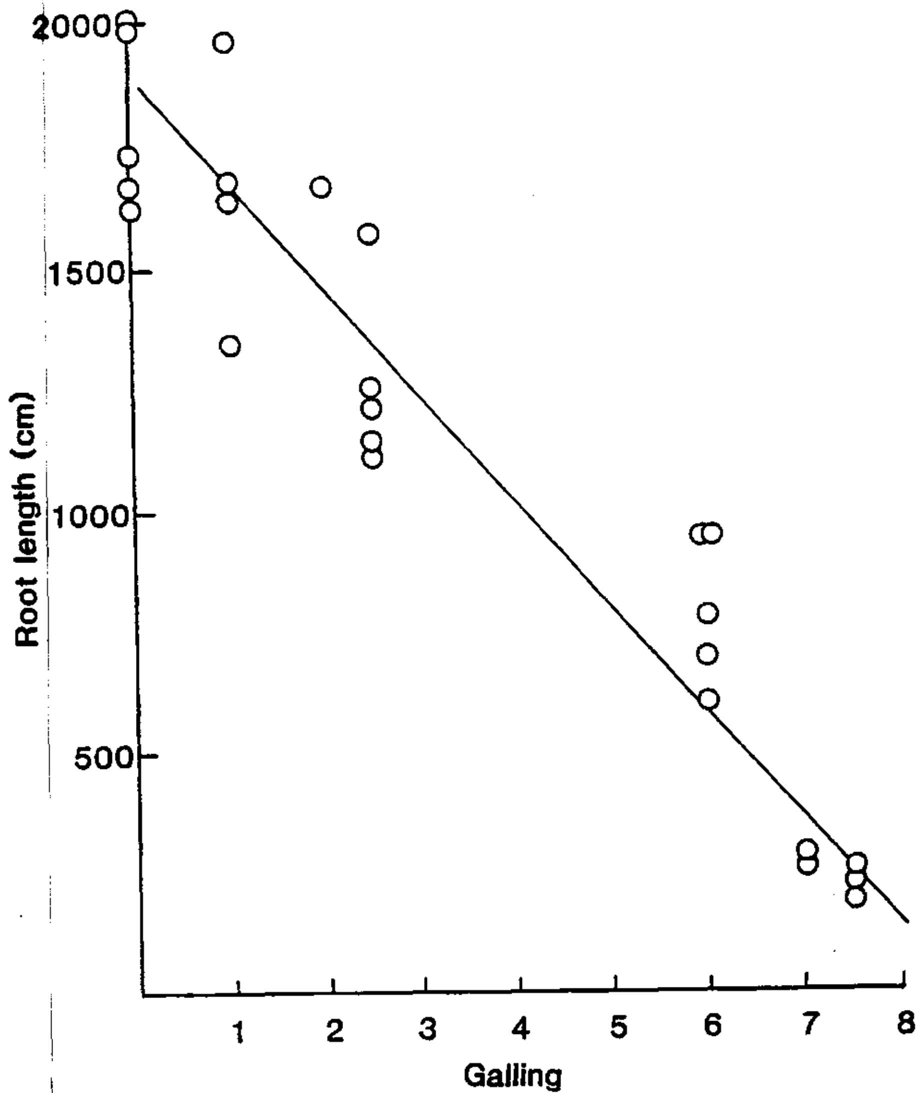

Fig. 1: Relationship between galling-index and root length of tomato roots infested with *Meloidogyne incognita*. X=-0.004531Y + 8.602 (r=-0.90); X=Galling, Y=Root length (cm).

Effect of fungal inoculum rate and nematode density on fungal establishment. The amount of *V. chlamydosporium* present in the soil was measured immediately after inoculation of the experiment and at both harvests, using dilution series of soil suspensions spread on Petri-dishes with semi selective medium (de Leij & Kerry, 1991). Root colonisation by *V. chlamydosporium* was measured at both harvests. Each root system was cut up and mixed, and a 1 g sample was crushed at room temperature in 9 ml of agar solution (0.05%) with a sterilised pestle and mortar. A dilution range of this suspension was plated out onto semi selective medium as described for the soil. Soil and root dilution plates were incubated at 25°C for one week before the *V. chlamydosporium* colonies were counted using a low power microscope (6 x). The number of colonies counted per plate was usually between 20 and 200.

Results

Effect of nematode density and fungal density on nematode invasion of roots. Invasion was linearly related to nematode inoculum density (fig 2) This relationship was not significantly affected by the fungus ($F<1$). All the nematodes in the roots at inoculum densities of 100 and 500 juveniles/plant developed into females; at inoculum densities of 5,000 and 25,000 juveniles/plant, a proportion of the nematodes in the roots developed into males (12% and 67% respectively) (Fig. 3).

Effect of nematode density and fungal density on the establishment of *V. chlamydosporium* in soil. Eleven weeks after inoculation with *V. chlamydosporium*, the numbers of cfu estimated in soil were mainly dependent on the initial inoculum level (Table 1). This relation could be expressed by the regression equation: log soil colonisation = 0.6206 x log initial inoculum + 2.8705 ($r = 0.98$). There was a small but significant ($P < 0.001$) positive effect of galling caused by *M. incognita* and fungal establishment in soil (Table 1; Table 5).

Effect of fungal inoculum rate and nematode density on establishment of *V. chlamydosporium* on the root surface. Establishment of *V. chlamydosporium* on the root surface was equally dependent on initial fungal inoculum rate as on the severity of galling caused by *M. incognita*; the more inoculum and the higher the galling index,

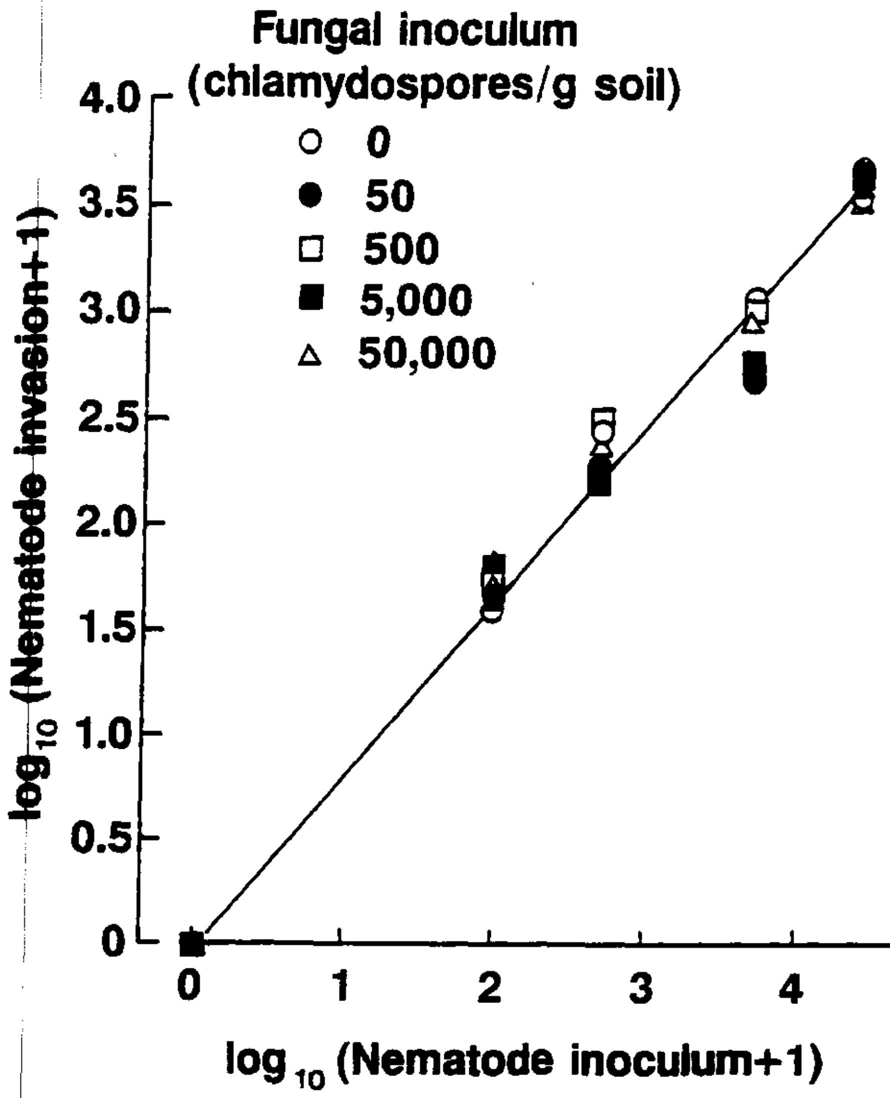

Fig. 2: Relationship between larval inoculum density/plant and the resulting numbers of developing nematodes (males and females) in the roots. Y=0.8134X + 0.03 (r=0.996); X= log10(invasion +1), Y=log10(larval inoculum + 1)

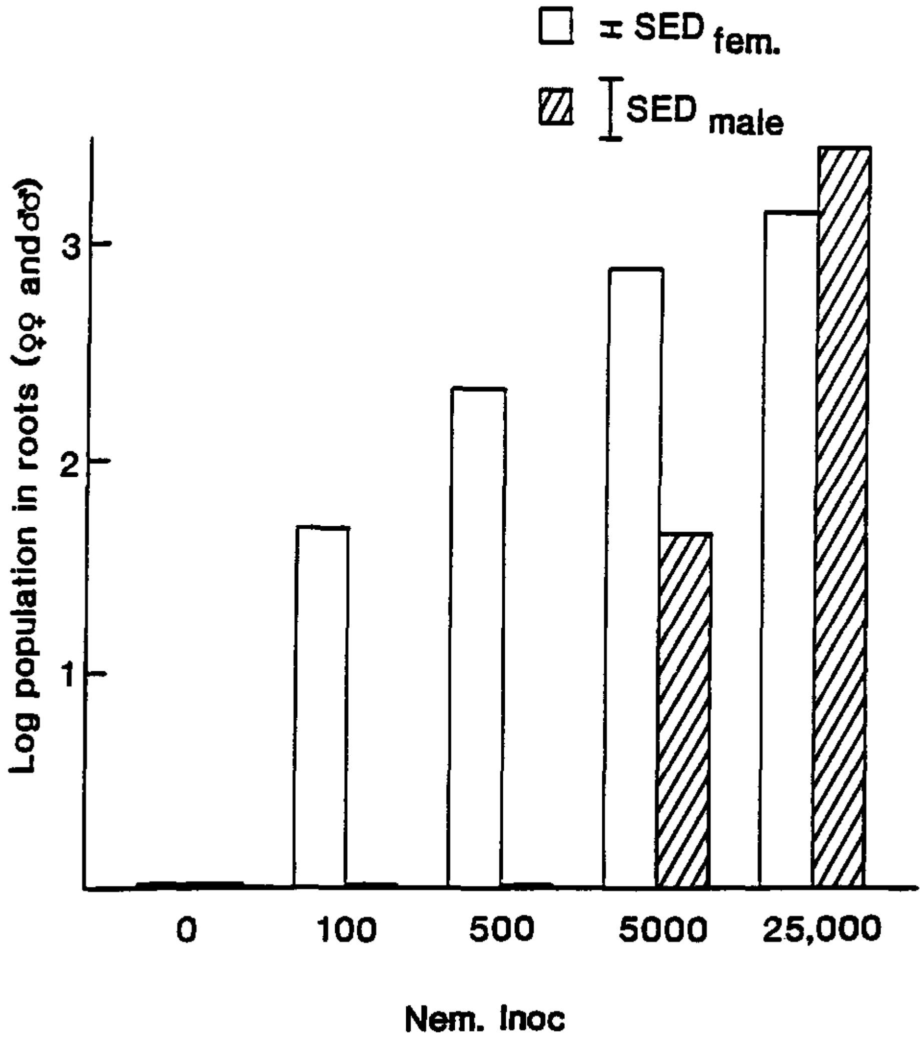

Fig. 3: Proportions of males and females developing in tomato roots inoculated with 100, 500, 5000 or 25,000 *Meloidogyne incognita* juveniles/plant.

TABLE I

Effect of fungal inoculum rate (chlamydpsores g^{-1} soil) and root galling on the establishment of *Verticillium chlamydosporium* (cfu x 1000) in soil eleven weeks after soil inoculation.

		Fungal inoculum				
		0	50	500	5,000	50,000
Galling rate (0-10)	0	0.0 (0.0)*	3.3 (0.62)	32.3 (1.52)	168.0 (2.22)	863.5 (2.92)
	3	0.0 (0.0)	2.8 (0.55)	32.3 (1.51)	215.5 (2.32)	508.5 (2.70)
	4	0.0 (0.0)	4.5 (0.73)	57.4 (1.75)	158.3 (2.19)	924.0 (2.95)
	6	0.0 (0.0)	6.2 (0.83)	53.8 (1.74)	153.5 (2.13)	619.5 (2.79)
	8	0.0 (0.0)	4.7 (0.75)	73.8 (1.86)	280.2 (2.45)	882.0 (2.93)

Values in brackets transformed to log10(x + 1); SED for transformed values = 0.21

TABLE II

Effect of fungal inoculum rate (chlamydospores/g soil) and nematode density on the establishment of Verticillium chlamydosporium *(cfu/cm^2) on the rhizoplane eleven weeks after soil inoculation.*

		Fungal inoculum				
		0	50	500	5,000	50,000
Nematode inoculum density	0	0.0 (0.0)	12 (1.06)	25 (1.41)	53 (1.67)	649 (2.77)
	100	0.0 (0.0)	19 (1.18)	78 (1.64)	80 (1.83)	645 (2.78)
	500	0.0 (0.0)	12 (1.22)	70 (1.81)	530 (2.56)	1238 (3.08)
	5,000	0.0 (0.0)	464 (2.56)	555 (2.54)	1948 (3.22)	1608 (3.20)
	25,000	0.0 (0.0)	1942 (2.26)	2735 (3.29)	2572 (3.32)	3819 (3.55)

Values in brackets transformed to log10(x + 1); SED for transformed values = 0.21

TABLE III

Effect of fungal inoculum rate (chlamydospores/g soil) and nematode density on the capacity of Verticillium chlamydosporium *to control populations of* Meloidogyne incognita *(junveniles and eggs x 1000).*

		Fungal inoculum				
		0	50	500	5,000	50,000
Nematode inoculum density	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	100	44 (1.64)	25 (1.41)	28 (1.46)	15 (1.06)	14 (1.15)
	500	160 (2.21)	138 (2.13)	120 (2.07)	89 (1.90)	51 (1.70)
	5,000	860 (2.93)	701 (2.83)	681 (2.83)	623 (2.77)	682 (2.81)
	25,000	1396 (3.13)	1486 (3.16)	1490 (3.15)	961 (2.98)	822 (2.91)

Values in brackets transformed to $\log_{10}(x + 1)$; SED of transformed values = 0.11

the more extensively the roots were colonised by *V. chlamydosporium* (Table 2; Table 5).

Effect of fungal inoculum rate and nematode density on control of *M. incognita*. Although *V. chlamydosporium* colonised galled roots more extensively than healthy roots, percentage control of *M. incognita* with *V. chlamydosporium* was negatively affected by galling (Table 3; Table 5). Control was greatest (70%) at large fungal inocula and low galling indices. There was no significant increase in control when more than 5000 chlamydospores/g soil were used, but there was significantly less control at rates below this application rate (Table 5).

Effect of nematode density on root weight, shoot weight and galling. Root weight, shoot weight and galling were all significantly ($P < 0.001$) influenced by nematode density. Roots of heavily galled plants were three times heavier than those of healthy plants (Table 4).

TABLE IV

The effect of nematode inoculum density on root weight, shoot weight and galling of tomato plants (n = 20)

Parameter	Nematode inoculum density (J2 plant^{-1})					
	0	100	500	5,000	25,000	SED
Rootweight (g)	6.84	9.72	10.97	13.39	19.11	0.73
Shootweight (g)	51.5	55.6	58.6	55.8	49.2	2.1
Galling (0-10)	0.0	3.2	4.0	6.0	7.9	0.15

TABLE V

*Summary of the results from the analyses of variance of the effects of nematode density and fungal density on root weight, shoot weight, galling, soil colonisation (soil col), root colonisation (root col.), eggs per root system and juveniles per root system. (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; N.S. = not significant)*

Treatment	Root-weight	Shoot-weight	Galling	Soil col.	Root col.	Eggs root syst.$^{-1}$	Juveniles root syst.$^{-1}$
Nematode Density (N)	***	***	***	***	***	***	***
Fungal Density (F)	N.S.	N.S.	*	***	***	***	***
N x F	N.S.	N.S.	N.S.	***	***	**	*

Discussion

Verticillium chlamydosporium did not affect nematode invasion, and therefore the fungus cannot be used to prevent initial nematode damage. At high nematode levels, smaller percentages of nematodes developed into females or males than at lower inoculum densities. Furthermore, at higher nematode densities, the competition for available nutrients in the roots meant that an increasing proportion of nematodes developed into males (Triantaphyllou, 1960).

In this experiment, *V. chlamydosporium* reduced nematode populations by about 40% when fungal inoculum densities were as low as 50 chlamydospores/g soil. The fungus may not be as effective at such low levels in natural soils, which might support less fungal growth.

Establishment in soil was described by the equation: log cfu in soil = 0.6206 x log inoculum + 2.8705. This means that *V. chlamydosporium* multiplies fastest in

soil at lower rates of application. It must therefore be assumed that the germinating chlamydospores not only use internal food sources for growth and proliferation, but that they also depend to some degree on externally available nutrients. If the level of available nutrients in soil is important for germination and proliferation of chlamydospores, soil-type and/or fungal formulation may greatly affect the establishment of *V. chlamydosporium* in soil.

Adult female nematodes require considerable amounts of nutrients for egg production, and compete with the host for the pool of nutrients in the root (Hussey, 1985). The increased metabolic activity of giant cells on which the nematode feeds, stimulates mobilisation of photosynthates from shoots to roots and in particular to the giant cells where they are removed and utilized by the feeding nematode (Bird & Loveys, 1975; McClure, 1977). Mobilisation and accumulation of substances in the giant cells reaches a maximum when the adult females commence egg laying and declines thereafter (Meon, Fisher & Wallace, 1978). It appears that *V. chlamydosporium* proliferates more extensively on nematode damaged roots. This may therefore be due to excessive leakage of root exudates from galled roots or due to photosynthates directly excreted by the developing nematodes. The direct relation between leakage of nutrients from the roots and the ability of the fungus to colonise the rhizoplane seems to be of great advantage for the development of *V. chlamydosporium* as a biological control agent because it would decrease dependency on other soil factors and therefore increase the reliability of *V. chlamydosporium* as a biological control agent. Despite the more extensive colonisation of galled roots by *V. chlamydosporium*, control was negatively correlated with heavily galled root systems. De Leij, Davies & Kerry (in press) showed that, in large galls, many egg-masses stay embedded in the gall tissue and are therefore physically protected from fungal parasitism. *Verticillium chlamydosporium* was most effective at low nematode densities, which means that the fungus could be very useful in situations where a farmer wants to prevent nematode build up, i.e. after treatment of soil with nematicides or after soil sterilisation, or when plants which form small galls in response to nematode invasion are cropped in heavily infested soils, so that egg-masses stay exposed on the root surface.

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CHAPTER 5

THE EFFECT OF TEMPERATURE AND NEMATODE SPECIES ON INTERACTIONS BETWEEN THE NEMATOPHAGOUS FUNGUS *VERTICILLIUM CHLAMYDOSPORIUM* AND ROOT-KNOT NEMATODES(*MELOIDOGYNE* SPP.)

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Summary

Optimal growth and sporulation on agar of an isolate of *Verticillium chlamydosporium* occurred at 32°C and 22°C, respectively. Differences in the numbers of colony forming units isolated from the root surface of tomato plants grown at different temperatures reflected the rate of hyphal growth on agar. However, the numbers of colony forming units in soil at different temperatures was related to the effect of temperature on sporulation *in vitro*; the fungus was most abundant in soil at 22°C, which was the optimal temperature for sporulation. After one nematode generation the most extensive root colonisation occurred at 20°C followed by 25 and 30°C. As a result, egg-masses exposed on the root surface were most extensively colonised at 20°C followed by 25 and 30°C. Total egg and juvenile counts showed that greatest control of *Meloidogyne* was achieved at 25°C (*c* 90%), while control at 20 and 30°C was usually around 60-70%. The lower level of control by *V. chlamydosporium* at 20°C may result from a reduced proportion of nematode egg-masses exposed on the root surface at this temperature. Eggs of *M. arenaria, M. incognita* and *M. javanica* were equally susceptible to parasitism by *V. chlamydosporium.* Of these three species, *M.arenaria* induced large galls and formed large egg-masses while *M.incognita* and *M.javanica* induced small galls and formed small egg-masses at 20°C. The end result was that control with *V. chlamydosporium* was approximately the same for the three nematode species.

Introduction

Verticillium chlamydosporium Goddard is a facultative parasite of eggs and females of cyst and root-knot nematodes (Willcox & Tribe, 1974; Kerry, 1975; Morgan-Jones *et al.*, 1981; Freire & Bridge, 1985). De Leij & Kerry (1991) showed that an isolate of *V. chlamydosporium* effective against *Meloidogyne arenaria* Neal was able to establish and proliferate in non-sterile soil, and colonise the rhizosphere of tomato plants, especially those sites where gall formation as a result of nematode invasion had occurred. This enabled the fungus to colonise the egg-masses of *M. arenaria* when they appeared on the root-surface. At this stage the nematode eggs were still immature and, therefore, more susceptible to infection by *V. chlamydosporium* than eggs containing second-stage juveniles (Irving & Kerry, 1986).

Temperature is known to affect the rate of production, development and hatching of *Meloidogyne* eggs as well as the rate of development from juvenile to egg-laying female (Tyler, 1933; Wallace, 1971; Bird, 1972; Ferris *et al*, 1978). In general, there are lower and upper threshold temperatures, between which the rate of development is usually linearly correlated with temperature (Allee, *et al.*, 1949). The rate at which nematode eggs are laid and develop and the rate at which *V. chlamydosporium* can infect immature eggs depend, therefore, on the growth rate of the fungus in relation to the rate of development of the nematode species involved. Stirling (1979) found that the egg-parasite *Dactylella oviparasitica* Stirling & Mankau was more effective in parasitising eggs inside egg-masses of *M. incognita* Kofoid & White on agar at temperatures which were sub-optimal for egg-production, egg-development and hatch.

Meloidogyne arenaria, M. incognita and *M. javanica* Treub are regarded as thermophiles and are widespread in the warmer regions of the world (Van Gundy, 1985). The temperature requirements of these three species are almost the same (Guiran & Ritter, 1979). It is, however, not known whether the species differ in their susceptibility to particular isolates of *V. chlamydosporium*. When 103 *V. chlamydosporium* isolates were tested for their capacity to infect eggs of *Heterodera avenae* Woll., *Globodera rostochiensis* (Skarbilovich [Woll.]) and *M. incognita* on water agar, isolates differed greatly in virulence, but the three nematode species did

not differ in susceptibility (Kerry, 1990). Although susceptibility of nematode eggs to infection by *V. chlamydosporium* greatly influences the efficacy of *V. chlamydosporium* as a biological control agent, little is known about nematode-host plant interactions in terms of gall-size and fecundity, both of which can have implications for the efficacy of nematophagous fungi (Stirling *et al.*, 1974; de Leij & Kerry, 1991; de Leij *et al.*, 1992).

The experiments presented in this paper include *in vitro* tests and pot-tests designed to study the effect of temperature on the interactions between *V.chlamydosporium* and *Meloidogyne* species on tomato plants.

Materials and methods

Fungal isolate: An isolate of *V. chlamydosporium* (CMI cc 334168) originally from *M. incognita* eggs and effective against *M. arenaria* and *M. incognita* in pot tests was used (de Leij & Kerry, 1991; *de Leij et al.*, 1992). The isolate was stored at 5°C on silica gel (Smith & Onions, 1983). A few silica gel crystals were sprinkled onto a Petri dish containing water agar (0.8%) and incubated at 25°C for 2 weeks, allowing the fungus to grow away from the crystals onto the agar. Plugs taken from the fungal colonies that developed were used for experiments.

In vitro experiments

Growth-rate on agar at different temperatures: Agar plugs (7 mm diam.) were placed in the middle of 9 cm diameter Petri dishes containing corn meal agar (Oxoid). Each of three dishes were incubated at 5, 10, 14, 18, 22, 25, 30 and 35°C. Because growth of *V. chlamydosporium* on agar is constant (Kerry *et al*, 1986), colony diameters were measured only after fifteen days incubation, and fungal growth/day estimated for the different temperatures.

Sporulation on agar at different temperatures: Czapek Dox broth was inoculated with two agar plugs, and grown in shaken liquid culture for 5 days at 23°C (Kerry *et al.*, 1986). A 0.2 ml aliquot of a 10^{-3} dilution (equivalent to one conidium/2mm^2) of this culture was spread onto each Petri dish containing corn meal agar. Each of four plates were incubated at 15, 18, 23, 25, 28 and 30°C. After 5 days the plates were

flooded with 5 ml sterile distilled water and the conidia released into the water by scraping the agar gently with a glass-rod. The suspension of conidia was collected in a small bottle and the number of conidia/plate estimated from counts made in a haemocytometer.

Pot experiments

General methods: The fungus was cultured on a moist, autoclaved mixture of sand and milled barley grain (1 : 1 $^{v}/_{v}$). A 250 ml conical flask containing 150 g of this medium was inoculated with two 7 mm agar plugs containing fungus and incubated at 25°C. After 2 weeks the cultures were washed through a 50 μm aperture sieve with a fine water spray to remove the sand and barley, and the fungal propagules were collected on a 10 μm sieve. The residue from this sieve was further washed to remove conidia and small hyphal fragments, leaving mainly chlamydospores and some hyphal fragments. The chlamydospores were counted using a haemocytometer. Inoculum was prepared by mixing the residue from the 10 μm sieve with fine sand to give a concentration of 10^7 chlamydospores/g sand. This inoculum could be further mixed with an unsterilised potting compost of fertilised peat and sand (3 : 5 $^{v}/_{v}$).

Experimental design: For the pot tests, six temperature controlled water tanks, set at different temperatures were used (the exact temperature range depended on the experiment). Each tank contained twelve 1 l plastic beakers, each filled with 750 g of compost.

The fungal application rate was 2000 chlamydospores/g compost, which is equivalent to about 0.15 g of inoculum/pot. Control pots received no treatment. The pots were planted with 3 week old tomato seedlings (cv. Pixie), which were allowed to establish in the pots for 2 weeks before 1000 nematode juveniles (< 1 week old) were pipetted with 10 ml water into three holes in the soil. Plants were watered daily as needed.

Fungal establishment: The compost from the different treatments was sampled immediately after inoculation and at 2 week intervals. At each sampling two cores (7 mm diam.) were taken from each pot with a cork borer. The sample was well mixed, and a 1 g subsample was used to estimate numbers of colony forming units

in the compost. This was done by spreading serial dilutions of the compost suspension onto a semi-selective medium in 9 cm diam. Petri dishes (de Leij & Kerry, 1991). The plates were incubated for 1 to 2 weeks at 25°C, after which time *V. chlamydosporium* colonies were readily recognised and counted at 6 x magnification.

Fungal colonisation of the root surface was assessed after the tomato plants were harvested. The root systems were washed carefully, blotted dry, weighed, cut into small segments (*c* 1 cm length) and mixed thoroughly. From each root system a 1 g sample was taken and crushed in 9 ml agar solution (0.05%) with a pestle and mortar at room temperature. Dilutions of this suspension were spread onto semi-selective medium, incubated and counted as for the soil.

Estimation of nematode damage, nematode populations and egg infection by V. chlamydosporium: *Before the roots were cut up into segments,* root galling was estimated using a gall-rating chart of 0-10 described by Bridge & Page (1980). After cutting the roots and mixing the segments thoroughly, eggs and juveniles were extracted from a 5 g sample (Coolen & d'Herde, 1973). Mature eggs are much more resistent to fungal infection than are immature eggs (Irving & Kerry, 1986) and it was assumed that most fully matured eggs and juveniles had escaped infection by *V. chlamydosporium*. The percentage control of the nematodes by *V.chlamydosporium* was estimated by dividing the numbers of fully matured eggs and juveniles in the treated plants by those in the untreated checks. From the remaining root segments ten egg-masses per treatment were randomly selected. Each egg-mass was crushed in a drop of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix, and examined at 100 x or 200 x magnification. The proportions of infected eggs, immature eggs and mature eggs and juveniles were estimated for each egg-mass.

Experiment I. *Effect of temperature on establishment of V. chlamydosporium in soil, on the root surface of tomato plants and on the control of M. incognita*: The six water tanks were set at 22, 25, 28, 32, 35 and 38°C. Each tank contained three *V. chlamydosporium* treated pots and three untreated pots. All plants were harvested 5 weeks after inoculation with *M. incognita* juveniles.

Experiment II. *Effect of nematode species and temperature on V. chlamydosporium*: The water tanks were set at 20, 25 and 30°C (two tanks for each temperature). At

each temperature, twelve pots were treated with *V. chlamydosporium* and twelve were not treated. *Meloidogyne incognita, M. arenaria* or *M. javanica* were added to four replicate pots per temperature.

The time needed from nematode invasion to reinvasion of the second nematode generation is 540 day-degrees for the three nematode species tested (de Guiran & Ritter, 1979; Ferris & van Gundy, 1979). The plants were harvested after the nematodes had gone through a full generation. This meant that the plants in the 20, 25 and 30°C tanks were harvested after 60, 40 and 30 days respectively.

Gall weights were measured in the untreated controls at 20 and 25°C by cutting out single galls from the roots (fifteen galls per root system) and weighing them after they were blotted dry. The numbers of exposed egg-masses and numbers of galls were counted on a 1 g subsample of roots from the untreated checks. Numbers of galls were only counted at 20 and 25°C. All results were analyzed using multivariate analyses of variance; data were transformed when necessary.

Results

Growth rate and sporulation on agar at different temperatures. At 10°C *V. chlamydosporium* colonies increased in diameter by less than 0.1 mm/day. The growth rate at temperatures between 15 and 25°C increased from 0.2 mm/day at 15°C to 1.2 mm/day at 25°C. Between 25 and 32°C fungal growth rate remained constant at 1.2 mm/day and at 35°C there was no growth (Fig. 1). The optimal temperature for conidia production was 22°C, at which temperature it was some 50 times greater than at 15°C. Above 22°C conidia production dropped sharply, and at 30°C it was similar to that at 15°C (Fig. 1).

Establishment in compost: Establishment of *V. chlamydosporium* was fastest and reached the greatest numbers of cfu/g compost at 22°C; above 22°C the rate of establishment decreased as temperature increased (Fig. 2). The rate of establishment at 20°C was not significantly different from that at 25°C (Fig. 2).

Establishment on the root surface of tomato plants: Between 22 and 32°C cfu/g root tended to increase with increasing soil temperatures (Fig. 3). Population densities of *V. chlamydosporium* on the root surface were low at 35 and 38°C (Fig. 3; Exp.I).

After completion of one nematode generation (540 day degrees), establishment on the root surface was most extensive at 20°C followed by 25 and 30°C (Fig. 3; Exp.II).

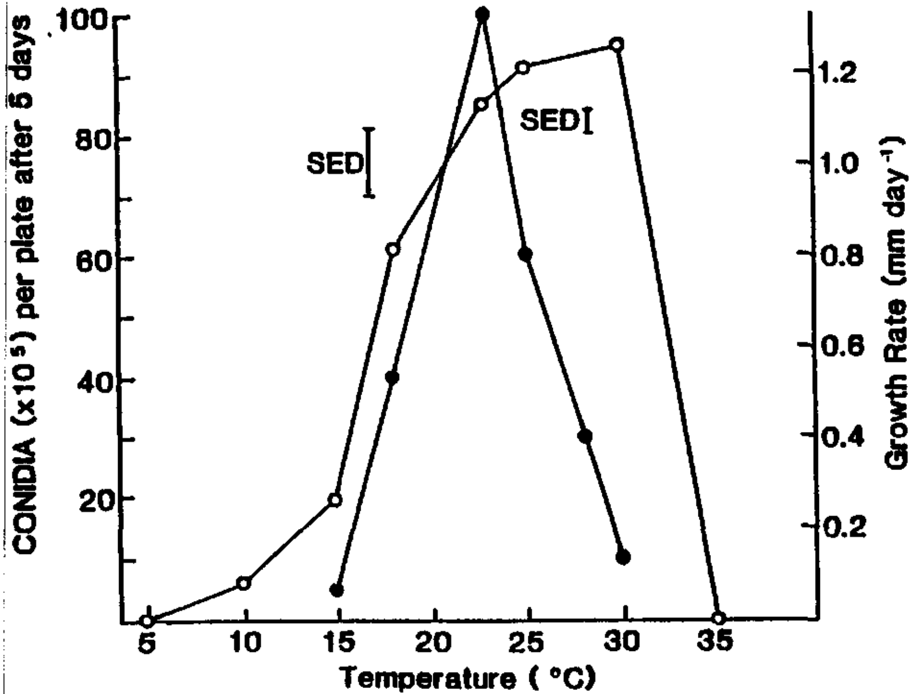

Fig. 1: Growth rate (O) and sporulation (●) of *Verticillium chlamydosporium* on corn meal agar at different temperatures. (n=3)

<u>Egg-infection by V. chlamydosporium in exposed egg-masses on the root-surface.</u> The percentages of fully matured and immature eggs were not significantly different at harvest-time (after one nematode generation) for any of the untreated controls (Fig. 4). A higher ($P < 0.001$) percentage of eggs in colonised egg-masses was infected at 20 and 25°C than at 30°C, leaving smaller ($P < 0.001$) proportions of immature and fully matured eggs in those colonised egg-masses which had developed at 20 and

25°C compared with those developed at 30°C (Fig. 4). Differences in percentage infected, immature and mature eggs in the colonised egg-masses were small between the three nematode species (Fig. 4).

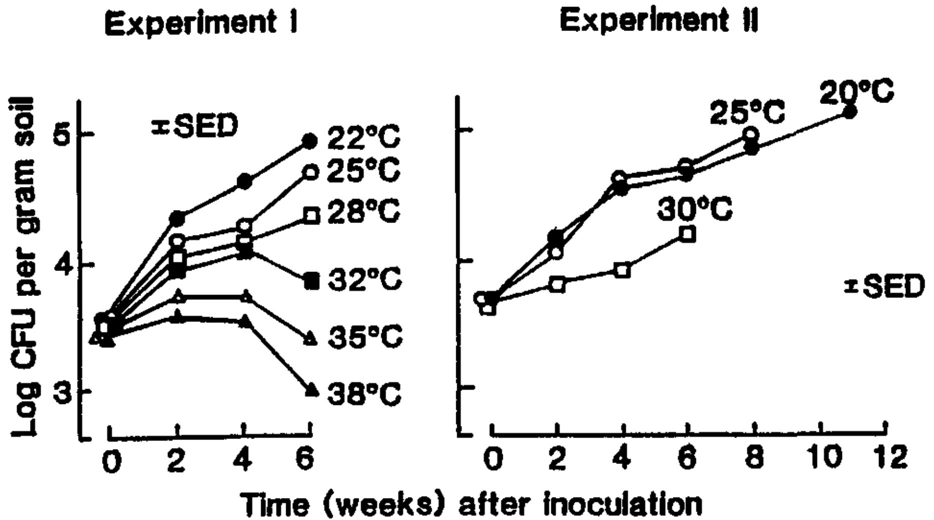

Fig. 2: Establishment and survival of *Verticillium chlamydosporium* in compost at different temperatures in two experiments. (n=4)

Nematode multiplication as affected by temperature, V. chlamydosporium and nematode species. After 5 weeks (Experiment I), greatest control of *M. incognita* with *V. chlamydosporium* was achieved at 25°C (82%) followed by 28°C (72%) and 32°C (64%) (Table 1). At 22°C few eggs had been produced and the eggs had not yet developed into fully matured eggs or juveniles. At 35 and 38°C, *V. chlamydosporium* had no significant effect on nematode populations. *Meloidogyne incognita* populations were small and variable at those temperatures (Table 1). After 540 day degrees nematode development time (experiment II), temperature, fungal inoculum and nematode species as weel as most of their interactions affected numbers of

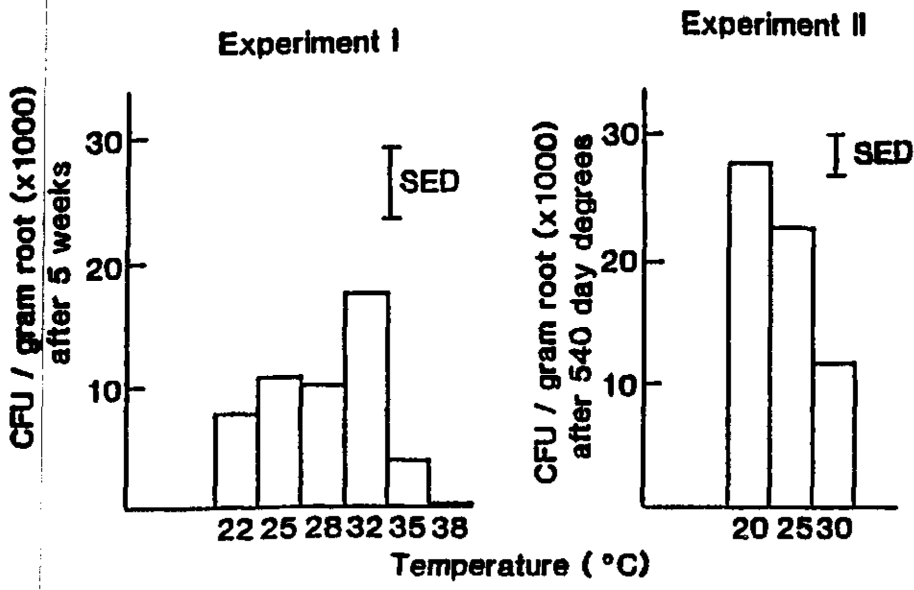

Fig. 3: Establishment of *Verticillium chlamydosporium* on the root surface of tomato plant roots at different temperatures; experiment I was harvested seven weeks after fungal inoculation; experiment II was harvested after one nematode generation. (n=4)

immature eggs, fully matured eggs and juveniles per root system (Table 3). Numbers of eggs and juveniles per root-system in the untreated checks were largest at 25°C followed by 30°C and 20°C for all three nematode species (Fig. 5). *Verticillium chlamydosporium* reduced numbers of immature eggs by 48%, numbers of fully matured eggs by 77% and numbers of juveniles by 76% averaged over the three temperatures and nematode species.

The best control was achieved at 25°C (*c* 90%); control achieved at 20°C was around 70% and at 30°C it was between 50 and 60%, with only small differences between the nematode species (Table 2). Population sizes of *M. arenaria*

TABLE I

Numbers of immature eggs, fully matured eggs and juveniles and percentage control as affected by an initial V. chlamydosporium *inoculum of 2000 chlamydospores/g soil.* (n=4)

Treatments temp. (°C)	Inoc.	immature eggs($x10^3$)	fully matured eggs ($x10^3$)	juveniles ($x10^3$)	% control
22	non	189	3	0	
	Vc	116	0	0	
25	non	534	93	116	82%
	Vc	156	11	27	
28	non	698	132	412	72%
	Vc	218	34	119	
32	non	1015	167	478	64%
	Vc	525	87	147	
35	non	80	9	30	
	Vc	107	20	32	
38	non	14	2	2	
	Vc	3	1	0	
SED		105	30	81	
F PROB		***	*	**	

were similar at the three temperatures while populations of *M. incognita* and *M. javanica* were often more than twice the size at 25°C compared with 20 and 30°C (Fig. 5).

Galling and exposed egg-masses: In general, galling-indices varied between 3.5 and 2.1 (Table 2). At 30°C the galling index was lowest (2.6) in comparison with 3.1 at 20°C and 3.3 at 25°C. Inoculation with *V. chlamydosporium* reduced galling from 3.2 to 2.8. Plants inoculated with *M. arenaria* had the highest galling-index (3.1) compared with plants inoculated with *M. incognita* or *M. javanica* (both 2.9).

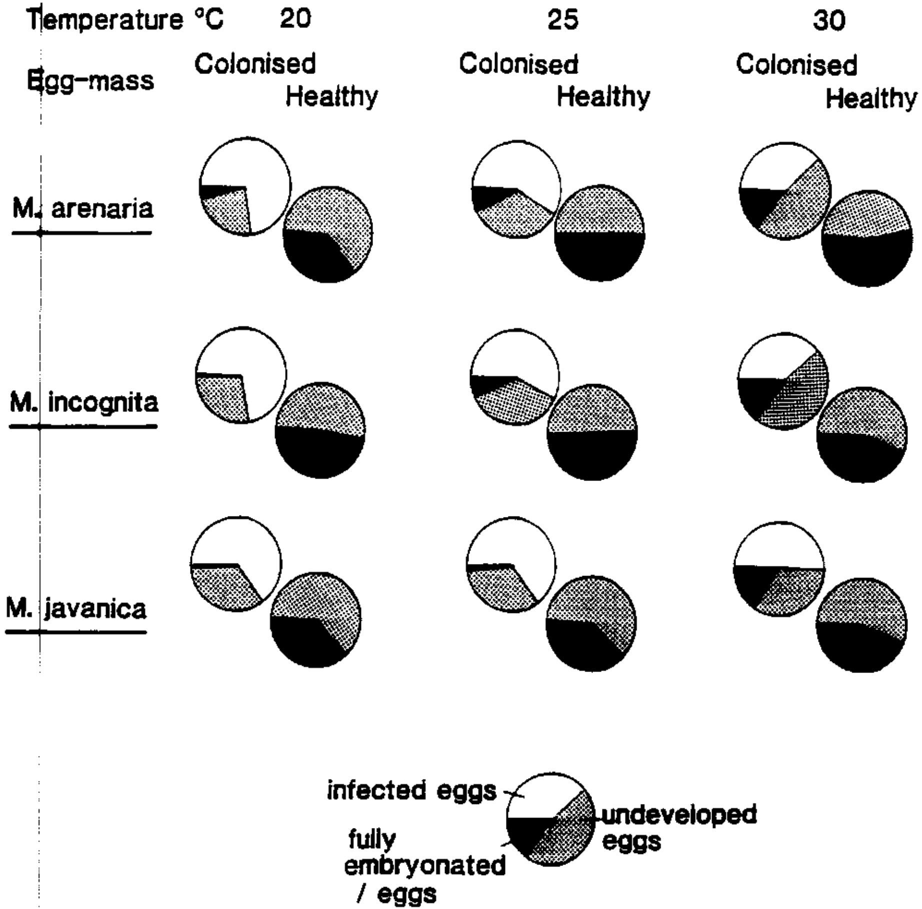

Fig. 4: Proportions of infected eggs, undeveloped eggs and fully embryonated eggs in healthy and colonised egg-masses (n = 10) of *Meloidogyne arenaria, M. incognita* and *M. javanica* at different temperatures. The egg-masses were studied after 540 day-degrees nematode development time.

TABLE II

Immature eggs, fully matured eggs, juveniles, galling and % control of Meloidogyne arenaria (MA), M. incognita (MI) *and* M. javanica (MJ) *after 540 day degrees nematode development time on treated* (+Vc) *and untreated* (-Vc) *tomato plants.* (n=4)

Parameter	temp. (°C)	*M. arenaria*			*M. incognita*			*M. javanica*			SED
		control (*-Vc*)	treated (*+Vc*)	% control	control (*-Vc*)	treated (*+Vc*)	% control	control (*-Vc*)	treated (*+Vc*)	% control	
Imature eggs (x 1000)	20	128.0	78.1		72.1	49.5		68.2	47.8		
	25	131.4	56.9		186.8	44.4		140.9	54.4		15.1
	30	121.9	73.1		141.3	92.2		78.0	55.9		
Fully matured eggs (x 1000)	20	20.0	7.5	63%	14.8	4.5	70%	14.8	3.9	74%	
	25	37.2	4.4	88%	63.5	3.7	94%	33.0	3.6	89%	4.7
	30	34.2	13.6	60%	31.3	12.7	59%	13.1	6.8	48%	
Juveniles (x 1000)	20	37.7	10.7	72%	29.0	12.4	57%	45.5	6.8	85%	
	25	62.9	9.7	85%	71.5	5.1	93%	125.5	13.0	90%	8.7
	30	49.2	20.7	58%	52.1	27.1	48%	48.0	18.2	62%	
Galling (0-10)	20	3.5	3.3		3.0	2.8			3.0	3.0	
	25	3.3	3.0		3.8	3.0			3.5	3.0	0.15
	30	3.0	2.8		2.8	2.4			2.6	2.1	

TABLE III

Summary of the results from the analyses of variance of the effect of temperature, treatment with Verticillium chlamydosporium *and nematode species on numbers of immature eggs, fully matured eggs, juveniles and galling in the tomato plant roots. (***, P < 0.001; **, P < 0.01; *, P < 0.05; NS = not significant)*

Treatment	immature eggs	fully matured eggs	juveniles	galling
Temperature (T)	***	***	***	***
Fungal inoculum (F)	***	***	***	***
Nematode species (N)	***	***	**	***
T X F	***	***	***	*
T X N	***	***	***	***
F x N	N.S.	**	***	N.S.
T x F x N	*	**	*	N.S.

Verticillium chlamydosporium reduced galling more at 25°C than at 30 and 20°C. (Table 2). On average, individual galls weighed less ($P < 0.01$) at 25°C than at 20°C (Fig. 6). *Meloidogyne arenaria* induced heavier ($P < 0.001$) galls than *M. incognita* or *M. javanica* (Fig. 6).

Numbers of exposed egg-masses on the root surface per root system were smaller ($P < 0.05$) for *M. arenaria* (282) than for *M. incognita* and *M. javanica* (362 and 370 respectively). Temperature affected ($P < 0.001$) numbers of exposed egg-masses; smallest numbers were recorded at 20°C (235) followed by 30°C (359) and 25°C (422) (Fig. 7). There was no significant difference in the numbers of galls/root system at 20°C (562) compared with 25°C (677) for the three nematode species.

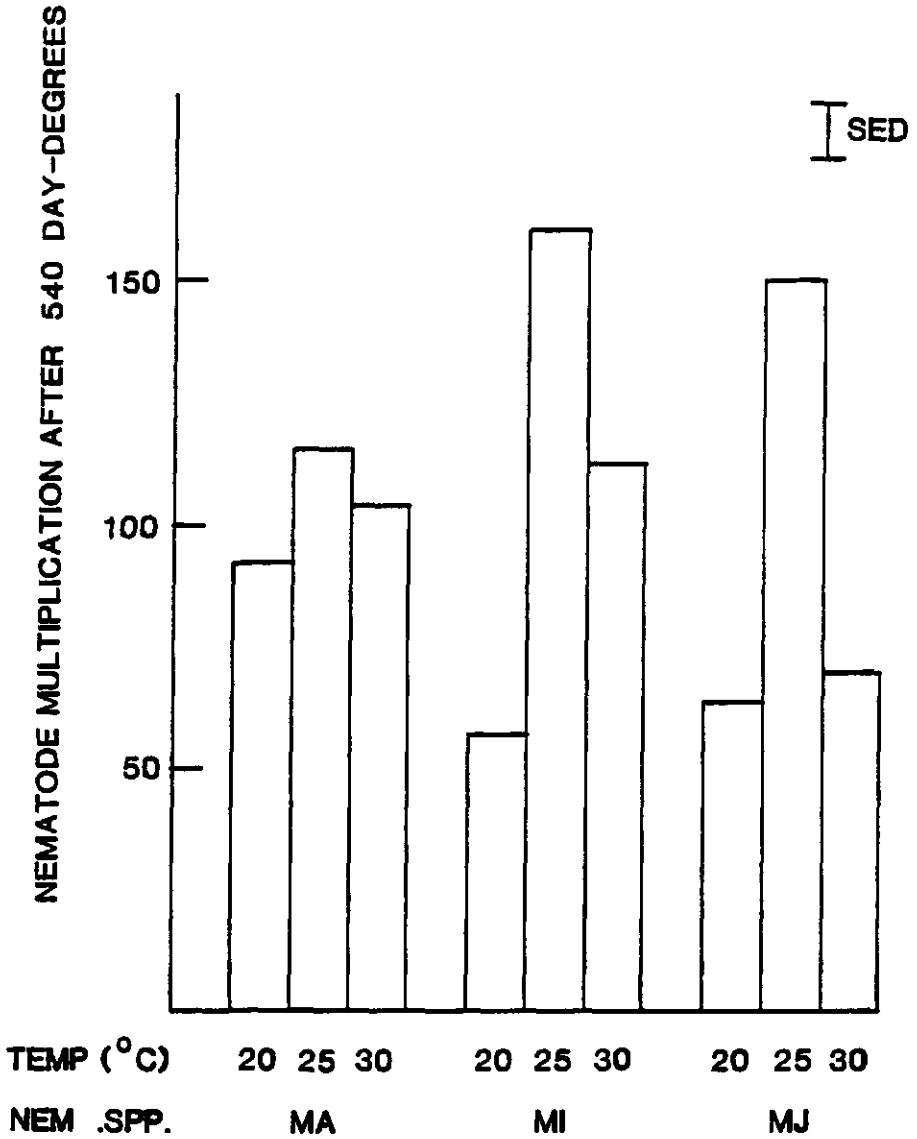

Fig. 5: Multiplication of *Meloidogyne arenaria* (MA), *M. incognita* (MI) and *M. javanica* (MJ) on untreated tomato plants at 20, 25 and 30°C, after 540 day degrees nematode development time. (n=4)

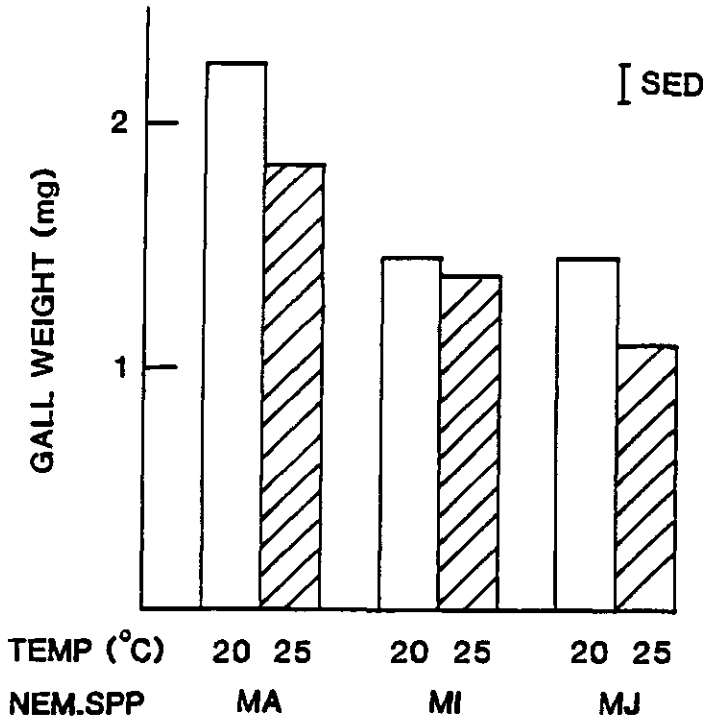

Fig. 6: Gall weights (n = 15) induced by *Meloidogyne arenaria* (MA) *M. incognita* (MI) and *M. javanica* (MJ) on untreated tomato roots at 20 and 25°C, after 540 day degrees nematode development time.

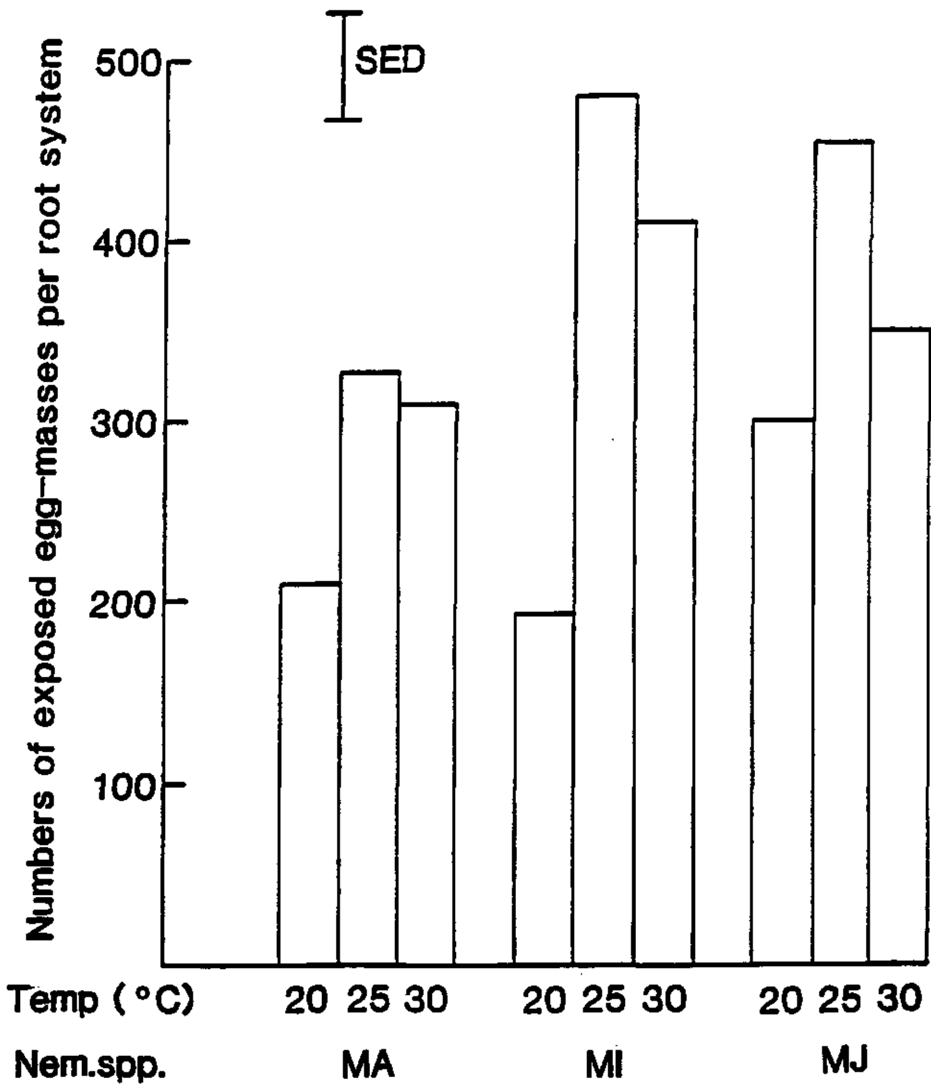

Fig. 7: Total number of exposed egg-masses of *Meloidogyne arenaria* (MA), *M. incognita* (MI) and *M. javanica* (MJ) on untreated tomato root systems at 20, 25 and 30°C, after 540 day degrees nematode development time. (n=4)

Discussion

The growth rate of *V. chlamydosporium* on agar and establishment of the fungus on the rhizoplane both increased with increasing temperatures, up to 32°C, and decreased drastically above 32°C. The effect of temperature on the establishment of *V. chlamydosporium* in soil, however, was not related to hyphal growth of the fungus, but was related to the effect of temperature on the production of conidia on agar.

Verticillium chlamydosporium colonised the root surface of tomato plants more rapidly with increasing temperatures; this increase in growth rate, however, did not compensate for the shorter development time of *Meloidogyne*. Most extensive colonisation of the root surface, after completion of one nematode generation occurred, at 20°C, followed by 25°C and 30°C. It might therefore be expected that best control of *Meloidogyne* would be achieved at 20°C. The proportion of infected eggs in exposed egg-masses on roots treated with *V. chlamydosporium*, suggested that nematode control of the three nematode species was dependent on temperature; after 540 day degrees, more eggs were infected at 20°C than at 25 and 30°C. The result was that almost no eggs escaped fungal infection at 20°C; a small proportion escaped at 25°C (*c* 10%), and a large proportion (*c* 30%) escaped fungal infection at 30°C. Nematode control estimated from total extractions of juveniles and eggs was around 70% at 20°C, around 90% at 25°C and around 60% at 30°C for all three nematode species. The effect of temperature on fungal growth rate and nematode development gives therefore a good explanation for the observed nematode control at 25 and 30°C. However, the relatively poor nematode control (60-80%) at 20°C was unexpected. When egg-masses stay embedded in the gall, *V. chlamydosporium* cannot colonise them (de Leij *et al.*, 1992). The proportion of egg-masses embedded in galls may be influenced by two factors; the size of the gall, and the size of the egg-mass. Larger galls and/or smaller egg-masses would result in a greater proportion of egg-masses staying embedded. *Meloidogyne arenaria* not only induced larger galls than *M. incognita* and *M. javanica*, but galls induced by *M. arenaria* were much larger at 20°C than at 25°C. The size of *M. arenaria* egg-masses, which can be derived from the number of galls/root system and the population size/root system

at that temperature in the untreated checks, was not influenced by temperature. However, *M. incognita* and *M. javanica* induced smaller galls at 20°C than *M. arenaria*, but the egg-masses of these two species were much smaller at 20°C than at 25°C. The result may have been that a significant proportion of the egg-masses stayed embedded in the galls at 20°C for all three nematode species resulting in poorer control than at 25°C.

Verticillium chlamydosporium does not seem to affect initial nematode invasion (de Leij, Davies & Kerry, 1992). Root systems treated with *V. chlamydosporium* were less galled than untreated ones, because at harvest time more second generation juveniles had invaded the roots in untreated soil. This is because *V. chlamydosporium* reduces reinvasion as a result of population control in the first generation. Because *V. chlamydosporium* was most effective at 25°C and reinvasion must have been smallest, galling reductions with *V. chlamydosporium* were greater at 25°C than at 20 and 30°C. Galling of root-systems infested with *M. arenaria* was slightly greater than galling of roots infested with the other two species. The fact that *M. arenaria* induced larger galls than the other two species might have resulted in an overestimation of the galling for roots infested with *M. arenaria*.

Eggs of the three *Meloidogyne* species were equally susceptible to parasitism by *V. chlamydosporium*, but the species differed slightly from each other in terms of the effect of temperature on fecundity, and the size of the galls induced on tomato roots. At 20°C, the three nematode species either formed large galls with relatively large egg-masses (*M. arenaria*), or smaller galls with relatively small egg-masses (*M. incognita* and *M. javanica*). The end result was that there were only small differences in the efficacy of *V. chlamydosporium* against the three nematode species studied.

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CHAPTER 6

THE EFFECT OF WATERING ON THE DISTRIBUTION OF *VERTICILLIUM CHLAMYDOSPORIUM* IN SOIL AND THE COLONISATION OF EGG MASSES OF *MELOIDOGYNE INCOGNITA* BY THE FUNGUS

F.A.A.M. de Leij, J.A. Dennehy and B.R. Kerry

Summary

The distribution and establishment of the nematophagous fungus *Verticillium chlamydosporium* as affected by watering was investigated in three different experiments. A small proportion (c. 1%) of the conidia produced in soil were transported over distances greater than 50 cm. However, most conidia formed in soil moved less than 10 cm during nine weeks when water was applied weekly. Establishment of *V. chlamydosporium* was in general better when the soil was well aerated. Most (90%) of the root weight of tomato plants was concentrated in the top 30 cm of the soil profile. After nine weeks, most nematode damage was also found in the top 30 cm even though the nematode inoculum was evenly distributed to a depth of 60 cm. Inoculation of the top 10 cm with *V. chlamydosporium* at a rate of 14,000 chlamydospores/g soil was sufficient to achieve high levels of root colonisation and the colonisation of 100% of exposed *Meloidogyne incognita* egg-masses in the top 30 cm of the soil profile. Watering from below slowed the development of eggs of *M. incognita* and reduced the establishment of *V. chlamydosporium*, probably as a result of oxygen depletion due to the saturation of the soil with water. Control of *M. incognita* with *V.chlamydosporium* was greater (80-90%) in well aerated soil than in soil which was less well aerated (control around 30%).

Introduction

The introduction of beneficial microorganisms for the control of soil-borne plant pathogens has considerable potential in agriculture (Cook & Baker, 1983). Seed treatments provide the most efficient method of applying biological control agents to many field crops, provided that the organism is capable of spreading along the developing root system. Incorporation of microbial agents with standard machinery into field soils will never provide a sufficiently even microbial spread through the soil profile that no further spread of the organisms involved is necessary. Only in the case of seed treatments which aim to protect the hypocotyl against damping-off diseases is extensive microbial spread not necessary (Kommedahl & Windels, 1981).

The movement of water has been reported to be influential in the distribution of Rhizobia and Pseudomonads through soil and along the rhizosphere (Bahme & Schroth, 1987; Parke *et al.*, 1986). Burges (1950) found that fungi which produce wettable conidia were easily transported over great distances through sand columns, while those fungi which produce dry, non-wettable conidia were not. Hepple (1960) confirmed these findings as long as the pF of the soil was kept near field capacity. When the water was allowed to drain, subsequent addition of water to the soil did not affect further movement of the spores. Such lack of movement may have been caused by the bonding between the mucilaginous walls of the fungal spores and soil particles; once formed, such bonds were difficult to break (Hepple, 1960). Because water-logged conditions in well drained soils are relatively rare, it was concluded that movement of soil-borne fungi by water was important only over small distances.

De Leij & Kerry (1991) found that extensive colonisation of the rhizoplane by the nematophagous fungus *Verticillium chlamydosporium* Goddard was an essential characteristic for effective control of *Meloidogyne* spp. Because the whole root system of a plant is susceptible to nematode attack, efficient colonisation of the root system in a relatively short time by the fungus is essential if effective control is to be achieved. Colonisation by hyphal extension along the roots is unlikely to be adequate, because hyphae of *V. chlamydosporium* grew only up to 1 cm/month in non sterile soil (Kerry, 1988), and even on corn meal agar growth was not more than 3 cm/month (de Leij *et al.*, 1992). When *V. chlamydosporium* was introduced as chlamydospores in non

sterile peat-sand compost a rapid increase in the numbers of colony forming units (cfu) /g soil took place and the propagules formed were mainly conidia (de Leij, *et al.*, 1992) Because conidia of *V. chlamydosporium* are 2-4 μm in size (Gams, 1988) and are easily dispersed in water, it is likely that water movement is an important means of dispersal. Basic understanding of the way in which *V. chlamydosporium* spreads through soil, and how this affects root colonisation and subsequent colonisation of egg-masses, is essential to devising means of introducing the fungus as a biological control agent to field soils.

This paper describes some of the factors which determine spread of *V.chlamydosporium* by water movement through soil and along the root system of tomato plants and the subsequent colonisation of egg-masses of *M. incognita* Kofoid & White by this fungus.

Materials and methods

Fungal inoculum: An isolate of *V. chlamydosporium* (CMI cc 334168) originally isolated from *M. incognita* eggs was used as it had previously been shown to parasitise *Meloidogyne arenaria* Neal, *Meloidogyne javanica* Treub, *Meloidogyne hapla* Chitwood and *M. incognita* in pot tests (De Leij & Kerry, 1990). The isolate was stored on silica gel at 5°C (Smith & Onions, 1988). For experimental use, a few silica gel crystals bearing the fungus were sprinkled onto a water agar plate and incubated at 25°C for two weeks, allowing the fungus to grow away from the crystals onto the agar. The fungus was mass-produced by inoculating a sterile mixture of moist milled barley grain and sand (1 : 1 $^{v}/_{v}$) with two 7 mm agar plugs colonised by *V. chlamydosporium*. After two weeks incubation at 25°C the cultures were washed through a 50 μm aperture sieve with a fine water spray to remove the culture medium, and collected on a 10 μm sieve. The residue on the 10 μm sieve was further washed to remove conidia and small hyphal fragments, leaving mainly chlamydospores. The chlamydospores were counted using a haemocytometer. Fungal inoculum was prepared by mixing this residue with fine sand to a concentration of 10^7 chlamydospores/g sand. This mixture of sand and chlamydospores was used to inoculate the compost, further refered to as soil.

Estimation of fungal establishment in soil and on the rhizoplane: The amount of *V. chlamydosporium* present in soil was measured by taking a 1 g sample from a well mixed soil. From this sample a dilution series was prepared which was spread onto Petri dishes containing a semi-selective medium developed for *V.chlamydosporium* (De Leij & Kerry, 1991). Root colonisation by *V. chlamydosporium* was measured by taking a 1 g sample from washed, well-mixed, chopped up roots. This sample was crushed at room temperature in 9 ml of agar solution (0.05%) with a sterilised pestle and mortar. A dilution range of this suspension was plated out onto the semi-selective medium. Soil and root dilution plates were incubated at 25°C for one to two weeks before the *V. chlamydosporium* colonies were counted at 6x magnification with the aid of a low power microscope.

Estimation of nematode damage and colonisation of egg masses by *V. chlamydosporium*: Before the roots were cut up into segments, root galling was estimated using the gall-rating chart described by Bridge & Page (1980). Colonisation of egg-masses on a root sample was estimated by taking ten randomly selected egg-masses. Each egg-mass was crushed in a droplet of 0.01% sodium hypochlorite solution to dissolve the gelatinous matrix, and examined at 200x magnification. The proportions of infected eggs, immature eggs, mature eggs and juveniles were estimated and the presence or absence of chlamydospores in each egg-mass noted. Because immature eggs are much more susceptible to fungal infection than mature eggs and juveniles (Irving & Kerry, 1986), it was assumed that mature eggs had escaped fungal infection. Nematode control was therefore estimated by comparing the percentage of mature eggs in the untreated checks with the percentage of mature eggs in the corresponding *V.chlamydosporium* treatments. Experiment 1. *Spread of V. chlamydosporium through soil and along the root systems of tomato plants from an unevenly distributed inoculum of chlamydospores*: To test the effect of water movement on the spread of *V. chlamydosporium*, five pairs of divided pots as illustrated in Fig. 1 were set up. The pots used were 12.5 cm in diam. and cut in half vertically. The two halves were placed in another similar pot. Each system contained *c* 1.6 kg of unsterilised sand-peat compost (3 : 5 $^{v}/_{v}$). The bases

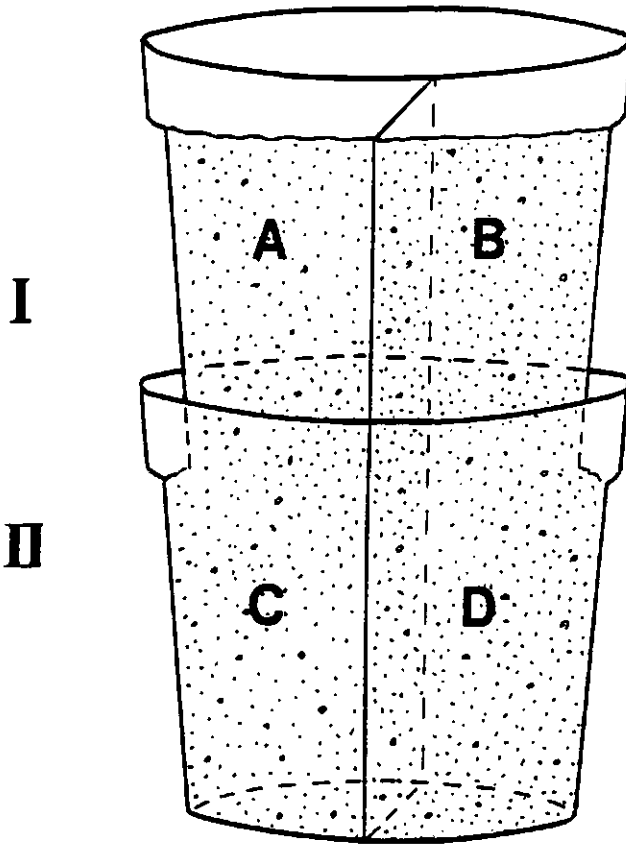

Fig. 1. Experimental set up to investigate the effect of water movement on the spread of *Verticillium chlamydosporium* from an uneven distribution of chlamydospores. (compartment A contained 50,000 chlamydospores/g soil, compartments B, C and D were not inoculated with fungus)

of the top pots were removed. After filling the bottom pots with uninoculated compost, the empty top pots were placed on top of the compost, and a removable screen was placed along the cuts. Compartment B was then filled with uninoculated compost and compartment A was filled with compost inoculated with 50,000 chlamydospores/g soil. After treating a system in this way, the screen was removed so that there were no barriers between the different compartments. Each system was sown with a single

tomato seed (cv. Pixie) and placed in the glasshouse at temperatures between 25° and 30°C. After one month each of the four compartments were inoculated with 500 *M. incognita* juveniles (< one week old). The juveniles were pipetted with 10 ml water into a hole made in the soil. Plants were watered daily according to need.

Seven weeks after nematode inoculation the systems were dismantled. First the upper pots were cut free from the bottom ones with a sharp knife. Then the outer pots were removed, and soil and roots were divided into the separate compartments with a sharp knife. The soil from each separate compartment was well mixed before a subsample was taken. Numbers of cfu/g soil were estimated as described before. The roots inside each compartment were washed carefully in a bucket of water, blotted dry, weighed, and cut into small segments (c. 1 cm). Numbers of cfu/g root were estimated as described before.

From each root sample, ten egg-masses were picked off randomly and each egg-mass was examined at high magnification (200x) for presence of chlamydospores and infected eggs.

Experiment 2. *The effect of two different watering regimes on spread and establishment of V. chlamydosporium through soil and its establishment along the root systems of tomato plants from an application rate of 14,000 chlamydospores/g soil in the top 10 cm compared with an application rate of 2,000 chlamydospores/g soil through the whole soil profile.* Transparent plastic tubes (length 60 cm; diam. 10 cm) were filled with a mixture of peat and sand (3 : 5 $^{v}/_{v}$; 5 kg mixture/tube) evenly infected with *M. incognita* infested tomato roots (density c. 1 nematode/g compost). The bottom of each tube was covered with a coarse nylon mesh. Each tube was planted with a one month old tomato seedling (cv. Pixie) and put in the glasshouse at a temperature of 25°C. Treatments differed in fungal inoculum and watering regimes (Table 1). Watering from the bottom of the tubes was done weekly by sinking the tubes in a deep container filled with water till the water level in the tube reached the soil surface. The tube was then lifted out, and excess water drained away through the nylon mesh. Watering from the top was given weekly with a watering can till water started to drain through the bottom of the tube. To prevent algae growing on the inside of the tubes, light was excluded by covering each tube with a sheet of black polythene.

Three replicate tubes of each treatment had rings of holes (five holes/depth; diam. 1 cm) made at depths of 5, 15, 25, 35, 45 and 55 cm below the soil surface. To estimate the spread of *V.chlamydosporium* in the different treatments in time and depth, one hole at each depth was sampled immediately and thereafter at weekly intervals for nine weeks with a cork borer (every hole was therefore sampled twice). Numbers of cfu/g soil were estimated as described before. Background populations of *V. chlamydosporium* were estimated at the beginning and end of the experiment by sampling the untreated checks.

TABLE I

Treatments and replication used in experiment II

Treatments	Watering from bottom	Watering from top
Vc in top 10 cm (14,000 spores/g soil)	6 reps (3 for soil; 3 for roots)	6 reps (3 for soil; 3 for roots)
Vc evenly spread (2,000 spores/g soil)	6 reps (3 for soil; 3 for roots)	6 reps (3 for soil; 3 for roots)
Control (no fungal inoculum)	3 reps	3 reps

After nine weeks, three tubes from each treatment used for root sampling were cut open, and the soil carefully washed away from the roots. The roots were then cut according to depth in six, 10 cm sections. From each section, root weight, galling, and the proportions of infected, immature and mature eggs in the egg-masses deposited on the root surface, were estimated as described before.

From the different root sections, the length of a 1 g root sample was estimated using the intercept method described by Tennant (1975). From this and the estimation of cfu/g root, the colonisation of the roots could be presented in terms of surface area (De Leij & Kerry, 1991).

Experiment 3: *Spread of V. chlamydosporium conidia through soil by different amounts of water.* Conidia of *V. chlamydosporium* were obtained by washing a ten

day old *V.chlamydosporium* culture over a 10 μm sieve onto a collection tray. A dilution series of the conidia suspension was plated out onto 9 cm diam. Petri dishes containing sorbose agar and antibiotics (De Leij & Kerry, 1991) to estimate numbers of viable conidia/ml suspension. Twenty ml of conidia suspension containing 1.32 x 10^7 conidia/ml was poured onto the surface of three columns similar to those described in experiment 2, followed by three waterings equivalent to 25, 125 and 250 mm of rainfall. Immediatly after each watering the holes made at different depths along the side of each tube were sampled with a cork borer (All soil-layers were sampled three times). Colony forming units in the different soil samples were measured using the semi-selective medium for *V. chlamydosporium* as described before.

Results

TABLE II

Colonisation by Verticillium chlamydosporium *of soil, roots and egg-masses of* Meloidogyne incognita *from an uneven distributed inoculum of 50,000 chlamydospores/g soil, seven weeks after nematode inoculation*

Compartment	cfu/g soil (x 1000)	cfu/g root (x 1000)	% egg-masses infected
A	633 (5.72)*	200 (5.23)*	80 (0.76)**
B	138 (5.01)	20 (4.30)	7 (-3.37)
C	4 (3.71)	1 (3.30)	8 (-3.62)
D	2 (3.48)	6 (3.69)	10 (-3.20)
SED	(0.18)	(0.17)	(1.1)
F prob.	$P < 0.001$	$P < 0.001$	$P < 0.001$

* = log10 transformation
** = logit transformation

Experiment I. *Spread of V. chlamydosporium through soil and along the root systems of tomato plants from an unevenly distributed inoculum of chlamydospores.* Eleven weeks after soil inoculation there was a twelve fold increase of fungal propagules in compartment A. Fungal propagules had mainly spread from compartment A to compartment B, while spread to the bottom compartments was limited (Table II). Colonisation of the roots in the different compartments reflected the spread in soil; roots were most extensively colonised in compartment A, followed by B, C and D (Table II). Even though there was extensive root colonisation in compartment B, colonisation of egg-masses of *M. incognita* was limited and was not significantly different from that in compartments C and D (Table II). Approximately 80% of the egg-masses were colonised in compartment A.

Experiment 2. *The effect of two watering regimes on spread and establishment of V. chlamydosporium through soil and its establishment along the root systems of tomato plants from a top and an evenly mixed application of chlamydospores.*

Application of chlamydospores in the top 10 of the soil profile: In nine weeks cfu in the top 10 cm of soil increased from 1.9×10^4 cfu/g soil to 4×10^5 cfu/g soil, when the tubes were watered from the top. When the tubes were watered from the bottom the numbers of cfu in the top 10 cm only increased to 2×10^5 cfu/g soil (Fig. 2). After four weeks *V. chlamydosporium* could be found through the whole soil profile in significant numbers (> 10^3 cfu/g soil) when the tubes were watered from the top. Thereafter, further increases in cfu took place when the tubes were watered from the top (Fig. 2). Spread of cfu took longer (c. six weeks) and was not as even when the tubes were watered from the bottom (Fig. 2). From all the cfu formed in the top 10 cm, only 1% was found 50 cm away from the inoculum source and most propagules moved less than 10 cm. Nine weeks after planting, *V. chlamydosporium* could be isolated from the roots in all soil layers in significant numbers, irrespective of the watering regime. Establishment on the rhizoplane was significantly higher ($P < 0.01$) when water was added from the top than with watering from the bottom of the tubes (Fig. 3). *Verticillium chlamydosporium* established in large numbers (> 1000 cfu/cm^2 root surface) in the first 30 cm of the soil profile when the tubes were watered from the top. When water was applied from the bottom, only the top 20 cm had > 1000 cfu/cm^2 root surface (Fig. 3).

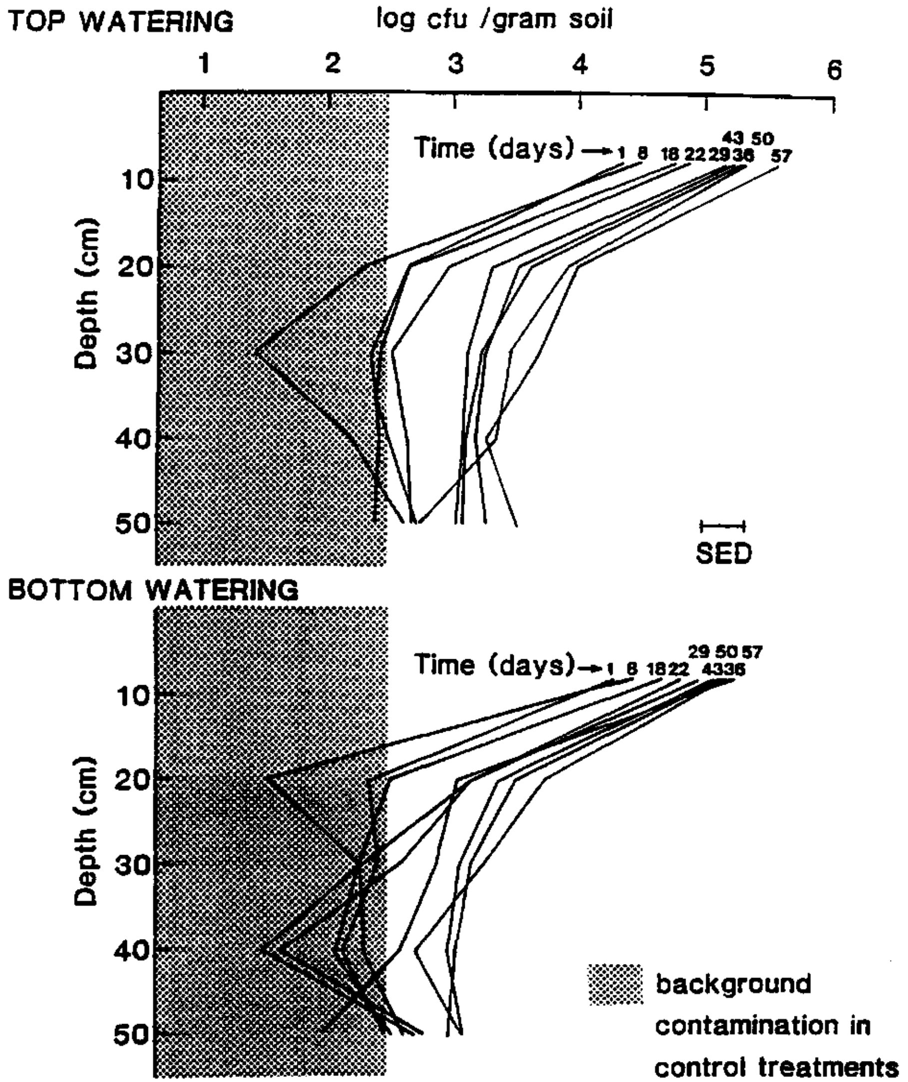

Fig. 2. Effect of two watering regimes on spatial-temporal distribution patterns of *Verticillium chlamydosporium* through a peaty-sand compost from an inoculum of 14,000 chlamydospores/g soil in the top 10 cm of the soil. (n=3)

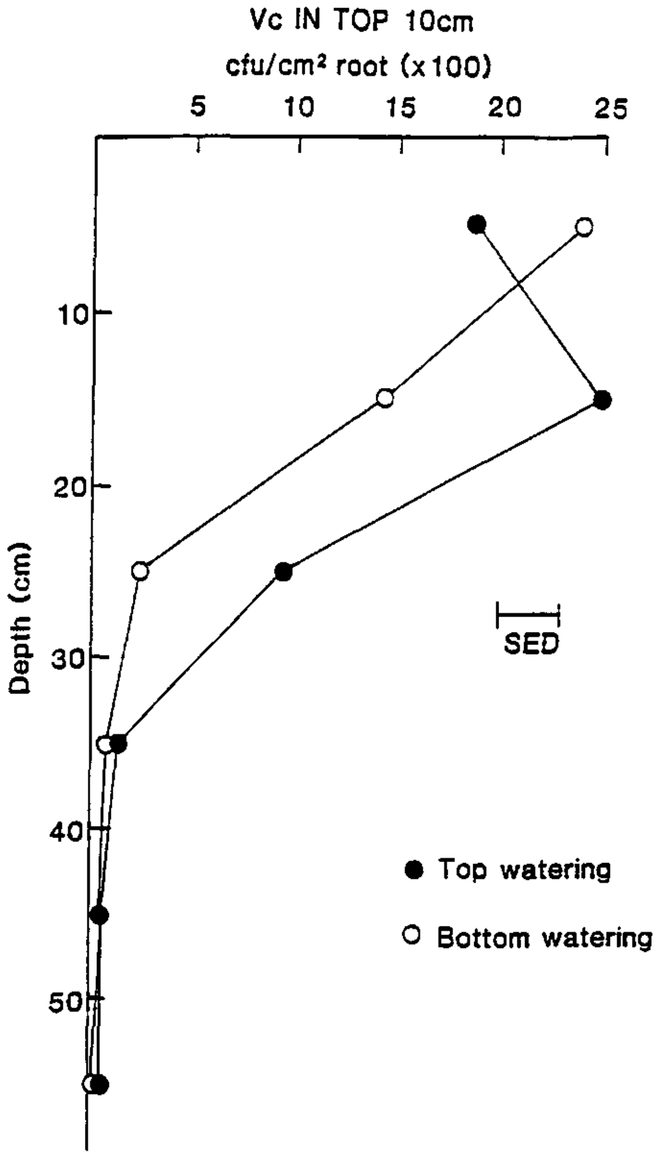

Fig. 8. Effect of two watering regimes on the spatial distribution of *Verticillium chlamydosporium* on the rhizoplane of tomato roots from an inoculum of 14,000 chlamydospores/g soil in the top 10 cm of the soil. (n=3)

Vc evenly spread through profile (2000 chl. sp/g soil)

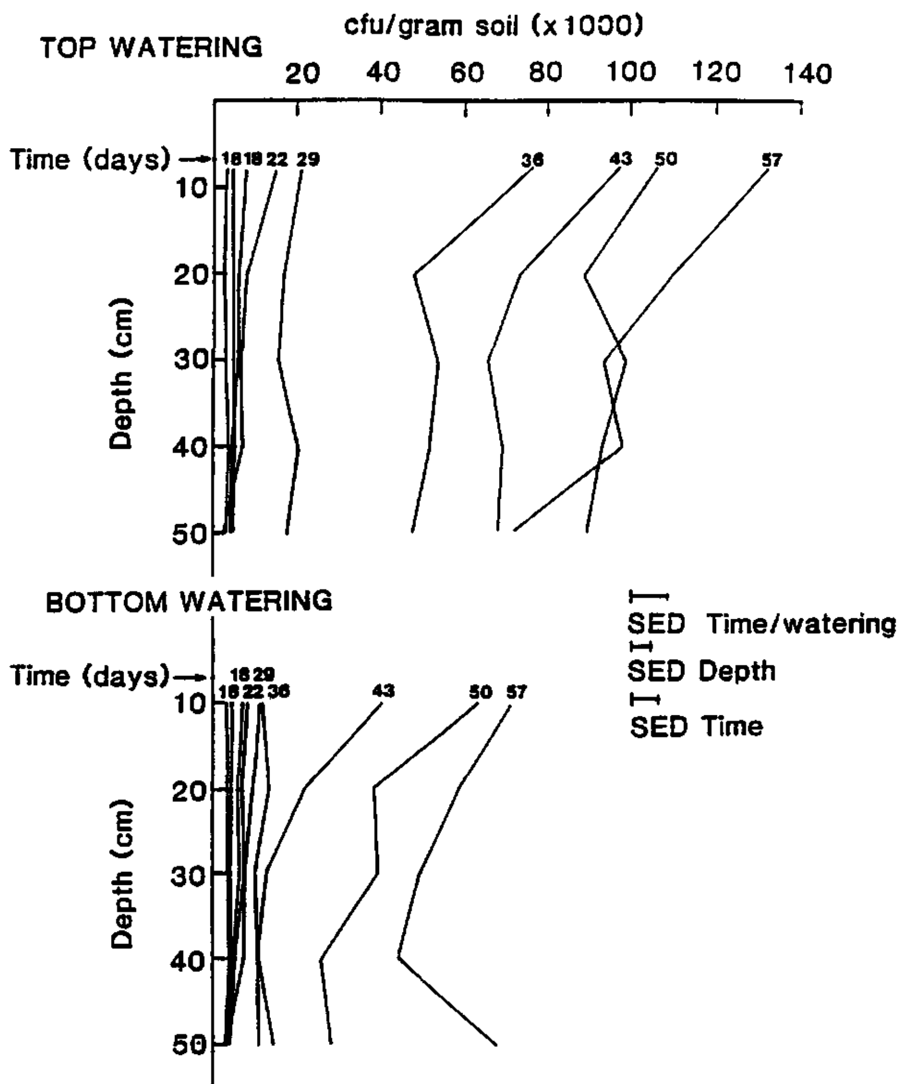

Fig. 4. Effect of two watering regimes on spatial-temporal distribution patterns of *Verticillium chlamydosporium* through a peaty-sand compost from an evenly distributed inoculum of 2000 chlamydospores/g soil. (n=3)

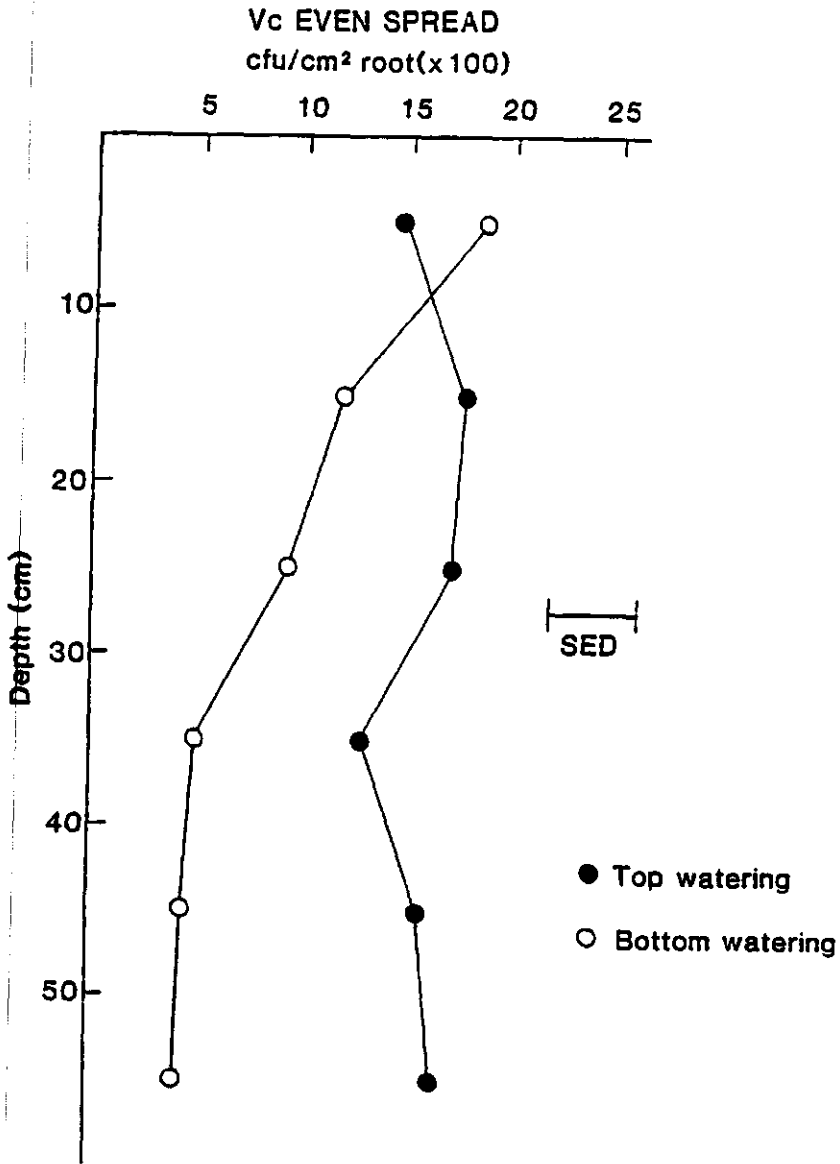

Fig. 5. Effect of two watering regimes on the spatial distribution of *Verticillium chlamydosporium* on the rhizoplane of tomato roots from an evenly distributed inoculum of 2000 chlamydospores/g soil. (n=3)

Chlamydospores evenly spread through the whole soil profile: In nine weeks cfu of *V. chlamydosporium* increased in the top 10 cm from 4 x 10^3 cfu/g soil to 1.3 x 10^5 cfu/g soil when the tubes were watered from the top. When the tubes were watered from the bottom, cfu in the top 10 cm increased to only 7 x 10^4 cfu/g soil. This difference could be seen through the whole soil profile and was highly significant ($P < 0.001$; Fig. 4) throughout the experimental period. In general, establishment of *V. chlamydosporium* was significantly ($P < 0.001$) better in the top 10 cm than in the rest of the soil profile (Fig. 4). Establishment of *V. chlamydosporium* on the roots after 9 weeks was not significantly different in the different soil layers when the tubes were watered from the top (Fig. 5). When water was applied from the bottom of the tubes cfu on the root surface decreased significantly ($P < 0.001$) with depth (Fig. 5).

Root growth, nematode damage and nematode control

It took the tomato roots less than three weeks to reach the bottom of the tubes, which is equivalent to a growth rate of 3.5 cm/day. However, nine weeks after planting more than 50% of the root weight was still concentrated in the top 10 cm of the soil and about 90% in the top 30 cm (Fig. 6). Galling induced by *M. incognita* was severe in the first 20 cm of the soil profile, moderate in the 20-30 cm layer and very slight below 30 cm (Fig. 7). Nematode control by *V. chlamydosporium* was therefore estimated only in the top 30 cm of the soil profiles. All the egg-masses which were extracted from tubes inoculated with chlamydospores were colonised by *V.chlamydosporium*, while none of the exposed egg-masses in the untreated checks were colonised by fungus. When the untreated checks were watered from the bottom a significantly ($P < 0.001$) greater percentage of eggs (Table 3) in the exposed egg-masses was immature (> 85%) than when tubes were watered from the top (*c* 60%). This effect was observed in tubes in which chlamydospores were incorporated in the top 10 cm of the soil, but not when the chlamydospores were evenly spread through the whole soil profile. The proportion of infected eggs in the top 10-30 cm in the tubes inoculated with fungus in the top 10 cm was significantly smaller ($P < 0.001$) when water was added from beneath than when water was added onto the surface. However there was no effect of watering on the proportion of eggs infected when *V.chlamydosporium* was evenly mixed through the soil profile. The result was that the proportion of mature eggs which escaped fungal infection, in the tubes inoculated with

V.chlamydosporium, was approximately the same in all soil layers, irrespective of the watering regime or fungal distribution (Table III). Development rate was greater and therefore there was a larger percentage fully matured eggs in the untreated checks when water was added from the top.

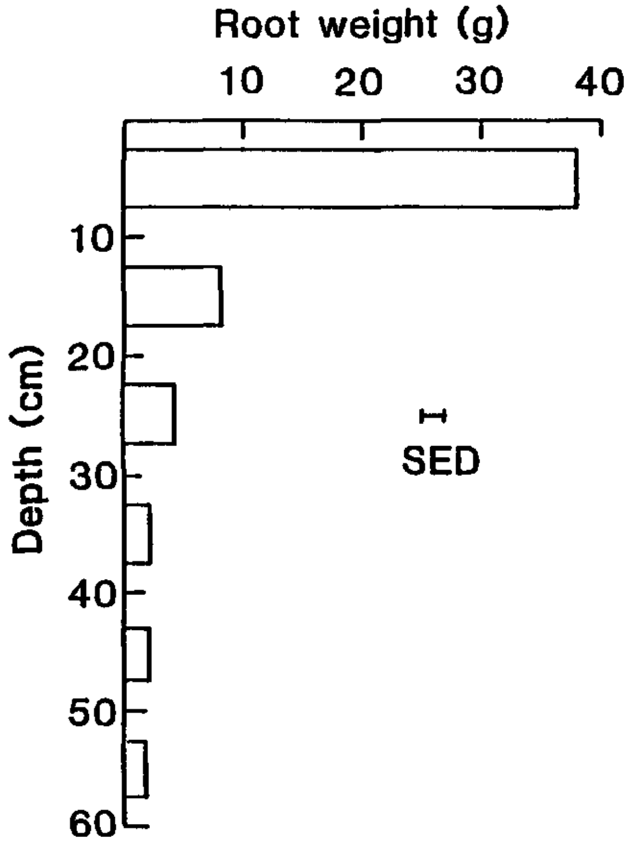

Fig. 6. Relation between root weights of tomato plants and soil depth. (n=3)

This meant that nematode control was between 80 and 90% when tubes were watered from the top. When tubes were watered from the bottom, control was similar in the top 10 cm, while at greater depths fewer eggs were infected and control was only around 30% (Table 3).

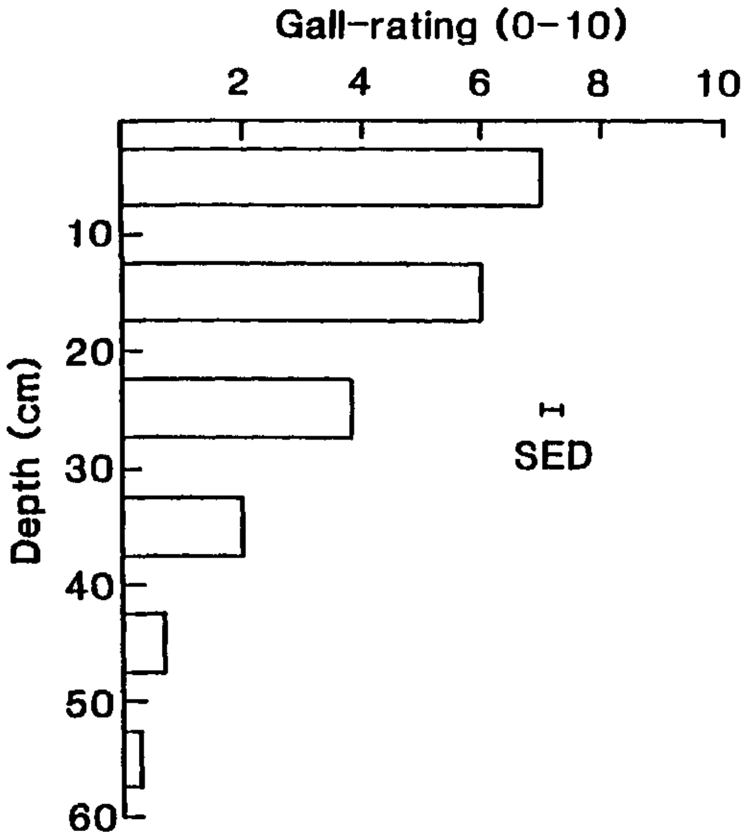

Fig. 7. Relation between nematode damage (galling) and soil depth from an evenly distributed inoculum of *Meloidogyne incognita*. (n=3)

TABLE III

Percentage immature, fully matured and infected eggs in egg masses exposed on the root-surface of tomato plants in the first 30 cm of the soil profile as affected by two different watering regimes in untreated soil, treated with 14,000 chlamydospores/g soil in top 10 cm, and treated with 2000 chlamydospores/g soil evenly distributed through the soil

Treatments	Untreated				*V. chlamydosporium* in top 10 cm						*V. chlamydosporium* evenly spread					
eggs (%)	Immature eggs		fully matured eggs		Immature eggs		fully matured eggs		Infected eggs		Immature eggs		fully matured eggs		Infected eggs	
watering	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
Depth (cm) 0-10	71.0 (1.08)	80.0 (1.60)	29.0 (-1.01)	20.0 (-1.81)	27.6 (-1.36)	34.9 (-0.86)	6.5 (-3.4)	2.6 (-3.75)	65.8 (0.88)	62.8 (0.81)	23.9 (-1.64)	21.0 (-1.64)	4.7 (-3.53)	2.2 (-3.78)	73.9 (1.43)	76.3 1.41)
10-20	50.9 (0.21)	90.7 (2.21)	41.1 (-0.66)	9.3 (-3.26)	9.6 (3.03)	48.6 (-0.03)	3.9 (-3.55)	6.2 (-3.46)	86.5 (2.38)	45.2 (-0.21)	34.2 (-0.86)	38.4 (-0.71)	4.8 (-3.37)	8.6 (-3.04)	61.0 (0.49)	52.9 (0.18
20-30	55.0 (0.07)	90.4 (2.24)	45.0 (-0.47)	9.6 (-3.06)	19.8 (-2.21)	62.4 (0.34)	10.4 -2.30)	6.7 (-3.77)	69.8 (1.12)	30.9 (-1.35)	40.6 (-0.41)	22.8 (-1.96)	2.9 (-3.55)	6.7 (-3.49)	56.6 (0.18)	70.5 (0.61)
SED	(0.583)		(0.708)		(0.497)		(0.352)		(0.530)		(0.468)		(0.401)		(0.488)	

( ) = logit transformations

Spread of *V. chlamydosporium* conidia through soil by different amounts of water. After adding 125 mm water, conidia could be recovered from all soil layers in significant numbers (Fig. 8). The addition of an extra 125 mm water did not cause any further spread of conidia (Fig. 8). From all the conidia applied to the soil surface only 10% were moved 10-15 cm away down the soil profile with waterings equivalent to 125 and 250 mm rainfall and only 1% of the conidia were recovered from below 15 cm depth.

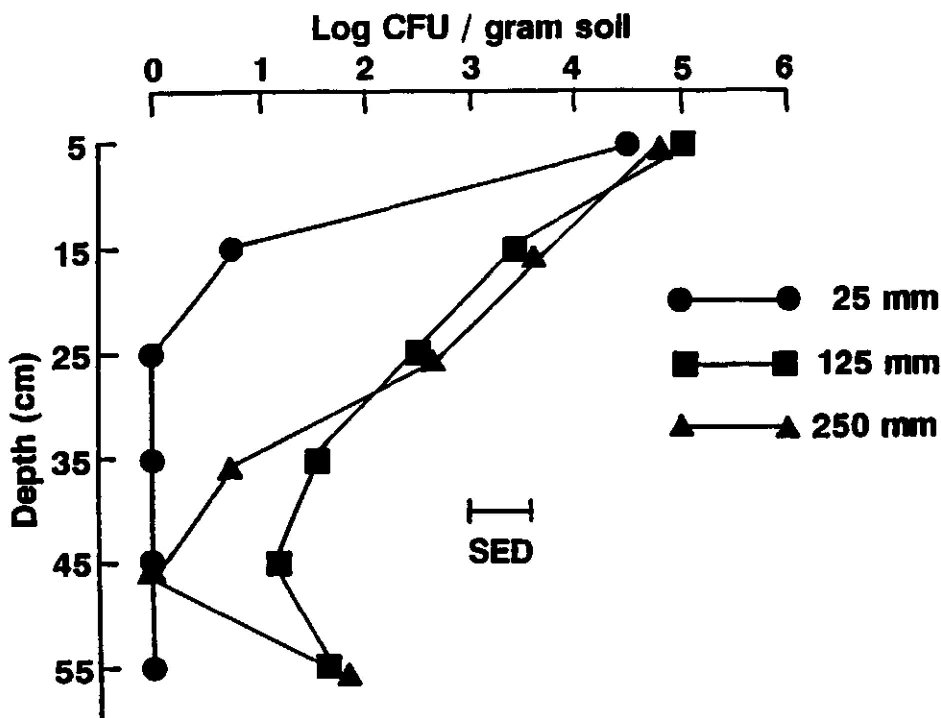

Fig. 8. Effect of three consecutive waterings on the spatial distribution of conidia of *Verticillium chlamydosporium*. (n=3)

Discussion

The results from the three experiments reported on in this paper, show that *V. chlamydosporium* can be transported considerable distances by water. De Leij *et al* (1992), considered that the increases of cfu in soil after an application of chlamydospores were caused by the formation of conidia. Transport of fungal propagules did not occur directly after inoculation of the first 10 cm with chlamydospores. Significant spread to all the deeper soil layers occurred only after the fungus had substantially increased in the top 10 cm. This cannot be explained in terms of hyphal extension (Kerry, 1988), but must be the result of conidia being transported to the deeper soil layers by water. The results from experiment 3 support this theory. Although *V. chlamydosporium* conidia seem to be capable of being transported considerable distances (> 50 cm) by water, only a relatively small proportion (< 1%) was found to have moved such a distance. Most conidia moved less than 10 cm away from the inoculum source.

Because roots grow at a rate of 3-5 cm/day, while hyphal extension of *V. chlamydosporium* in soil is at most 1 cm/month (Kerry, 1988), it is impossible to imagine root colonisation by hyphae growing alongside the growing root tips. The most likely mechanism for root colonisation is therefore that conidia formed in soil are transported by water which percolates along the root surface and through soil pores. When the soil drains, the conidia probably attach themselves with their mucilaginous layer to soil particles or to the root surface, where they germinate under favourable conditions to give rise to hyphae and new conidia. The process can then repeat itself when pores become filled with water again. It is therefore likely that most conidia do not move more than a few cm along the root surface under normal field conditions. Application of *V. chlamydosporium* as a seed dressing therefore seems unlikely to achieve the necessary quick colonisation of the whole root system.

Although colonisation of the whole root system is unlikely to occur from a point inoculum, it does not seem to be necessary to achieve a completely even distribution of fungal inoculum through all the soil layers where nematodes occur. An uneven distribution of inoculum through the top layers of soil (Experiment 1) or chlamydospores only applied to the top 10 cm of soil (Experiment 2) resulted in high

levels of root colonisation through the whole top layer and top 30 cm of the soil profile respectively. In the last experiment exposed *M. incognita* egg-masses in those soil layers were also successfully colonised. Although the amounts of water applied and the very loose texture of the soil used in these experiments might be somewhat unrealistic, limited spread of *V. chlamydosporium* is likely to occur under field conditions, provided that the chlamydospores introduced into the soil germinate and give rise to conidia. A possible way of introducing *V. chlamydosporium* to field crops would therefore be the addition of conidia to irrigation water, provided that the conidia produced on artificial media have the same characteristics as those produced in soil.

Verticillium chlamydosporium did not establish as well when tubes were watered from the bottom compared with those tubes watered from the top. Baxter & Blake (1969) found that hatch of *M. javanica* eggs was suppressed by oxygen levels less than 10%. They also found that egg development was slowed down when oxygen was in short supply. In the tests reported, the percentage of immature eggs was significantly greater in the untreated tubes watered from the bottom than those watered from the top, indicating that egg development was slower in tubes watered from below. This may have been because less oxygen was available in the tubes watered from the bottom than in those watered from the top. *Verticillium chlamydosporium* is an aerobic organism, and lower oxygen levels would cause the fungus to establish in smaller numbers in soil and on root surfaces. If the availability of oxygen is a limiting factor for fungal growth this would also explain why establishment of *V. chlamydosporium* in the soil and on the root surface was significantly greater in the top layers than the bottom layers of the soil profile when the fungus was evenly distributed through the soil. Lack of oxygen affected the ability of *V. chlamydosporium* to control *M. incognita*. Even though the development of both fungus and nematode was inhibited by lack of oxygen, control of *M.incognita* with *V.chlamydosporium* was greater in well aerated soil than in soil which was less well aerated.

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CHAPTER 7

THE EFFECTIVENESS OF *VERTICILLIUM CHLAMYDOSPORIUM* AS A BIOLOGICAL CONTROL AGENT FOR *MELOIDOGYNE INCOGNITA* IN POT TESTS IN THREE SOILS AND FOR CONTROL OF *M.HAPLA* IN A MICRO-PLOT TEST

F.A.A.M. de Leij, B.R. Kerry and J.A. Dennehy

Summary

An isolate of *Verticillium chlamydosporium* which parasitised *Meloidogyne* spp., multiplied in peaty-sand from inoculum rates of 500, 1,000, 5,000 or 10,000 chlamydospores/g soil to a maximum level of 5.5 x 10^4 cfu/g soil and survived for at least 8 weeks. The fungus survived, but did not multiply in loamy sand or sand. Establishment of *V. chlamydosporium* on the rhizoplane of tomato plants was significantly greater in peaty sand than in loamy sand or sand. Nematode control was in general better in peaty sand (average 59% control) than in the other two soil types (average control in loamy sand 51% and in sand 39%) In a micro-plot experiment on sandy loam, *V. chlamydosporium* controlled populations of *M. hapla* on tomato plants by more than 90%. The fungus multiplied and survived in soil, for at least 123 days. Significantly more fungus could be found in rhizosphere soil than in non-rhizosphere soil. Combining *V. chlamydosporium* with an aldicarb treatment equivalent to 2.8 kg a.i/ha did not affect the activity of the fungus, and gave better control (98%) when both treatments were applied together.

Introduction

An isolate of *Verticillium chlamydosporium* Goddard gave effective population control of four different *Meloidogyne* spp. when the fungus was thoroughly mixed through a non sterilised sand-peat potting compost (de Leij & Kerry, 1990, 1991). *Verticillium chlamydosporium* is a facultative parasite of cyst nematodes (*Heterodera* spp. and *Globodera* spp.) and root-knot nematodes (*Meloidogyne* spp.), and has a parasitic (Willcox & Tribe, 1974; Kerry,, 1975; Morgan-Jones, Godoy & Rodriguez-Kábana, 1981; Freire & Bridge, 1985), as well as a saprophytic phase. In the saprophytic phase its growth and survival are dependent on nutrients other than those provided by the nematode host. Fungal inoculum applied to soil must grow and colonise the rhizosphere where it multiplies, particularly on root-galls induced by the nematode (de Leij & Kerry, 1991). Here the fungus infects eggs laid by root-knot nematodes in egg-masses laid on the root surface. Although growth of the fungus on the rhizoplane can, to some extent, compensate for a low inoculum density in soil (de Leij, Davies & Kerry, 1992), the soil environment may be important for the initial establishment of the fungus on the rhizoplane.

In this paper the effects of three different soil types on the establishment of *V.chlamydosporium* in soil and on the rhizoplane were tested at five different inoculum rates and the resulting population control of *Meloidogyne incognita* Kofoid & White was examined in a pot test. The fungus was also tested against *Meloidogyne hapla* Chitwood, in a micro-plot experiment in a plastic tunnel-house.

Materials and methods

Fungal isolate and production of inoculum: An isolate of *V. chlamydosporium* (CMI cc 334168) originally isolated from *M. incognita* eggs was used because it had previously been shown to be effective against *Meloidogyne arenaria* Neal, *M. incognita*, *Meloidogyne javanica* Treub and *M. hapla* (de Leij & Kerry, 1990). The isolate was stored at 5°C on silica gel using the methods of Smith & Onions (1983). For experimental use, a few silica gel crystals bearing the fungus were sprinkled onto a Petri dish containing water agar (0.8%) and incubated at 25°C for two weeks,

allowing the fungus to grow away from the crystals onto the agar. Two plugs (7 mm diam.) taken from the fungal colonies that developed were used to inoculate 250 ml conical flasks, each containing 150 g of a moist, autoclaved mixture of sand and milled barley grain (1 : 1 $^{v}/_{v}$). After two weeks incubation at 25°C the cultures were washed on a 50 μm aperture sieve with a fine water spray to retain the sand and grain, and fungal propagules were collected on a 10 μm aperture sieve held beneath. The residue on this sieve was further washed to remove conidia and small hyphal fragments, leaving mainly chlamydospores and some larger hyphal fragments. The chlamydospores were counted in a haemocytometer. Inoculum was prepared by mixing the residue from the 10 μm sieve with fine sand to give a concentration of 10^7 chlamydospores/g sand. This inoculum was then further mixed with the soils used for the different experiments.

<u>Pot experiment</u>

<u>Soil types and inoculum rates</u>: Three different soil types were selected: an unsterilised peat based potting compost (37% peat, 19% fine sand, 44% coarse sand), a loamy sand soil from Woburn, England, mixed with 25% coarse sand (22% silt, 47% fine sand, 31% coarse sand) and a sharp sand mixed with 25% Woburn soil (7% silt, 53% fine sand, 40% coarse sand). Pots (12.5 cm diam.) were filled with 750 ml of the different soil-types and each of four replicate pots were inoculated with 0, 500, 1000, 5,000 or 10,000 chlamydospores/g soil. Each pot was planted with a four week old tomato seedling (cv. Pixie) which was allowed to establish for two weeks before 1000 *M. incognita* juveniles (< 1 week old) were pipetted with 10 ml water into three holes around the roots. The pots were placed in a glasshouse at 25-30°C in four completely randomized blocks. Each plant received 0.75 g Phostrogen fertiliser at planting, and was watered daily according to need.

<u>Fungal establishment</u>: Three of the four replicates of each treatment were sampled immediately after inoculation with chlamydospores and at two-weekly intervals throughout the experiment. At each sampling occasion two cores (7 mm diam.) were taken from each pot with a cork borer. The samples were well mixed and a 1 g subsample was used to estimate numbers of colony forming units in the soil. This was done by spreading serial dilutions of the soil suspensions onto a semi-selective

medium (de Leij & Kerry, 1991). The Petri dishes (9cm diam.) were incubated for one to two weeks at 25°C, after which time *V. chlamydosporium* colonies could readily be recognised and counted at 6 x magnification.

Plants were harvested seven weeks after addition of nematodes. Colonisation of the root surface by the fungus was assessed after the tomato plants were harvested. The root systems were washed carefully, blotted dry, weighed, cut into small segments (c 1 cm length) and mixed thoroughly. From each root system a 1 g sample was taken and crushed with a pestle and mortar in 9 ml of agar solution (0.05%) at room temperature. A dilution range of this suspension was spread onto semi-selective medium, incubated and counted as described.

Estimation of nematode control: After cutting up the roots and mixing the segments thoroughly, a 5 g sample was taken from which the eggs and juveniles were extracted using the method described by Coolen & d'Herde (1973). Because immature eggs are much more susceptible to fungal infection than mature eggs and juveniles (Irving & Kerry, 1986), it was assumed that mature eggs and juveniles had escaped fungal infection. Nematode control was therefore estimated by comparing the numbers of fully matured eggs and juveniles in the untreated checks with those extracted from treated plants. To confirm egg-infection by *V. chlamydosporium* in the different soil types, ten egg-masses were picked off randomly from each root system treated with 10,000 chlamydospores/g soil and from the untreated roots. Each egg-mass was examined at 200 x magnification for infected eggs and presence of chlamydospores.

Micro-plot experiment

Treatments: The micro-plot experiment was done in a plastic tunnel house at Shinfield (University of Reading) on a sandy loam soil (29% silt, 62% fine sand, 9% coarse sand). Each plot was 1.60 m long and 0.85 m wide. The plots were divided by narrow paths (0.3 m wide) and arranged in six blocks, each block consisting of four randomised treatments. The following treatments were mixed thoroughly with the soil to a depth of 30 cm:

(a) no treatment; untreated control treatment.

(b) 5000 chlamydospores/g soil; *V.c*-treatment

(c) aldicarb granules equivalent to a final concentration of 2.8 kg a.i./ha; aldicarb treatment

(d) *V. chlamydosporium* and aldicarb equivalent to the concentrations in (a) and (b); *V.c* + aldicarb-treatment.

Before the treatments were applied to the micro-plots, inocula were first mixed with 20 kg soil. Each plot was planted with four, six-week-old, tomato seedlings (cv. Pixie), which were allowed to establish for ten days before each plant was inoculated with 1000 *M. hapla* juveniles. The juveniles were suspended in 25 ml water and this was poured into three holes made around the tomato roots. Plants were watered three times/week and received fertilisation with Phostrogen weekly (c 5 g/plot/week) for the first eight weeks. Soil temperatures were measured every week or every other week at depths of 10 and 20 cm.

Fungal establishment: Soil samples were taken at weekly or two-weekly intervals for 123 days from all the treatments in the first four blocks. At every sampling occasion, from each plot, three soil cores were taken on a diagonal across the plot to a depth of 30 cm with a cheese corer (1 cm diam.) and combined. After mixing, a 1 g subsample was taken, a dilution series prepared, plated out on semi-selective medium, incubated, and counted as described before. From every plot, two plants were harvested 50 days after inoculation with *M.hapla* juveniles. The remaining two plants were harvested 113 days after inoculation. At each harvest, root systems were carefully dug out and excess soil was shaken off. All roots taken from one plot at one time were cut into small segments (c 2 cm) and mixed together. Establishment of *V. chlamydosporium* in the rhizosphere soil was estimated by taking from every plot an unwashed, chopped up root sample (c 15 g). This sample was divided into two equal portions. One of the portions was carefully washed on a 53 μm sieve to remove all soil. The roots were then blotted dry and reweighed. From this measurement the weight of soil attached to 1 g of root could be calculated. From the washed, as well as from the unwashed roots, a 1 g sample was taken. Each of the sub-samples was crushed with a pestle and mortar and plated out on semi-selective medium, incubated and counted as described before. From the two counts numbers of colony forming units/g root (rhizoplane) as well as colony forming unit/g rhizosphere soil could be estimated. Numbers of colony forming units in the rhizosphere soil were estimated only after the first harvest, while numbers of colony forming units on the rhizoplane were estimated after both harvests.

Estimation of nematode populations on the roots and in the soil: Because root galling estimated with the gall rating chart described by Bridge & Page (1980) was insignificant in all treatments at both harvests, a large root sample (> 10 g) was taken to extract eggs and juveniles, using the method described by Coolen & d'Herde (1973).

After both harvests, the soil from which the roots were taken was thoroughly dug over before 1.5 kg soil was taken at random from all the plots treated with *V. chlamydosporium* alone and the untreated checks. The 1.5 kg of soil taken from each plot was mixed with 0.5 kg coarse sand to make it suitable for use in pots. The soil mixtures were put in 15 cm diam. pots and each pot was planted with five, four week old marigold (*Calendula officinalis*) seedlings. The pots were placed in the glasshouse at 20°C for six weeks, after which time the root ball was washed carefully to remove the soil, blotted dry, and cut into small segments (c 2 cm). The chopped up roots were then digested in Pectinex (Novo Enzyme Products Ltd, Farnham, U.K.) for 24 h at room temperature. The slurry was washed through a 0.8 mm screen with a strong water jet onto a 53 μm aperture sieve. The residue was suspended in 100 ml water, and *Meloidogyne* females in two samples of 10 ml were counted with the aid of a low power microscope (x 6). Numbers of females extracted from the different pots were used to compare the nematode populations in the micro-plot soils.

Results

Pot test

Establishment of *V. chlamydosporium* in three different soil types:
In the peaty sand, numbers of cfu/g soil increased rapidly at all inoculum rates during the experiment. Increases in the numbers of cfu/g soil were greater when less inoculum was mixed into the soil and, eight weeks after application, there were approximately 5.5 x 10^4 cfu/g soil irrespective of the inoculum rate used (Fig. 1). However, in loamy sand as well as in the sand, the initial number of cfu/g stayed the same throughout the experiment (Fig. 1).

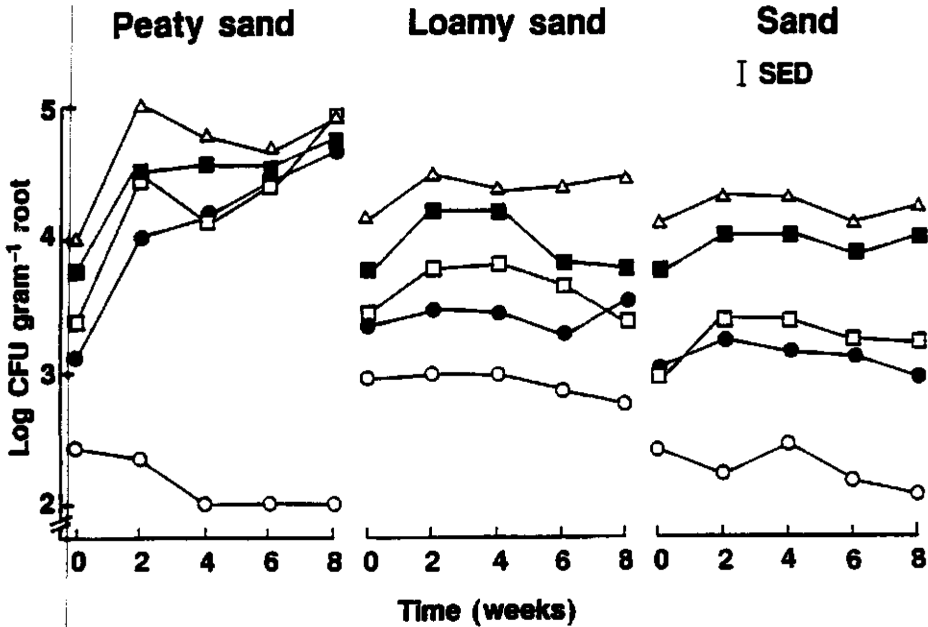

Fig. 1. Establishment of *Verticillium chlamydosporium* in three different soil types from inoculum rates equivalent of 0(O), 500(●), 1000(□), 5000(■) and 10,000(△) chlamydospores/g soil. (n=4)

<u>Establishment of *V. chlamydosporium* on the root surface of tomato plants</u>: At the end of the experiment fungal establishment on the roots in peaty sand was significantly ($P < 0.001$) greater than in loamy sand or sand (Fig. 2). In the peaty sand, only an initial inoculum rate of 500 chlamydospores/g soil resulted in significantly ($P < 0.001$) smaller numbers of cfu/g root compared with the other inoculum rates (Fig. 2). In the sandy loam and sand, cfu/g root increased with increasing inoculum rates in the soil, resulting in significant differences in rhizoplane colonisation (Fig. 2).

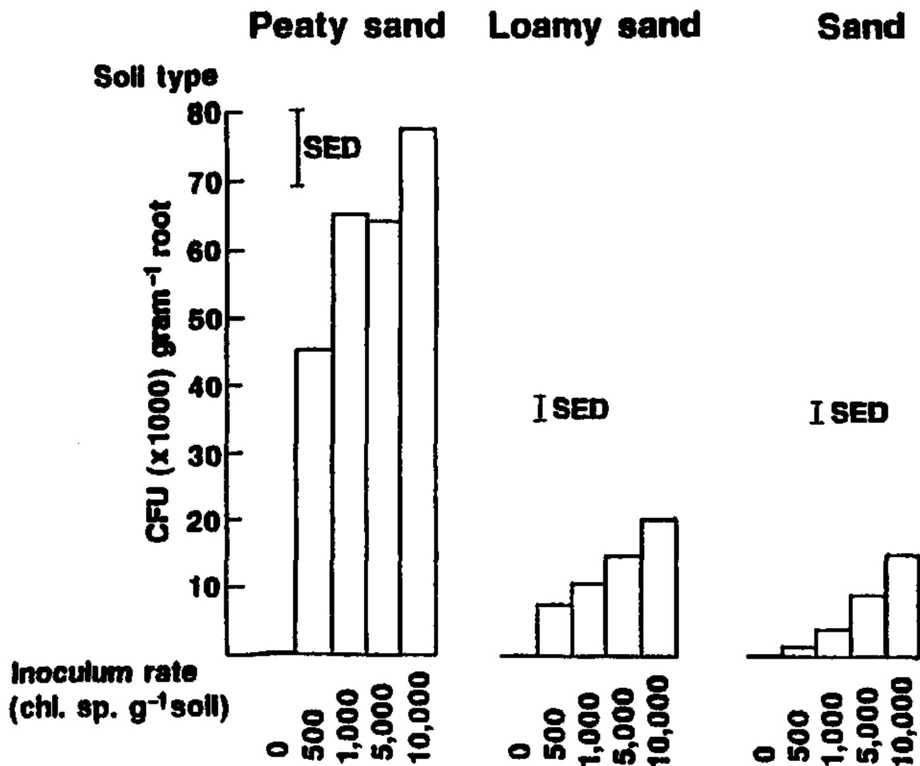

Fig. 2. Establishment of *Verticillium chlamydosporium* on the rhizoplane of tomato plants grown in three different soil types inoculated with 0, 500, 1000, 5000 or 10,000 chlamydospores/g soil, six weeks after planting. (n=4)

<u>Effect of *V. chlamydosporium* on juvenile populations of *M. incognita* in the roots</u>: At inoculum rates of 10,000 chlamydospores/g soil, control of *M. incognita* in the three soil types was between 50 and 70%, and significantly different from the untreated checks (Table 1). On average, there was greater control (Table 1) in peaty sand (58.5%) than in loamy sand (51.3%) and sand (39%). However, the nematode reproduced less well in the sand than in the other two soils (Table 2). There was no

significant effect of increasing the application rate of the fungus on the final nematode population, except in the peaty-sand. In this experiment the level of nematode control was less than previously reported. Chlamydospores and infected eggs were found in egg-masses picked from the different root systems in all soil types inoculated with 10,000 chlamydospores/g soil. Egg-masses taken from the untreated checks contained neither chlamydospores nor infected eggs.

TABLE I

Numbers of M. incognita *juveniles and fully matured eggs per root system (x 10^3) in peaty-sand, loamy-sand and sand, as affected by* V. chlamydosporium *at inoculum rates of 0, 500, 1,000, 5,000 and 10,000 chlamydospores/g soil. (n=4)*

Soil-type / rate of inocul.	Peaty-sand	loamy-sand	Sand	SED
0	147.0	141.9	79.2	
500	72.5	65.3	51.7	
1000	67.0	94.8	58.5	15.2
5000	61.1	68.2	44.1	
10000	43.2	48.6	38.8	

Micro-plot experiment

Soil temperatures: Soil temperatures during the first 60 days of the experiment were in general between 20 and 25°C but, shortly after inoculation with *M. hapla* juveniles, soil temperatures rose to above 30°C in the top 10 cm of the soil profile (Fig. 3). After the first harvest soil temperatures fell gradually to around 17°C (Fig. 3).

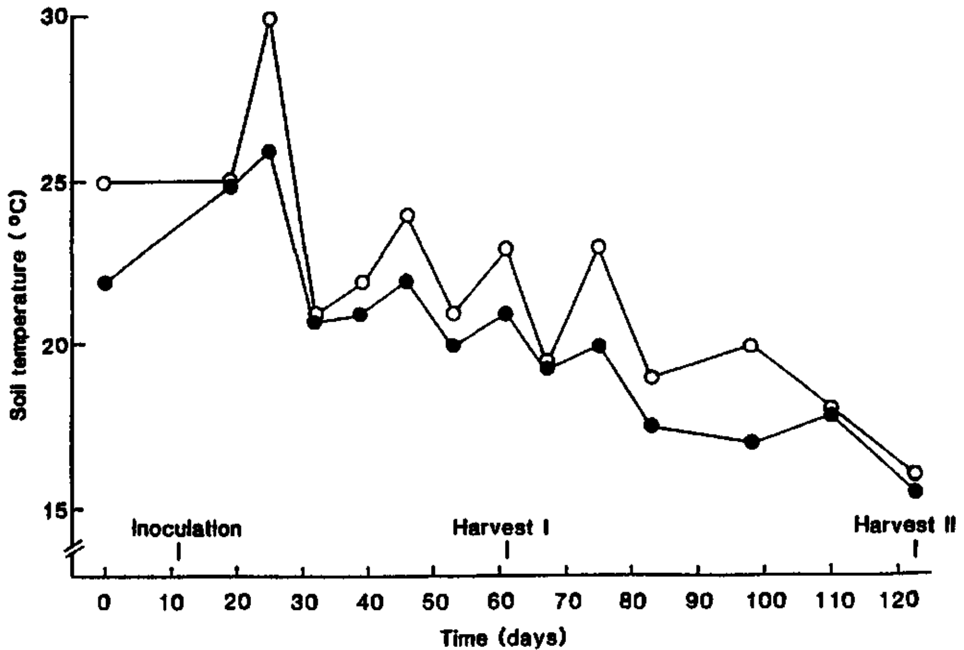

Fig. 3. Soil temperatures at 10 cm (O) and 20 cm (●) depth during the course of the micro plot experiment.

Fungal establishment: During the course of the experiment *V. chlamydosporium* survived and proliferated in soil, increasing from c 7,000 to c 50,000 cfu/g soil (Fig. 4). The numbers of cfu/g soil were consistently greater ($P < 0.001$) in plots treated with the fungus than in those which were treated with aldicarb alone or in the untreated checks. Combining *V. chlamydosporium* with aldicarb had no significant effect on fungal establishment in soil (Fig. 4).

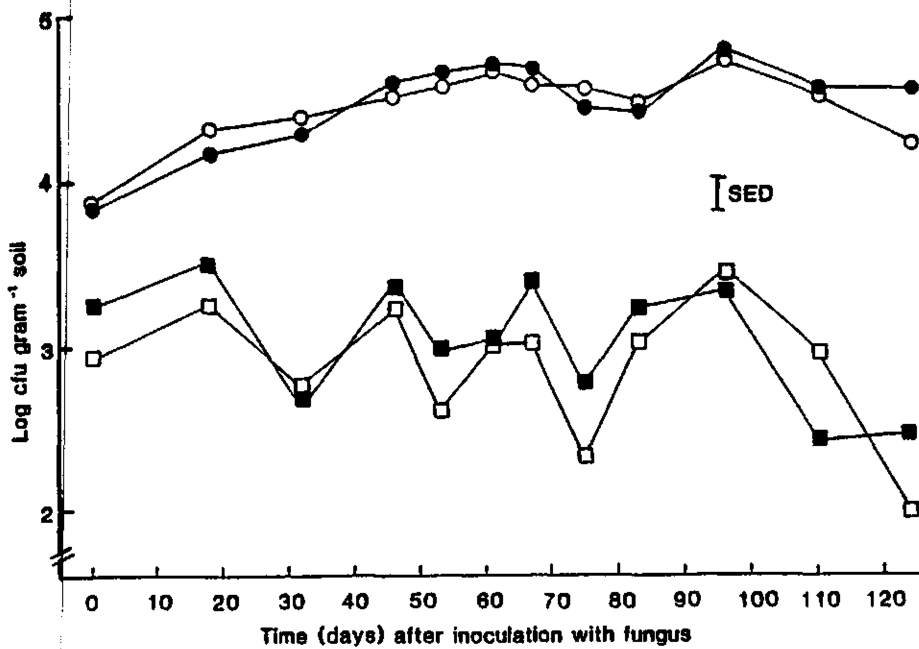

Fig. 4. Colony forming units of *Verticillium chlamydosporium* in soil during the course of the micro plot experiment in the different treatments: (untreated control: □ ; aldicarb: ■ ; *V. chlamydosporium*: 0; *V. chlamydosporium* + aldicarb: ●). (n=4)

The numbers of cfu/g rhizosphere soil were on average four times greater ($P < 0.001$) than in the non-rhizosphere soil (Fig. 5). Establishment of *V. chlamydosporium* on the rhizoplane was not significantly different for the two harvest times. Approximately 13 x 10^3 cfu/g root were counted on those roots which had grown in

the plots treated with *V. chlamydosporium* or a combination of *V. chlamydosporium* and aldicarb. In the plots which were not treated with the fungus, no *Verticillium* could be detected on the rhizoplane (Fig. 6). Combining aldicarb with *V. chlamydosporium*, had no significant effect on fungal establishment on the rhizoplane (Fig. 6)

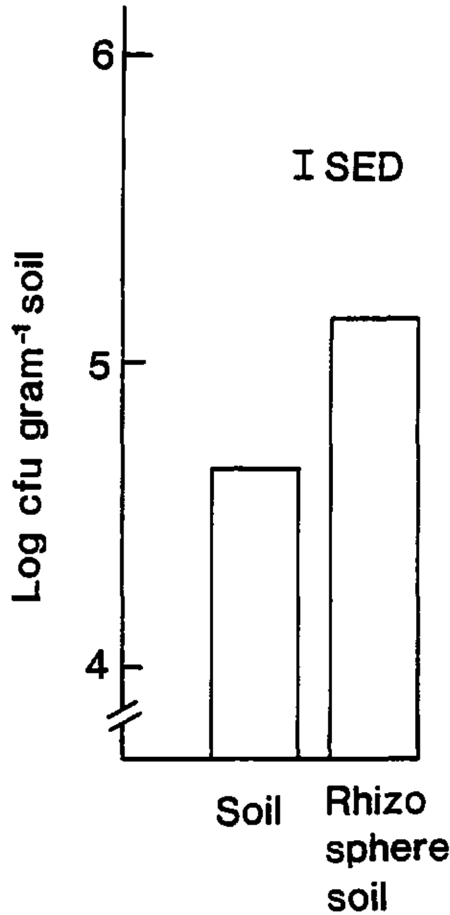

Fig. 5. Colony forming units of *Verticillium chlamydosporium* in rhizosphere soil, and non-rhizosphere soil seven weeks after planting. (n=6)

<u>Effect of *V. chlamydosporium* and aldicarb on control of *M. hapla*</u>: Nematode populations on the roots, 50 days as well as 113 days after inoculation with *M. hapla* juveniles, were small (< 100 eggs + juveniles/g root) (Table 2). Fifty days after

inoculation with *M. hapla* juveniles, eggs as well as juveniles on the tomato roots were significantly reduced by all treatments compared with the untreated checks. Control of juveniles with *V. chlamydosporium* was similar to the control level achieved with aldicarb (87%); When the two were combined, control was increased to 98% (Table 2). At the second harvest the effect of the different treatments on nematode populations in the roots was similar to that observed after the first harvest. Nematode control of juveniles invading the roots with aldicarb, *V. chlamydosporium* and the combination of *V. chlamydosporium* and aldicarb were 89, 92 and 100% respectively (Table 2).

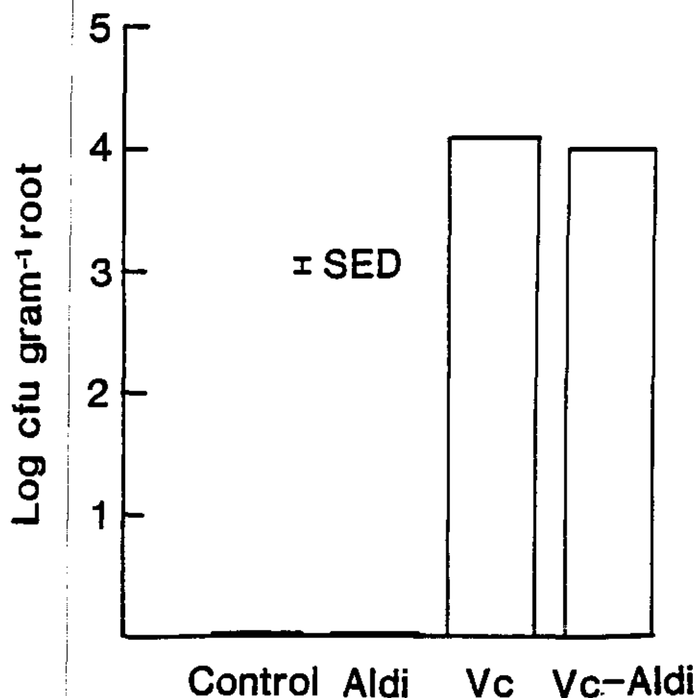

Fig. 6. Colony forming units of *Verticillium chlamydosporium* on the rhizoplane of tomato plants grown in untreated soil, or soil treated either with aldicarb, *V. chlamydosporium* or *V. chlamydosporium* + aldicarb, seven weeks after planting. (n=6)

The numbers of females developing in the roots of *Calendula officinales* grown in pots filled with untreated soil and soil treated with *V. chlamydosporium*, 113 days after nematode inoculation, showed that populations of *M. hapla* in soil were also significantly ($P < 0.001$) reduced by 82% in soil treated previously with *V. chlamydosporium* (Table 2). Numbers of females extracted from soil 50 days after nematode inoculation were too small to be analyzed (between 0 and 10 females/pot; results, therefore, are not presented).

TABLE II

Numbers of eggs and juveniles of Meloidogyne hapla *in tomato roots 50 and 113 days after nematode inoculation in the different treatments, and the number of* M. hapla *females developing on marigold plants grown in 1.5 kg soil treated with* Verticillium chlamydosporium *or untreated soil. The soil was taken at the end of the experiment.* (n=6)

Harvest Treatment	50 days		113 days		no. of females per pot
	eggs g^{-1} root	juveniles g^{-1} root	eggs g^{-1} root	juveniles g^{-1} root	
Control	64.2	14.1 (1.12)	28.4 (1.45)	23.2 (1.34)	186.0
Aldicarb	12.8	1.9 (0.33)	15.0 (1.01)	2.6 (0.46)	
V. chlam	24.8	1.9 (0.41)	9. 2 (0.78)	1.8 (0.37)	32.6
Vc + Aldic	27.1	0.3 (0.10)	12.7 (1.06)	0.0 (0.00)	
SED	12.9	(0.15)	(0.22)	(0.13)	30.0
Significance level (*P*)	$P < 0.01$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$

*data between brackets not normally distributed and transformed to $y = \log10(x + 1)$

Discussion

Although *V. chlamydosporium* survived in the three soil types tested, only peaty sand provided conditions which allowed the fungus to multiply. Eight weeks after inoculation of the peaty sand with four different application rates, the numbers of cfu in the peaty sand soils reached a similar level. The peaty sand may have an upper limit to its carrying capacity of about 10^5 cfu/g soil. It is most likely that the carrying capacity of a soil is determined by the amounts of nutrients available for fungal germination and subsequent growth (Linderman & Toussoun, 1967). It appeared that the chlamydospores mixed into the loamy sand and sand did not germinate unless they were triggered by nutrients leaking from the roots. This kind of relationship is well known in plant pathogens which respond to nutrients exuded by roots to form infectious structures to overcome host defences (Mangenot & Diem, 1979).

The initial fungal inoculum that will establish on the rhizoplane is derived from that present in soil. As a consequence, inoculum rates may have to be greater in soils which do not provide suitable conditions for chlamydospore germination and fungal growth than in those which do, to ensure a level of establishment on the rhizoplane that gives nematode control. Another approach would be to amend the soil with suitable nutrients. Linderman & Toussoun (1967) stimulated up to 58% of chlamydospores of *Thielaviopsis basicola* (Berk & Br.) Ferraris to germinate in soil when carrot juice or raw carrot slices were added to the soil. A similar approach might help to stimulate germination of chlamydospores of *V. chlamydosporium* in mineral soils.

Verticillium chlamydosporium was much more prevalent on the rhizoplane in peaty sand than in loamy sand or sand at all inoculum rates. Although concentrations of around $2x10^4$ cfu/g root have provided more than 90% control of *M.incognita* in peaty sand (de Leij *et al.*, 1992), nematode control in this experiment never exceeded 70%, even though colonisation of the rhizoplane was often greater than $2x10^4$ cfu/g root in all soil types. However, soil temperatures in this experiment were relatively high (c 30°C) which meant that a proportion of the nematode eggs developed faster than the fungus could infect them (de Leij, *et al.*, 1992) and often poor control did not result from lack of inoculum on the rhizoplane.

For the first time it was shown that *V. chlamydosporium* could give nematode population control of more than 90% when introduced into field soil, rather than in pot experiments in the glasshouse. Because the isolate of *V. chlamydosporium* chosen for this experiment is a typical egg-parasite (de Leij & Kerry, 1991) it was encouraging that a combination of this fungus with a nematicide did not restrict fungal establishment in the soil, in the rhizosphere, on the rhizoplane, or affect nematode control by the fungus. This was consistent with findings of Crump & Kerry (1986) who found that incorporation of aldicarb in corn meal agar up to a concentration of 100 ppm had no significant effect on fungal growth. In this experiment nematode population densities on roots were small (probably as a result of the high soil temperatures immediately after nematode inoculation). This meant that *M.hapla* did not cause any initial plant damage and *V.chlamydosporium* was able to prevent further nematode population build up. However, in situations with large nematode populations a combination of nematicide with *V. chlamydosporium* could be very useful; the chemical would prevent initial damage, while *V. chlamydosporium* would give long term nematode control.

Although the results are encouraging, much more research is needed before the potential of this fungus as a biological control agent can be assessed. Fermentation technology is required to advance from laboratory (solid) culture to produce the fungus in large scale fermenters (Kenney & Couch, 1981). Also, the fungal inoculum needs to be formulated in such a way that it is easy to incorporate in soil and that fungal multiplication and survival in soil is ensured. More tests under realistic conditions need to be done to build up the necessary confidence that biological control with *V. chlamydosporium*, is likely to be a successful method for the control of plant parasitic nematodes.

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CHAPTER 8
GENERAL DISCUSSION

When the key factors which determine the efficacy of a biological control agent are known, it is possible either to:

(a) manipulate the organism to overcome certain environmental constraints, or

(b) integrate the organism into a control programme, so that certain shortcomings of the organism can be compensated for, or

(c) use the organism under those circumstances where it is most likely to be effective.

In this chapter the use of *V. chlamydosporium* as a reliable bio-pesticide is discussed, using the information which has been reported in the previous chapters. Included is also a section discussing the selection of potential biological control agents and the prospects of biological control in the management of nematode pests.

The interactions which occur between fungus, soil, host plant and nematode can be visualised as follows (Fig. 1; Kerry & de Leij, 1991).

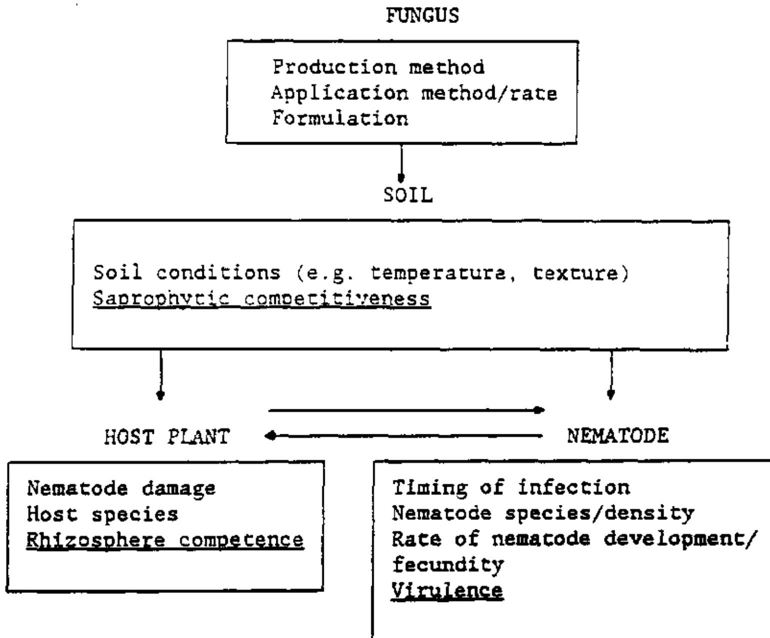

Fig. 1 Factors affecting the establishment of *Verticillium chlamydosporium* in soil and on the roots and the numbers of nematodes infected

These factors can be divided into four main groups:

1. *Factors where the organism itself can be directly manipulated*. These include production method, application method, application rate and formulation.
2. *Factors which can be selected for*. These include saprophytic competitiveness, rhizosphere competence and virulence.
3. *Factors which cannot be manipulated directly, but can be adapted by agricultural practice*. These include soil conditions, nematode damage and nematode density, and plant host.
4. *Factors which are very difficult to manipulate*. These include temperature and nematode species.

The greatest control of *Meloidogyne* can be expected in those situations where:

(a) The fungus is present in large numbers in soil, either because it multiplies fast or because the inoculum rate is high.

(b) The host plant allows for a high level of root colonisation.

(c) The nematode produces its egg-masses on the root surface either because the host plant is not very susceptible to galling, or nematode populations are small, or the nematode species itself does not induce large galls (e.g. *M. hapla*).

(d) The development rate of the eggs is slower than the infection rate of the fungus (e.g. temperatures $< 25°C$).

However, under most conditions it will be necessary to integrate *V. chlamydosporium* into a nematode control programme. Because *V. chlamydosporium* is a typical egg-parasite (Chapter 2) initial economical damage caused by *Meloidogyne* can only be avoided when nematode populations are small (Chapter 4). When nematode populations are large, economic losses may be avoided by using a tolerant plant variety in combination with *V. chlamydosporium*. In this way crop losses can be avoided, while nematode populations can be checked using the fungus. The third option would be to use a nematicide to reduce initial nematode populations to an economically non damaging level and use *V. chlamydosporium* to keep nematode infestations low, so that in the next cropping cycle it is not necessary to use a nematicide.

Different host plants might react very differently to *Meloidogyne* in terms of

invasion, multiplication rate and gall size, all of which have an influence on the effectiveness of *V. chlamydosporium* (this thesis). A small experiment was set up to investigate the effect of different host plants on *V. chlamydosporium*. Some prelimenary results on the ability of the fungus to colonise the rhizoplane and control *M. incognita* are presented in table 1. These results show that there are great differences in the ability of the fungus to colonise the rhizoplane of different host plants and that this affects nematode control. It remains to be seen, whether a low affinity of the fungus to certain host-plant roots can be compensated for by using a higher inoculum rate.

TABLE I

Colonisation of the rhizoplane (cfu/cm^2 root) on a range of plants grown in soil treated with 5,000 chlamydospores/g soil (n=4) and the resulting effect on nematode control. (* = missing data).

Hostplant	Root-colonisation cfu/cm^2 root	Control (%)
Tomato	229	58
Potato	88	79
Pepper	29	36
Tobacco	43	40
Cabbage	199	68
Sorghum	25	0
Wheat	15	5
Maize	216	71
Soybean	14	*
Pigeon pea	36	*
Cotton	22	*
SED	49	

Soil conditions, i.e. availability of nutrients for chlamydospore germination and fungal growth, may be crucial for the level of establishment in soil (Chapter 7). A possible way of overcoming such limitations would be to formulate chlamydospores in such a way (e.g. by coating the spores with a low concentration of nutrients), that

when they are incorporated into the soil germination and growth will take place irrespective of the nutrient status of the soil. However, care has to be taken that the nutrient carrier does not increase the mycostasis of the soil (Chapter 2) by over-stimulation of the indigenous soil microflora.

The form (conidia, chlamydospores or hyphal fragments) in which *V. chlamydosporium* is introduced into soil has obvious consequences in terms of survival, spread through soil and storage characteristics of the fungal product. Conidia are easy to mass-produce in liquid culture (Kerry *et al.*, 1986) but it was found that conidia produced in liquid medium were larger in size than those produced on agar (Kerry *et al.*, 1986). When conidia which are produced in liquid culture are incorporated into soil their survival is, in general, poor and a nutrient carrier is necessary to ensure establishment in soil (Kerry, 1988). This might be due to the formation of conidia with thin, unpigmented cell-walls in liquid culture, while the conidia formed on solid culture, have thicker and pigmented cell-walls (Davies *et al.*, 1991). When conidia can be produced in such a way in liquid culture that their cell-walls are toughened so that they can withstand adverse soil conditions, inundation techniques might be feasible (Chapter 6). Chlamydospores are the natural survival structures of the fungus, so would appear to be the ideal propagule to be introduced into the soil, or formulated into a product with a long shelf life. However, production of chlamydospores is difficult in liquid fermentation (Kerry *et al.*, 1986). Furthermore, chlamydospores are quite large structures (Gams, 1988) and their spread through soil is, as a consequence, poor (Chapter 6). Uniform distribution through soil relies therefore on thorough incorporation techniques or on the formation of conidia which can be spread through soil by water movement (Chapter 6). The process of chlamydospore germination, formation of conidia and subsequent spread by water movement might be too slow and nematode eggs are liable to escape fungal infection. Hyphal fragments seem to have both the negative aspects of conidia (poor survival) and chlamydospores (poor distribution) and are unlikely to be of much value to be used in a biological control product against nematodes.

The selection of potential biological control agents for nematodes poses a few difficult dilemmas. Jatala (1986) lists 16 criteria which are desirable for biological control agents. But it is unlikely that an organism exists which survives and multiplies

in a wide range of soil types, kills a wide range of nematode pests and other pests and is adapted to a range of abiotic factors (to quote only three of his criteria). Such an organism, if it existed, would not be rare, but extremely common. Furthermore nematode pests would probably not exist. It seems therefore a waste of time trying to find the ideal biological control agent. However, some selection is necessary to avoid wasting time and money on organisms which are unlikely to be usefull. It seems, for example, reasonable to select organisms which have the ability to reduce plant damage and/or suppress nematode populations. Also care has to be taken that the selected organism is not harmful to non target organisms including host-plants and humans. And last but not least, the organism must have enough commercial potential to be further developed as a bio-pesticide (i.e. large market, cheap to produce and reliable under a range of conditions).

But even these three criteria pose many problems. It makes sense to select useful biological control organisms from soils which are suppressive to a certain nematode pest (Kerry, 1990; Crump, 1987; Stirling, 1979; Deacon, 1991). But the isolation procedure itself determines which organisms are to be found, and this is no guarantee that the organisms selected, are those which cause the suppressiveness. Observation chambers as described by Crump (1987) can be used to select (fungal) parasites of females and eggs of cyst and root-knot nematodes, while parasites of juveniles go undetected. Agar plates sprinkled with soil and baited with nematodes (Barron, 1977) will only detect those parasites which destroy juveniles, while those that kill females go undetected. The isolation of the fungi from diseased nematodes on the surface of observation plates poses another problem. If a nematode female dies, and if a fungus can be isolated from the cadaver, this is no guarantee that the isolated fungus is a true nematode parasite. Plant pathogens in particular are known to invade and destroy the feeding cells upon which the nematode feeds (Fattah & Webster, 1983; Moussa & Hague, 1988) and so deprive the nematode of nutrients. When the nematode dies the plant pathogenic fungus might well be isolated from the dead female. If selection is aimed at true parasites, organisms which might prevent nematodes from invading the roots, for example mycorrhizae (Marx, 1972) and rhizobacteria (Oostendorp & Sikora, 1989) are left undetected. Whatever the way in which organisms with nematode suppressive properties are selected, it is likely that this

depends on: (a) Skills of the scientist(s) involved.

(b) The special interest of the scientist(s) involved.

(c) The isolation technique(s) used.

(d) Timing and soil samples used.

Because of this, it is important that a secondary screen is devised in which the selected organisms are first mass-produced and then brought back into the soil inoculated with nematodes to prove their ability to suppress nematode populations and reduce plant damage. This stage however, should be less subjective than the previous one. Stirling (1988) lists three criteria which should be satisfied if claims are to be made that an organism has nematicidal properties. These are:

- Assessment of survival of the organism in soil,
- Re-isolation of the organism from the nematode host
- Adequate check treatments.

The first one requires suitable selective media, genetic markers or other isolation techniques which are often not available at this stage of the screening programme. The second one is only of value when the selected organisms are true parasites of nematodes (which is not always the case). The third criterium seems of most value. But even this condition is often not satisfied. From 25 experiments purporting nematode control with the fungus *Paecilomyces lilacinus* (Thom) Samson, 50% failed this criterium (Kerry, 1990). If tests are to be done it is important that something is known about the ecology and epidemiology of the selected organism so that its "window of opportunity" can be defined (Deacon, 1991). In naturally suppressive soils different nematophagous fungi succeed each other (Crump, 1987). If an organism is selected which dominates late in the succession, it is useless to test its efficacy on young plants. The same problem occurs if organisms are tested in *in vitro* systems; these kind of studies ignore the ecological niche in which the organism has to be active and are therefore only of limited value (Deacon, 1991). Ecological understanding is therefore important from an early stage of the screening process, and a division into screening first, and ecological research later is very misleading and can lead to unnecessary failure. Ecological understanding is not only important in defining the ecological niche in which the selected organism might be active, but it is also crucial to satisfy the need to select an organism which is harmless to non target

organisms. If the biology of the organism is well understood predictions can be made about possible negative aspects which can then be further tested.

The need to select an organism with economical potential, is again a fairly grey area which depends on: (a) the economical damage caused by the nematode pests, (b) the availability of cheap alternatives, (c) the effectivity and predictability of the organism selected, (d) economics of large scale production, formulation, storage, application and costs of promotion of the product, and farmer supervision. All of which will determine the market size of the product. Even though nematodes can cause considerable economical losses to farmers (Sasser, 1979) the value of the nematicide market has been estimated at being only about 2.45% of the total pesticide market (Wybou & Homeyer, 1984). To be cost-effective, a biological pesticide against nematodes must therefore not only be effective against one or two of the major nematode pests in a wide range of soil-types, climates and on several major host-crops, but also cheap to produce and require little deviation from standard farm practices. As long as effective nematicides are on the market, it will be difficult to commercially develop biological pesticides like *V. chlamydosporium* which demand careful use. Only if further legislation forces the withdrawal of more nematicides, introduction of biological control agents against nematodes may be an economically feasible option.

This however, does not mean that biological control of nematodes in general is not economical and therefore not worth pursuing. Several components of the normal soil microflora serve naturally to regulate the activities of pathogens, including nematodes (Deacon, 1991; Kerry *et al.*, 1982). This natural control can be enhanced by management practices such as crop rotation, the use of soil amendments (Hoitink & Kuter, 1985), partial soil sterilisation (Munnecke & van Gundy, 1979) or solar heating (Katan, 1980). A thorough understanding of the soil ecology is necessary to devise further cultural practices which enhance soil suppressiveness to soil- borne pathogens and reduce dependency on chemical control. It is hoped that even if *V. chlamydosporium* is not developed commercially, this study will at least have contributed to the general understanding of the soil ecosystem and has highlighted some important factors affecting the development of a biological control agent for root-knot nematodes.

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SUMMARY

A thorough understanding of the interactions which occur between nematode parasites and nematode pests and the influence of biotic and abiotic factors on these interactions, is essential in the development of biological control agents for nematodes. The aim of this study was to develop a particular isolate of the nematophagous fungus *Verticillium chlamydosporium* as a biological control agent for root-knot nematodes. The work has gained insight into some of the key factors which govern the efficacy of the fungus as a biological control agent. The development of a semi-selective medium for *V. chlamydosporium* made it possible to study the growth, survival and spread of this fungus in non-sterile soil and on different parts of the root system as affected by soil-type, fungal density, nematode density, nematode species, temperature and watering.

<u>The *V. chlamydosporium* isolate</u>

The isolate of *V. chlamydosporium* used in these studies was effective against *Meloidogyne incognita, M. javanica, M. arenaria* and *M. hapla*, and had no plant pathogenic characteristics. The fungus can be regarded as a typical egg-parasite, a characteristic which made it unsuitable for preventing initial nematode damage. However, effective population control in excess of 80% gave significant damage control after more than one nematode generation. After one generation of nematodes, population control achieved with the fungus was comparable with a nematicide treatment of aldicarb equivalent to 3.75 kg a.i./ha.

<u>The importance of ecological factors on the efficacy of *V. chlamydosporium*</u>

The efficacy of *V. chlamydosporium* as a biological control agent against root-knot nematodes is governed by four key factors:

(a) Fungal establishment on the rhizoplane.

(b) The proportion of egg-masses of *Meloidogyne* spp. exposed on the rhizoplane.

(c) Rate of fungal growth relative to nematode development.

(d) Spread of the fungus through soil.

Fungal establishment on the rhizoplane: This is dependent on:

1. *Soil type.* Mineral soils support less fungal proliferation than organic ones. This results in a slower build up of fungus on the rhizoplane when plants are grown in mineral soil compared with plants grown in organic soil.
2. *Application rate.* This determines the initial fungal establishment on the rhizoplane. Presumably, because there are more nutrients available for the growth of a small initial fungal inoculum on the rhizoplane than for a large one, initial differences in fungal establishment on the roots tend to disappear after several weeks.
3. *Nematode density/galling.* Root galls induced by *Meloidogyne* spp. stimulate fungal growth on the gall surface.
4. *The host plant. Verticillium chlamydosporium* differs in its ability to colonise the rhizoplane of different host plants, with for example roots from tomato, maize, cabbage and potato plants being readily colonised by the fungus, while roots from pepper, sorghum, soy-bean, pigeon pea and cotton plants are far less readily colonised.

Exposure of egg-masses of *Meloidogyne* on the rhizoplane. This depends on:

1. *Nematode density.* Galling on tomato plants is much more severe when there is a high density of nematodes feeding in the roots, resulting in a proportion of of egg-masses staying embedded in the gall tissue at high nematode densities. These embedded egg-masses are physically protected from fungal attack.
2. *Temperature.* Low temperatures (around 20°C) result in the induction of larger galls compared with those produced at higher temperatures (25°C-30°C). At 20°C, the egg-masses produced by *M. incognita* and *M. javanica* are in general smaller than those produced between 25°C and 30°C. The combination of these effects results in fewer egg-masses being exposed at lower temperatures.
3. *Nematode species.* Galls induced by *M. arenaria* are larger than those induced by *M. incognita* or *M. javanica*. Depending on temperature this means that more egg-masses might stay embedded in gall-tissue when roots are infested

by *M. arenaria* than with infestations of *M. incognita* or *M. javanica*.

4. *Host plant*. There are marked differences in gall-size between different host plants infested with *Meloidogyne*. This undoubtedly has consequences for the proportion of egg-masses being exposed on the root-surface. However, data on this subject are not presented in this thesis.

Rate of fungal growth relative to nematode development. This depends on:

1. *Temperature*. At temperatures below 25°C *V. chlamydosporium* is able to infect eggs before they mature and contain second-stage juveniles. At temperatures above 25°C nematode eggs develop faster than the fungus can infect them. This results in a proportion of eggs developing into fully embryonated eggs and juveniles at those temperatures. Juveniles and fully embryonated eggs are far less susceptible to fungal infections and it can be assumed that they have escaped further fungal attack. At 30°C, this may result in more than 40% of eggs escaping fungal infection.
2. *Aeration*. Aeration influences the rate with which nematodes develop as well as the extend of fungal growth. It seems that, when oxygen is in short supply, the fungal growth rate is slower than nematode development. This factor however needs further investigation.

Spread through soil. Numbers of colony forming units (cfu) can increase rapidly in soil when *V. chlamydosporium* is introduced as a chlamydospore inoculum into non sterile soil. Increase in numbers of cfu in soil is related to the soil temperature, and is most likely explained by the formation of conidia. These conidia can be moved by water percolating through soil. Roots of tomato plants became extensively colonised in this way up to 20 cm away from the original inoculum source, nine weeks after inoculation. This resulted in 100% of exposed egg-masses being colonised by the fungus. However, spread of conidia is dependent on:

1. *Watering*. When the top 10 cm of the soil was inoculated with fungus, conidia spread more rapidly and more evenly when water was added from the top in comparison with watering from below.
2. *Water filled pores*. When surplus water was allowed to drain out of the water

saturated soil, subsequent waterings had no significant effect on further movement of conidia.

3. *Soil type.* This factor needs further investigation, but it is likely that movement of conidia is greater in soils with a coarse soil texture and large pores than in soils with a fine soil texture and narrow pores.

The practical implications of the study

The ecological factors which govern the efficacy of *V. chlamydosporium* as a biological control agent against *Meloidogyne* were studied in the glasshouse. It was encouraging that introduction of *V. chlamydosporium* into field soil in a micro-plot test resulted in more than 90% population reductions of *M. hapla* on tomatoes, showing that data obtained from pot-tests were relevant in the field. The nematicide aldicarb (application rate: 3.75 kg a.i./ha) applied in combination with the fungus had no detrimental effects on fungal establishment in soil or on the root-surface. Fungal efficacy was therefore not influenced by the nematicide, resulting in greater control (98%) when both control measures were combined.

The ecological studies presented in this thesis have practical implications for the use of *V. chlamydosporium* in the control of root-knot nematodes. The insights into the ecology of the soil might also be useful in the development of cultural practices to enhance soil suppressiveness in field soils.

SAMENVATTING

Een goed inzicht in de interacties tussen parasieten van nematoden, plantparasitaire nematoden en de invloed van biotische- en abiotische faktoren op die interacties, is essentiëel voor de ontwikkeling van biologische bestrijdingsmiddelen tegen deze nematoden. Dit proefschrift beschrijft het belang van een aantal bodem-ecologische faktoren voor het gebruik van de nematofage schimmel *Verticillium chlamydosporium* als een biologisch bestrijdingmiddel tegen wortelknobbelalen. De ontwikkeling van een semi-selectief medium voor *V. chlamydosporium* maakte het mogelijk om de groei, overleving en verspreiding van de schimmel in niet steriele grond onder invloed van grondsoort, schimmeldichtheid, nematodendichtheid, nematodesoort, temperatuur en water te bestuderen.

Het *V. chlamydosporium* isolaat

Het *V. chlamydosporium* isolaat dat gebruikt werd in deze studies was effectief tegen *Meloidogyne incognita, M. javanica, M. arenaria* en *M. hapla*. De schimmel moet gezien worden als een typische eiparasiet, en is daarom ongeschikt om initiële nematodeschade te voorkomen. Een nematodebestrijding van meer dan 80% na één nematodegeneratie bleek mogelijk en resulteerde in signifikante schadereductie tijdens volgende nematodegeneraties. De reductie van nematodepopulaties met *V. chlamydosporium* kwam overeen met een nematicide behandeling met aldicarb van 3.75 kg a.i./ha. *Verticillium chlamydosporium* beschermde de plant echter gedurende een lange periode, terwijl het nematicide slechts de eerste nematodegeneratie reduceerde. De schimmel penetreerde niet in de wortelcortex en had geen negatief effect op plantgewichten. *Verticillium chlamyodsporium* is daarom geen plantepathogeen.

Ecologische faktoren die van invloed zijn op de effectiviteit van *V. chlamydosporium*

De effectiviteit van *V. chlamydosporium* als biologisch bestrijdingsmiddel wordt bepaald door vier factoren:

(a) De hoeveelheid schimmel op de wortel.

(b) Het percentage eiproppen dat uit de wortel naar buiten komt.

(c) De verhouding tussen groei en ontwikkelingssnelheid van schimmel en nematode.

(d) De verspreiding van de schimmel door de grond.

De hoeveelheid schimmel op de wortel. Deze wordt bepaald door:

1. *Grondsoort*. De groei van *V. chlamydosporium* is in minerale grondsoorten minder dan in gronden met een hoog percentage organisch materiaal. Dit betekent dat onder experimentele omstandigheden kolonisatie van de wortels in eerstgenoemde gronden langer duurt.
2. *Inoculum dichtheid*. Dit is vooral belangrijk in het begin van het kolonisatieproces. Omdat op het worteloppervlak nutriënten een beperkende factor zijn, worden initiële dichtheidsverschillen op het worteloppervlak volledig opgeheven na ongeveer 7 weken.
3. *Nematodendichtheid/gallen*. De groei van *V.chlamydosporium* wordt sterk gestimuleerd door wortelgallen en eiproppen, gevormd door de nematoden.
4. *De waardplant*. De affiniteit van *V. chlamydosporium* voor verschillende waardplanten verschilt sterk. Het worteloppervlak van bijvoorbeeld tomaten, maïs, kool en aardappel wordt gemakkelijk gekoloniseerd, terwijl wortels van paprika, sorghum, sojaboon, duivenerwt en katoen nauwelijks gekoloniseerd worden door de schimmel.

Het percentage eiproppen aan de buitenkant van de wortel. Dit wordt bepaald door:

1. *Nematodendichtheid*. Galvorming in tomatenwortels is veel sterker wanneer de nematodendichtheid in de wortels hoog is. Het gevolg is, dat een aantal van de gevormde eiproppen in het galweefsel blijft zitten, waardoor deze eiproppen afgeschermd zijn van de schimmel en daardoor niet gekoloniseerd kunnen worden.
2. *Temperatuur*. *Meloidogyne* spp induceren bij lagere temperaturen (rond 20°C) grotere gallen dan bij hogere temperaturen (25°C-30°C). Daarentegen zijn eiproppen die geproduceerd worden bij een temperatuur van 20°C in het algemeen kleiner dan die welke gevormd worden bij temperaturen tussen 25 en 30°C. De combinatie grote gallen en kleine eiproppen betekent dat bij lage

temperaturen een kleiner percentage eiproppen aan het worteloppervlak gevormd wordt dan bij hogere temperaturen.

3. *Nematodesoort*. Gallen die geïnduceerd worden door *M. arenaria* zijn groter dan die, welke geïnduceerd worden door *M. incognita* en *M. javanica*. Dit betekent dat, afhankelijk van de temperatuur, een groter gedeelte van de eiproppen in het galweefsel blijft zitten wanneer wortels aangetast zijn door *M. arenaria*, dan bij aantastingen door *M. incognita* of *M. javanica*.
4. *Waardplant*. Er zijn grote verschillen gevonden in galgrootte bij verschillende waardplanten aangetast door *Meloidogyne* spp. Dit heeft ongetwijfeld gevolgen voor het percentage eiproppen dat aan het worteloppervlak gedeponeerd wordt. Dit aspect verdient nader onderzoek, en is niet opgenomen in dit proefschrift.

Groeisnelheid van de schimmel ten opzichte van de snelheid waarmee de nematode zich ontwikkelt. Deze hangt af van:

1. *Temperatuur*: Bij temperaturen lager dan 25°C is *V. chlamydosporium* in staat om nematode-eieren te infecteren voordat een larve zich kan ontwikkelen. Bij temperaturen hoger dan 25°C echter ontwikkelen de eieren van *M. incognita, M. javanica* en *M. arenaria* zich sneller dan de schimmel ze kan infecteren. Omdat volledig geëmbryoneerde eieren en larven vrijwel immuun zijn voor schimmelinfectie, betekent dit, dat een groot deel van de eieren zich kan ontwikkelen tot larven. Bij 30°C kan meer dan 40% van de eieren in door de schimmel gekoloniseerde eiproppen aan schimmelinfectie ontkomen.
2. *Doorluchting*. Doorluchting heeft zowel een effekt op de snelheid waarmee de nematode zich ontwikkelt als op de groeisnelheid van de schimmel. Het lijkt erop dat, de schimmel sterker benadeeld wordt door zuurstofgebrek dan de nematode. Deze factor verdient nader onderzoek.

Verspreiding van de schimmel door de grond. In niet steriele grond kunnen de aantallen kolonievormende eenheden (cfu) snel toenemen wanneer chlamydosporen van *V. chlamydosporium* in de grond gebracht worden. Deze toename is afhankelijk van de bodemtemperatuur, en kan verklaard worden door de vorming van conidia. Deze conidia kunnen verspreid worden met water dat door de grond sijpelt. Op deze manier konden ook tomatenwortels die 20 cm verwijderd waren van de inoculumbron, negen weken na schimmelinoculatie gekoloniseerd worden. Dit had tot gevolg dat alle

aan het worteloppervlak gevormde eiproppen gekoloniseerd werden door *V. chlamydosporium*. De verspreiding van conidia door de grond hangt af van:

1. *De water beweging.* Na inoculatie van de bovenste 10 cm van de bodem met chlamydosporen, was de verspreiding van conidia door de grond sneller en gelijkmatiger wanneer water toegediend werd van bovenaf dan wanneer water toegediend werd van beneden af.
2. *Water verzadiging van de grond.* Nadat overtollig water is afgevloeid, heeft extra toevoeging van water weinig effekt op de verdere verspreiding van conidia.
3. *Bodemtype.* Het is aannemelijk dat er een betere verspreiding van conidia plaats vindt in gronden met een grove textuur dan in gronden met een fijne textuur. Dit aspect verdient verder onderzoek.

Het praktisch belang van de studie

De ecologische factoren die van invloed zijn op de effectiviteit van *V. chlamydosporium* als een biologisch bestrijdingsmiddel tegen *Meloidogyne* spp. werden bestudeerd in de kas. Na introductie van *V. chlamydosporium* in normale veldgrond in een microplot-experiment, werden populaties van *M. hapla* op tomatenplanten voor 90% bestreden door de schimmel. Dit bevestigde de gegevens die verkregen waren in de potproeven. Een combinatie van het nematicide aldicarb (dosis: 3.75 kg a.i./ha) en *V. chlamydosporium* had geen nadelige gevolgen voor de kolonisatie van de grond en van het worteloppervlak van tomatenplanten door de schimmel. De effectiviteit van de schimmel tegen *M. hapla* werd dan ook niet nadelig beinvloed. Dit betekende dat er een nog betere nematode bestrijding was (98%) bij een gecombineerde behandeling met aldicarb en *V. chlamydosporium*.

De ecologische studies die gepresenteerd worden in dit proefschrift, zijn van praktisch belang voor het gebruik van *V. chlamydosporium* in de bestrijding van wortelknobbelalen. De inzichten in de ecologie van de bodem kunnen wellicht ook van belang zijn voor de ontwikkeling van cultuurmaatregelen die het natuurlijke antagonisme van de bodem tegen ziekten en plagen stimuleren.

CURRICULUM VITAE

Franciscus Antonius Anna Maria de Leij werd geboren op 5 februari 1959 te Geleen. Na het atheneum is hij in 1978 begonnen aan de studie Planteziektenkunde bij de Landbouw Universiteit te Wageningen. In juni 1986 studeerde hij af met het doctoraalpakket entomologie (verzwaard hoofdvak), alternatieve methoden in de land- en tuinbouw (hoofdvak), nematologie (bijvak) en dieroecologie (bijvak). Tevens werd bij het afstuderen de eerstegraads lesbevoegdheid in de biologie verkregen. In oktober 1986 werd hij aangesteld als wetenschappelijk medewerker op het Rothamsted Experimental Station in Harpenden (Engeland) met als onderzoekstaak de ontwikkeling van *V. chlamydosporium* als een biologisch bestrijdingsmiddel tegen cyste- en wortelknobbel-alen. Een gedeelte van dit onderzoek heeft geresulteerd in dit *proefschrift*.

Per 30 september 1991 is hij werkzaam als bodemmicro-oecoloog bij Horticulture Research International te Littlehampten (Engeland).

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