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THE EFFECT OF *VERTICILLIUM CHLAMYDOSPORIUM* AND OXAMYL ON THE CONTROL OF *MELOIDOGYNE JAVANICA* ON TOMATOES GROWN IN A PLASTIC HOUSE IN CRETE, GREECE

by

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Summary. The effect of the fungus *Verticillium chlamydosporium* (applied at the rate of 5,000 chlamydospores per g of soil) alone and in combination with oxamyl was assessed against *Meloidogyne javanica* infecting tomato in a microplot experiment on sandy loam soil in plastic house. The fungus survived in the soil and colonised the roots of tomato but did not reduce root galling, egg production and the remaining egg and juvenile inoculum in the soil after a six month period. A combined treatment with oxamyl did not have any additional effect.

Root-knot nematodes (*Meloidogyne* spp.) are major pests of vegetable production in Crete. Currently, fumigation of soil with methyl bromide and application of organophosphate or carbamate nematicides are the principal methods used for their control. The bacterial parasite *Pasteuria penetrans* has been evaluated in these crops under local conditions in combination with oxamyl, plant resistance or solarization, and has shown potential as a biological control agent (Tzortzakakis and Gowen, 1994). However, *P. penetrans* is very specific and is difficult to mass produce. *Verticillium chlamydosporium* is another parasite of root-knot nematodes with potential as a biological control agent as demonstrated in pot and microplot experiments (Davies *et al.*, 1991; Leij and Kerry, 1991; Leij *et al.*, 1993b).

An evaluation has been made of the potential of *V. chlamydosporium* as a control agent of root-knot nematodes in combination with oxamyl on tomato cultivated in a plastic house, this being typical of vegetable production systems in the Mediterranean region.

Materials and methods

The experiment was conducted at the Plant Protection Institute of Heraklion, Crete. The soil was a loamy sand (59% sand, 16% clay, 25% loam, electrical conductivity=3.3, pH=7.3, $CaCO_3$=44) and was prepared for the experiment by weeding, followed by disc ploughing, harrowing and methyl bromide fumigation (at a dosage of 68 g per m^2). Following fumigation, the soil was disc ploughed again and seedlings were transplanted along the lines of a trickle irrigation system. The 90 m^2 site was divided into five blocks each containing four plots. Each plot measured 1.6 m^2 and consisted of two rows of four plants, 0.8 m between rows and 0.4 m between plants. Adjacent plots were partitioned by 0.8-1 m wide paths.

Seedlings of the dwarf tomato *Lycopersicon esculentum* Mill cv. Tiny Tim were germinated in trays filled with commercial compost and transplanted individually at the two leaf stage into 50 ml plastic sockets in seedling trays filled

with the same compost. Each seedling was inoculated with approximately 300 second stage juveniles of *Meloidogyne javanica* (Treub) Chitw. that had originally been isolated from field soil in Crete. The plants were left in a growth room at 26-32 °C air temperature and 16 hr photoperiod until 42 days when egg masses appeared (30 May - 12 July 1997). They were then transplanted into the plastic house to establish large nematode populations in the soil, typical of those occurring in vegetable production in Crete. After 12 weeks the tops of the plants were removed and roots were left in the soil.

The fungus was mass produced in conical flasks containing a moist autoclaved mixture of coarse sand and milled barley (1:1 v/v). After c. three weeks incubation at 25 °C the cultures were washed through 250 and 53 µm sieves to remove the sand and barley and the fungal propagules were collected on a 10 µm sieve. The residue was further washed to remove conidia and hyphal fragments, leaving mainly chlamydospores. The inoculum was prepared by mixing the washed residue with fine, autoclaved sand and the concentration of chlamydospores was estimated in diluted samples using a haemocytometer (Leij and Kerry, 1991). The production was done at IACR-Rothamsted, UK by Dr J. Bourne using the fungal isolate *V. chlamydosporium* Goddard 10 originated from infected eggs of *M. incognita* (Kofoid *et* White) Chitw.

The inoculum was stored at Rothamsted in a refrigerator for 3-10 days, then transported by plane to Crete and after a further 10 hours at 4 °C was applied to the soil on 30 September. Application was at a rate of 25 g inoculum per 4.5 kg soil (equivalent to 5,000 chlamydospores per g of soil) at each plant site; soil was removed from the plant site, mixed thoroughly with the inoculum in a plastic bag and returned to the planting hole.

To check the viability of the chlamydospores, 1 g of inoculum was diluted in 0.05% agar solution and 0.2 ml of dilutions 10^{-2} and 10^{-3} plated on to 9 cm Petri dishes containing sorbose agar and antibiotics (50 mg per litre of each of streptomycin sulphate, chloramphenicol and chlortetracycline). After two days incubation at 25 °C the percentage of germinated chlamydospores was estimated.

One of four treatments was assigned to each block giving five replicate plots for each treatment in a completely randomised design. The soil was treated with *V. chlamydosporium*, oxamyl, a combination of the two or was untreated. The day after application of the fungus to the soil (1 October 1997) seedlings at the two leaf stage of the susceptible tomato cv. Early Pak were transplanted to each planting site. Some tomato seedlings succumbed to damping-off symptoms and were replaced. A mixture of propamocarb hydrochloride and benomyl was sprayed twice to plant stems and surrounding soil at two week intervals, six and 20 days after transplanting to contain and prevent further spread of the disease.

Oxamyl (Vydate L 24% a.i.) was applied as a soil drench using a watering can four days after planting at a dose rate of 0.6 ml (diluted in 100 ml water) per plant. The soil was lightly irrigated before and after nematicide application.

Fungicides to control powdery mildew, late blight, target spot and grey mold and insecticides against caterpillars were applied as required. At the end of December some plants in block 5 died due to a heavy infestation by *Erwinia carotovora* subsp. *carotovora* (Malathrakis and Goumas, 1987). Diseased plants were destroyed and the others were sprayed to prevent further spread of the disease. As a result there were too few plants in this block for analysis so only root galling was recorded. This block contained one replicate plot of each treatment and was excluded from the analysis.

Maximum and minimum air temperatures were recorded daily during the two crop cycles. Soil temperature was recorded at a depth of 10 cm beneath the canopy at 08.00 and 14.30 hr. Plants were watered and fertilised as required

according to local practice. Fruits were harvested and the yield recorded. After approximately 26 weeks (April 1998) the plants were uprooted, the roots were washed gently in water and blotted dry on tissue paper.

Root galling was estimated using the 0-10 index of Bridge and Page (1980). Six plants were analysed per plot, excluding the plants in the borders. Roots from plants within the same replicate plot were chopped into small pieces, mixed thoroughly and a representative subsample of 20 g was taken randomly. These subsamples were left in water for two days until the tissue had softened, then comminuted in a kitchen blender and washed through 150 µm and 38 µm sieves. Root-knot nematode eggs in the lower sieve were collected in a beaker and the numbers counted using a stereoscopic microscope. To estimate the number of nematodes remaining in the soil, 1.5 l pots were filled with soil (one pot per plot) and planted with a tomato seedling. The pots were maintained for 24 days at 25 °C and 16 hr photoperiod and after which the roots were washed and root galling estimated.

To prove the absence of any residual population of *Pasteuria penetrans* in the soil from a previous experiment several females were collected from the macerated root material and checked in the microscope for the presence of spores. Also, soil samples were air dried, rehydrated and inoculated with juveniles. After 24 hr incubation, they were placed in modified Baermann funnels and juveniles migrating in clean water were checked for attached spores.

Six weeks after planting, soil samples were collected from around the plant roots using a soil corer and combined in two groups: with or without *V. chlamydosporium*. The soil was then mixed thoroughly and a representative 1 g subsample from each was diluted (10^{-2} and 10^{-3} in 0.05% agar solution) and plated on to a semi-selective medium containing 37.5 mg each of carbendazim and thiabendazole, 75 mg rose bengal, 50 mg each of streptomycin sulphate, chlortetracycline, and chloramphenicol, 3 ml Triton X100, 17 g corn meal agar and 17.5 g NaCl in a litre of distilled water. After two weeks incubation at 25 °C the number of colonies of *V. chlamydosporium* on the plates were counted (Leij and Kerry, 1991).

After uprooting, a 1 g representative subsample from chopped roots was crushed in 9 ml 0.05% agar solution with a sterile pestle and mortar and a dilution series (10^{-2} to 10^{-3}) of the resulting suspension was made and 0.2 ml of each dilution plated on to 9 cm Petri dishes containing a semi-selective medium to estimate the amount of fungus colonising the rhizosphere. Also, soil samples were collected from each plot at 15-20 cm depth and soil colonisation by *V. chlamydosporium* was estimated in representative samples of 1 g as described before (Leij and Kerry, 1991).

To confirm the identity of the fungus, representative colonies were transferred on to Petri dishes containing either corn meal agar or water agar plus antibiotics (50 mg per litre of each of streptomycin sulphate, chloramphenicol and chlortetracycline). After two and one week incubation periods, respectively, the Petri dishes were checked for chlamydospore production.

Results and discussion

Temperature records are presented in Table I. Taking account of the date of planting (1 October), considered to be the first day of juvenile invasion, T basic for *M. javanica* approximately 13 °C and the minimum thermal time requirement (K) for one generation as 343 °C (Trudgill, 1995; Tzortzakakis and Trudgill, 1996) it can be assumed that mid November is the minimum time required for completion of the first generation. Based on accumulation of degree days it is estimated that the emergence of juveniles laid by the second generation females would occur at the beginning of April. As the average soil temperature was based on two daily records only and data were not recorded during some

of the weekends the above estimates can not be considered to be precise; however, they give an indication that at the week of harvesting (middle of April) eggs recovered from the roots represented mainly those laid by females of the second generation and probably a percentage of these produced by late females of the first generation.

Chlamydospore germination from the inoculum incorporated into the soil at the beginning of the experiment was greater than 80% and thus considered viable. However, fungal establishment in the soil was relatively poor. Six weeks after transplanting the number of colony forming units (cfu) per g of dry soil was approximately 4,500 and this did not increase significantly during the course of the experiment. At harvesting, the average cfu per g of soil in plots treated with *V. chlamydosporium* was 1-1.5 times larger than the initial application rate of chlamydospores. Although the fungus survived, conditions did not allow its multiplication as has previously been shown for loamy sand and sandy soils (Leij *et al.*, 1993b).

The fungus was not recovered from the soil of the untreated plots. Culturing techniques implemented during the crop cycles aimed to minimise soil spread between plots and since movement of the fungus due to watering is limited (Leij *et al.*, 1993a) no cross-contamination occurred. This was also confirmed by the absence of fungal colonization from roots of untreated plots (Table III). Colonization of roots by *V. chlamydosporium* showed marked differ-

TABLE I - *Average minimum and maximum air and average soil temperature (°C) at 10 cm depth recorded at 08.00 and 14.30 hr.*

Month	Max. air temperature	Min. air temperature	Soil temperature
July	41.5	21.1	29.8
August	40.5	20.8	30.5
September	37	17.3	26.3
October	33.4	12.9	22.8
November	27	11.1	18.2
December	25.7	10	15.6
January	26	6.7	13.2
February	25	8.4	15
March	29.4	7.2	16.8
April	37.8	11.9	22

TABLE III - *Colony forming units of* Verticillium chlamydosporium (V.c.) *per g of rhizosphere and dry soil and root galling index in final bioassay.*

Treatment	cfu g^{-1} soil x 1000	cfu g^{-1} root x 1000	Root galling
Nematodes only	0	0	4
Nematodes + oxamyl	0	0	4.3
Nematodes + *V. c.*	9.5±6.2	76.9±53.1	3.8
Nematodes + *V. c.* + oxamyl	3.5±4.4	18±22.2	4.3
SED	(0.72)	(1.14)	0.36

± standard deviation; ( ) ANOVA on ln transformed data.

TABLE II- *Plant yields and population densities of* Meloidogyne javanica *(1 October - 25 April).*

Treatment	Yield kg $plant^{-1}$	No of fruits $plant^{-1}$	Root galling	No of eggs g^{-1} root (x 1000)
Nematodes only	2.2	12	6.7	6.825
Nematodes + oxamyl	2.6	14	6.8	4.950
Nematodes + *V. chlamydosporium*	2.4	13	6.2	4.830
Nematodes + *V. c.* + oxamyl	2.4	13	6.8	5.200
SED	0.35	1.50	0.26	1.820

Average of four replicate microplots per treatment with an assessment of six plants in each.

ences between replicated blocks as has also been observed in pot experiments (Kerry and Bourne, 1996) and analysis of the log transformed data did not reveal significant differences between the two treatments (Table III). Oxamyl did not affect fungal establishment in rhizosphere, as similarly demonstrated with aldicarb (Leij *et al.*, 1993b). It is not known whether the fungicide application for preventing damping-off symptoms had any significant effect on fungal growth and establishment.

Yields were not significantly different between treatments. Most of the plants in block 5 died due to bacterial soft rot and surviving plants (3-4 per plot) were uprooted and assessed only for root galling. The trend was similar to that presented in Table II. Oxamyl and *V. chlamydosporium* alone and in combination did not have a significant effect in reducing either root galling or number of eggs per g of root (Table II). As root weight did not differ between treatments it can be assumed that the total number of eggs per root system and therefore the residual egg and juvenile inoculum in the soil would be similar. This was proved by the root galling of bioassay plants (Table III).

The apparent inability of oxamyl to reduce the nematode population could be attributed to the single application. Probably a second application would attain better control as is recommended commercially. However, the aim of the treatment in the present experiment was to assess its synergistic effect with *V. chlamydosporium*. Ideally a combination of a non-fumigant nematicide preventing juvenile invasion, together with *V. chlamydosporium* which destroys eggs produced by surviving females, would give long term control of root-knot nematodes.

The fungal isolate used in this experiment had been found effective in the control of root knot nematodes in pots and microplots (Leij and Kerry, 1991; Leij *et al.*, 1992 and 1993b). However, it was not found effective in the control of *M. javanica* even with oxamyl application in a crop of tomato grown in a plastic house in Crete. While incompatibility of the fungal isolate with the tested nematode is unlikely, the lack of nematode control could be attributed to the large nematode densities. On infected roots where two generations had developed galls were large with all egg masses remaining inside the gall, unlike experiments done in protected glasshouse conditions in the UK. In the last case egg masses were mostly exposed on the root surface and so colonized efficiently by the fungus (Kerry and Bourne, 1996).

Interest in the use of *V. chlamydosporium* will increase in the future if the use of chemical soil treatments are restricted or banned. Whitehead (1998) suggested that 99.9% control of root-knot nematodes is required in order to prevent the subsequent build-up of damaging populations. It is clear from the data presented here that *V. chlamydosporium* does not achieve this level of control when susceptible host plants favourable to nematode reproduction in subtropical conditions are grown. A partially resistant plant or a poor host supporting low nematode reproduction such that egg masses will be exposed to *V. chlamydosporium* in the rhizosphere will be a key factor in development of integrated control strategies (Kerry, 1995; Bourne *et al.*, 1996).

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