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Interactions between *Fusarium oxysporum* f. sp. *tracheiphilum* and *Meloidogyne* spp. in *Vigna unguiculata*. 3. Pathogenesis by *F. o. tracheiphilum* as affected by *M. javanica* and host cultivar

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Experiments were conducted to determine the temporal and spatial effects of *Meloidogyne javanica* and host cultivar on pathogenesis by *Fusarium oxysporum* f. sp. *tracheiphilum* in cowpea. In the wilt-susceptible cowpea cultivar California Blackeye No. 5 (CB5), *F. o. tracheiphilum* proliferated rapidly in both the hypocotyl and first internode 6 weeks after inoculation. The fungus spread quickly upward as plants grew, colonized most tissues within 6 weeks, and caused severe wilt. In wilt-resistant cultivar CB3, there was little proliferation of *F. oxysporum* in any tissue, whether or not plants were infected by *M. javanica*. The fungus was found above the primary internode in 15% of all CB3 plants, but did not continue to spread upward after 4 weeks. Vascular discoloration was greatest when *M. javanica* was added 4 weeks before *F. o. tracheiphilum*, but simultaneous inoculation also increased wilt symptoms. Root wounding did not increase wilt. Split-root experiments provided no evidence that infection by *M. javanica* results in a translocatable factor that reduces wilt resistance. When hypocotyls were inoculated with *F. o. tracheiphilum* at different intervals after roots infected by *M. javanica* were removed, there was no evidence that the effect of the nematode on wilt susceptibility was translocated or persistent.

INTRODUCTION

A review of studies of infection and pathogenesis by *Fusarium oxysporum* in a variety of crops led MacHardy & Beckman (1981) to conclude that this fungus may be equally able to penetrate roots of resistant and susceptible plants. In resistant and non-host plants, however, *F. oxysporum* is restricted to the roots and lower stem. Systemic invasion of susceptible hosts by *F. oxysporum* occurs when host resistance responses are delayed or otherwise altered, allowing the pathogen to spread upward to the hypocotyl. Symptom expression results from plant–fungus interactions that occur once the pathogen has invaded the upper stem and petioles. Wilt develops as a response to severe internal water stress following vascular plugging, especially within the petioles where xylem resistance is normally much higher than in the rest of the plant (Duniway, 1971). Gels, gums, tyloses and vessel collapse all have been implicated in interference with water flow.

Anatomical studies of cowpeas (*Vigna unguiculata*) infected by *F. oxysporum* f. sp. *tracheiphilum* provided evidence to support the above hypotheses (Duniway, 1971; MacHardy & Beckman, 1981) concerning the penetration and spread of *F. oxysporum* in resistant and susceptible plants. A wilt-resistant cowpea cultivar produced more tyloses and consequential xylem occlusions after inoculation with *F. o. tracheiphilum* than a wilt-susceptible cultivar (Forrest, 1971). *Fusarium* increased within the tracheary elements of roots of the resistant plants for 8 days after inoculation, but declined and degenerated thereafter.

For many years, it was hypothesized that nematodes increased the severity of certain fungal diseases merely by wounding the roots during feeding, thus leaving an entry site for fungi (Hunger, 1901; Kawamura & Hirano, 1968; Shepherd & Huck, 1989). Other researchers suggested that nematodes play a more active and complicated role, at least in some disease complexes (Linford, 1931; Holdeman & Graham, 1954). Jenkins & Coursen (1957) showed that *Meloidogyne incognita acrita* and *M. hapla* increased and hastened Fusarium wilt in two wilt-resistant tomato cultivars, but repeated artificial root wounding after inoculation with *F. oxysporum* had no effect on host susceptibility to the fungus.

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Several researchers have attempted to determine whether the enhancement by *Meloidogyne* of plant susceptibility to *F. oxysporum* is a localized or a systemic effect. Resistant tomato plants in split-root containers exhibited wilt symptoms only when inoculated with both *M. incognita* and *F. oxysporum*, whether the fungus and nematode were together or separated (Bowman & Bloom, 1966). Bridging and grafting experiments (Sidhu & Webster, 1977) led to the hypothesis that a factor emanating from the interaction of *M. incognita* and *F. oxysporum* was translocated considerable distances to the upper foliage across a resistant scion. In cotton, however, *M. incognita* increased wilt incidence only in plants in which *F. o. vasinfectum* was either on the same part of the root system as the nematodes, or was inoculated into the hypocotyl (Hillocks, 1986). In a variation of the split-root technique with cowpea cultivar California Blackeye No. 3 (CB3), Swanson (1984) found that vascular discoloration at the cotyledonary node was more extensive in plants inoculated with both *F. o. tracheiphilum* and *M. javanica* than in plants inoculated with the fungus alone. The increase in vascular discoloration caused by the addition of *M. javanica* was greatest if both the nematode and the fungus were inoculated onto the same region of the root system.

Also there appears to be an optimum predisposition period by *Meloidogyne*. Fusarium wilt was shown to be much more severe when either *M. incognita*, *M. arenaria* or *M. javanica* were inoculated into wilt-resistant or susceptible tobacco 2 or 4 weeks before *F. o. nicotianae*, than when both nematode and fungus were added simultaneously, or when roots were wounded before adding the fungus (Porter & Powell, 1967).

In cowpea, Thomason *et al.* (1959) first reported that *M. javanica* increased xylem necrosis in stems of the wilt-tolerant cultivar Grant and the wilt-susceptible Chino 3 when grown in pots of loam infested with *F. o. tracheiphilum*. Swanson (1984) reported that *M. javanica* also reduced resistance of cultivars CB3 and California Blackeye No. 5 (CB5) to *F. o. tracheiphilum*. The nematode-enhanced wilt susceptibility was inoculum density-dependent (Harris, 1989; Harris & Ferris, 1988a, 1991a) and specific for certain nematode isolates and cowpea cultivars (Harris, 1989; Harris & Ferris, 1988a, b, 1991b).

The following studies were conducted to determine the temporal and spatial effects of *M. javanica* and host cultivar on pathogenesis by *F. o. tracheiphilum* in cowpeas. In particular, sequential inoculation, root wounding, dispersion and proliferation in the wilt-resistant cowpea cultivar CB3 were studied in an attempt to determine how *M. javanica* makes the plants more susceptible to the fungus (Swanson, 1984; Harris & Ferris, 1991a, b). Systemic spread and proliferation also were studied in the wilt-susceptible cultivar CB5 for comparison with CB3. Both cultivars are susceptible to *M. javanica* (Swanson, 1984).

Objectives were first to determine the effects of nematode–fungus inoculation intervals and root wounding on the incidence and severity of wilt. The following model was then proposed for the interaction between *M. javanica* and *F. o. tracheiphilum*: infection of Fusarium wilt-resistant cowpeas by *M. javanica* results in production or release of a factor(s) that is translocated upward and inhibits, delays, or reverses the formation of xylem occlusions in the hypocotyl region. This allows rapid spread of *F. o. tracheiphilum* up the stem and subsequent systemic invasion. Three hypotheses (stated under subheadings in Materials and Methods) derived from this model were tested. Subsequently, an alternative hypothesis was proposed and tested: infection of roots of wilt-resistant cowpeas by *M. javanica* facilitates proliferation of *F. o. tracheiphilum* in the roots and hypocotyl, but not in the lower stem.

MATERIALS AND METHODS

The following materials and methods were common to all experiments. Seeds of Fusarium wilt-resistant cowpea cultivar CB3 and wilt-susceptible cultivar CB5 were surface-disinfested and pre-germinated (Harris & Ferris, 1991a). Seedlings then were sown in aerated-steam-treated coarse sand in polystyrene cups, one plant per cup and 15 plants per treatment. Except where specified, cups were then placed in plastic pots in either constant-temperature water baths or environmental growth chambers (air baths) that maintained the soil temperature at 27 ± 3°C. Treatments in all but two experiments were arranged in randomized complete block designs with constant-temperature baths as blocks. All experiments were conducted in glasshouses (24 ± 5°C) at the University of California, Davis. Each plant was fertilized regularly with half-strength Hoagland's solution (Hoagland & Arnon, 1950) and sprayed for mites and insect pests as required. Where possible, attempts were made to imitate natural infection processes by using realistic inoculum levels, inoculating with chlamydo-

spores of *Fusarium*, and minimizing root damage, as described previously (Harris & Ferris, 1991a).

The isolate of *F. o. tracheiphilum* used in all experiments (160/s128) was race 3 (Harris, 1989; Harris & Ferris, 1991a). Chlamydospores were produced on straw (Harris & Ferris, 1991a), which then was milled (Wiley mill, 20 mesh [0·84-mm] or 60 mesh [0·25-mm] screen), and inoculum was quantified by dilution plating (Harris & Ferris, 1991a). Unless otherwise specified, dried chlamydospore-infested straw was mixed with aerated-steam-treated sand in a sterilized concrete mixer to give a density of 2×10^4 colony-forming units (c.f.u.) per cm^3 of sand.

Nematodes were obtained from single-egg-mass cultures of *M. javanica* (isolate J-7c-54) on tomato (*Lycopersicon esculentum* 'UC82'). This virulent nematode isolate was used in previous interaction experiments (Thomason *et al.*, 1959; Swanson, 1984; Harris, 1989; Harris & Ferris, 1991a, b). Infective juveniles were obtained by placing roots in a Seinhorst mist apparatus (Seinhorst, 1950) and collecting hatched juveniles at 8-h intervals (Harris & Ferris, 1991a). Eggs were extracted from roots by the bleach technique (Hussey & Barker, 1973). To determine viability, a sample of eggs was placed on a 500 mesh (0·026-mm) sieve in tap water in a glass dish, and hatched juveniles were collected and counted every few days.

Effect of nematode–fungus inoculation interval

The following experiment was designed to test the hypothesis that the time interval between inoculation with *M. javanica* and subsequent inoculation with *F. o. tracheiphilum* affects the incidence and severity of wilt. To determine the optimum nematode–fungus inoculation interval, germinating seeds of cowpea cultivar CB3 were planted in 970-cm^3 polystyrene cups. These cups were immersed in aerated-steam-treated sand in 20-cm-diameter plastic tubs (two cups per tub) in water baths. Juveniles of *M. javanica* were added at the rate of 1000 nematodes per cup (i.e. plant) either at planting or after 2, 4 or 6 weeks. Nematodes were applied with a canula syringe in two injections of 5 ml at a depth of 3 cm. Six weeks after planting, a suspension of straw milled to 20 mesh (0·84 mm) colonized by chlamydospores of *F. o. tracheiphilum* was injected into the sand. Each cup received eight injections with a 5-ml micropipette at a depth of 5 cm, 1 cm from the edge of the pot. Two control treatments received *F. o. tracheiphilum* but no *M. javanica*, and in one of these, roots were wounded by cutting around the soil in the pot with a knife. The cut was made up to 2 cm from the perimeter to a depth of 8 cm immediately before injecting *F. o. tracheiphilum*. The six treatments were replicated 15 times in a randomized complete block design with split plots, with the position of tubs within water baths as main plots. Subplots were treatments, and cups were completely randomized within tubs. Plants were grown for 40 days after inoculation with *F. o. tracheiphilum* and then were assessed for Fusarium wilt symptoms, according to a disease rating and a vascular discoloration rating of the primary node, as described earlier (Harris & Ferris, 1991a).

Data were subjected to analysis of variance, with a weighted least-squares regression procedure in SAS, as described in Harris & Ferris (1991a). Means were compared by *t*-tests and linear contrasts. Control treatments also were compared separately with the ANOVA procedure in SAS (Statistical Analysis System; SAS Institute Inc., 1985) and *t*-tests.

One stem section was sampled from each block for each treatment, surface-disinfested in 0·5% NaOCl, and placed in petri dishes of PCNB agar. Emergent colonies were examined microscopically, and the observations confirmed the presence of *F. oxysporum* in the stems of diseased plants. Root samples also were collected from each block, then galls were examined microscopically and the presence of root-knot nematodes confirmed.

Effects of root wounding

The effect of root wounding on the incidence and severity of Fusarium wilt was investigated further; care was taken to avoid any disturbance that might cause root wounding in control treatments (no wounding). The objective was to test the hypothesis that the effect of *M. javanica* in increasing wilt is not due merely to root wounds providing entry sites for *F. o. tracheiphilum*. Four treatments were: root wounding alone, *M. javanica* alone, wounding plus *M. javanica*, and neither wounding nor *M. javanica*. Seeds of cultivar CB3 were sown in 460-cm^3 polystyrene cups (one seed per cup) filled with sand infested with chlamydospores of *F. o. tracheiphilum*. After 6 days, seedlings in the two wounding treatments were removed from the sand, their root tips were excised with sterilized scissors, then the seedlings were replanted immediately. In the nematode treatments, 1000 eggs of *M. javanica* (67·7%

hatch) in 5 ml of water were pipetted immediately onto the soil surface around each emerging seedling, followed by 25 ml of deionized water to wash the nematodes into the soil. Cups were maintained on a glasshouse bench in a completely randomized design. Plants were rated for Fusarium wilt symptoms 9 weeks after wounding and/or adding nematodes, and results were subjected to two-way analysis of variance with the general linear models (GLM) procedure in SAS (SAS Institute Inc., 1985). Weighted least-squares regression was used for wilt ratings to minimize the residual sum of squares.

Persistence and dispersion of effects of *M. javanica*

An experiment was designed to test the hypothesis that infection of roots by *M. javanica* causes persistent physiological changes in other plant parts, which increase susceptibility to *F. o. tracheiphilum*. To determine whether effects of *M. javanica* on pathogenesis by *F. o. tracheiphilum* were only temporary and localized, seeds of cultivars CB3 and CB5 were sown in U. C.-type soil mix (1 sand: 1 black peat moss) (Baker, 1957) in 190-cm^3 polystyrene cups. Cups were embedded in sterilized sand in trays until a root emerged from the single drainage hole in the bottom of each cup. Each cup then was placed in a 460-cm^3 polystyrene cup, which was two-thirds full of sterilized sand. The sand in the lower large cups was watered daily with half-strength Hoagland's solution to encourage root growth. After approximately 1 week, 10^5 eggs of *M. javanica* (25·7% hatch) in 5 ml of sterile distilled water were pipetted into a 6-cm-deep hole in the sand in the large cups. Nematodes were allowed to develop to maturity (5 weeks), then the infected part of each root system was excised. The plants were repotted immediately into 530-cm^3 polystyrene cups of U. C.-type soil mix. One set of control plants was left with the nematode-infected roots attached, and the surrounding sand was not disturbed during re-potting. Another two sets of control plants (CB3 and CB5) had 5 ml of distilled water (without nematodes) added to a hole in the sand, and the roots in the lower large cups were excised as described above. Treatments were arranged in a completely randomized design on a glasshouse bench, with 15 replicate plants per treatment.

All plants were inoculated with *F. o. tracheiphilum* by stem inoculation either 1 day after cutting off roots and/or re-potting, or after 2 or 4 weeks. The inoculation method was modified from Hillocks (1986) and consisted of placing three 25-µl drops of conidial suspension (10^6 conidia per ml) onto the hypocotyl of each plant, then puncturing through each drop and through the hypocotyl with a no. 11 size sewing machine needle.

To delay senescence, flowers were removed daily and plants were re-potted into 970-cm^3 polystyrene cups of U. C.-type soil mix 2 weeks after roots were excised. Five weeks after re-potting, controlled-release fertilizer was added to each pot, and pots were watered daily with half-strength Hoagland's solution. Plants were assessed for Fusarium wilt symptoms 11 weeks after fungal inoculation. Results were analysed by both analysis of variance and analysis of covariance, with time as the covariate, using the GLM procedure in SAS (SAS Institute Inc., 1985).

Split-root experiments

A further two experiments were conducted to test the hypothesis that *M. javanica* reduces resistance to *F. o. tracheiphilum* when inoculated to the opposite side of a split-root system. Seeds of cowpea cultivar CB3 were planted in 190-cm^3 polystyrene cups (one seed per cup). The cups previously had two 7-mm-diameter holes punched in the bottom rim and were partially submerged in trays of steam-treated sand. When roots emerged from the two holes, the bottom half of each cup was split with a knife midway between the holes. The split was then divided over the lips of two 460-cm^3 polystyrene cups, which were three-quarters filled with steam-treated sand, and the protruding roots were placed in the sand (Fig. 1). The pair of larger cups was held together firmly within a 15·6-cm-diameter plastic pot inserted into a constant-temperature air bath. The top of each large plastic pot was sealed with a foam ring for temperature insulation.

In the first experiment, eight plastic drinking straws were inserted approximately 5 cm deep into each 460-cm^3 cup for later use as inoculation tubes through which inoculum could be introduced into the root zone without wounding roots. After 10 days, 1000 juveniles of *M. javanica* were added to each cup by injecting 5 ml of nematode suspension into each tube with a canula syringe. Four weeks after inoculation with nematodes, a suspension of cowpea straw, milled to 60 mesh (0·25 mm) and colonized by *F. o. tracheiphilum*, was added to the appropriate treatments by the same method. Control treatments were injected with deionized water instead of nematodes, and/

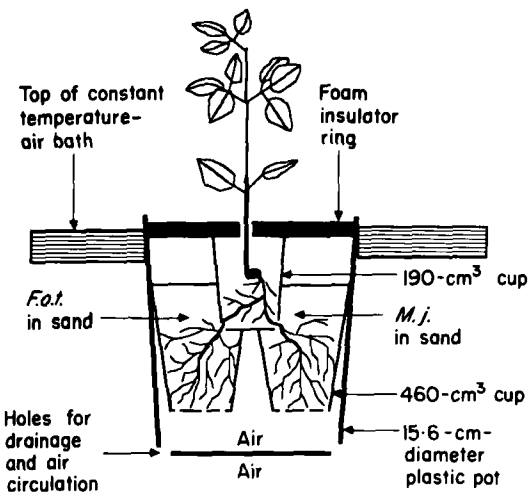

Fig. 1. Diagram showing arrangement of pots in split-root experiments; *F.o.t.* = *Fusarium oxysporum* f. sp. *tracheiphilum*, *M.j.* = *Meloidogyne javanica*.

or sterilized milled straw instead of colonized straw. In the second experiment, the split-root systems were planted directly into sand infested with *F. o. tracheiphilum*, instead of adding the fungus through inoculation tubes. The juveniles of *M. javanica* were pipetted immediately onto the sand above the root zone.

Five treatments were compared: *F. o. tracheiphilum* and *M. javanica* together on the same half of the root system, *F. o. tracheiphilum* and *M. javanica* on separate halves of the root system, *F. o. tracheiphilum* but no *M. javanica*, *M. javanica* but no *F. o. tracheiphilum*, and controls with neither *F. o. tracheiphilum* nor *M. javanica*. After inoculation, each upper (190-cm³) cup was watered with deionized water, but the two lower (460-cm³) cups were watered regularly with half-strength Hoagland's solution to encourage root growth into the infested sand. Plants were grown for 10 weeks after inoculation with *F. o. tracheiphilum*. Each plant was assessed for disease severity and vascular discoloration at the cotyledonary and primary nodes according to two rating systems described earlier (Harris & Ferris, 1991a). In the second experiment, plant stem length, number of seed pods, and number of seeds also were measured.

Because of outliers, results for the first experiment were analysed with a Kruskal Wallis test (Hollander & Wolfe, 1973) to compare all five treatments, then a Mann-Whitney test (Hollander & Wolfe, 1973) to compare each pair of treatments separately. Results for the second experiment were subjected to analysis of variance with a weighted least-squares regression procedure, as described earlier. Treatments were compared by protected least significant difference (Fisher, 1935), and linear contrasts with a Bonferroni adjustment (Neter & Wasserman, 1974).

Roots were examined for galls in both experiments to determine whether any cross-contamination had occurred. Hand sections of root, stem and petiole also were cut from each plant of the three treatments that had been inoculated with *F. o. tracheiphilum*. The sections were surface-disinfested in 0·5% NaOCl and placed on PCNB agar (Nash & Snyder, 1962) in petri dishes. Representative fungal colonies were transferred to petri dishes of yeast-dextrose agar (YDA) medium (Nelson *et al.*, 1986). All cultures were placed at 25 C under cool white fluorescent lights (approximately 1 W m⁻²), with one near-ultraviolet tube, set on a 12-h photoperiod. All colonies on YDA were examined microscopically to confirm the presence of *F. oxysporum* in diseased plants.

Systemic spread of *F. oxysporum*

This experiment was designed to test the hypothesis that *M. javanica* reduces resistance to *F. o. tracheiphilum* by allowing the fungus to become systemic and spread upward rapidly. Fungal spread was compared in cowpea with three putative responses to race 3 of *F. o. tracheiphilum*: susceptible (cultivar CB5), resistant (cultivar CB3), and nematode-predisposed (CB3 + *M. javanica*). Sand infested with chlamydospores of *F. o. tracheiphilum* was placed in 190-cm³ polystyrene cups, then a cowpea seed was sown in each cup. One thousand juveniles of *M. javanica* in 5 ml of water were pipetted immediately into each planting hole for half of the CB3 seeds. Cups were arranged in groups of three, and partially submerged in aerated-steam-treated sand in cm³ plastic tubs. The tubs (experimental units) were replicated five times and immersed in constant-temperature water baths. After 1 week, the roots of 15 plants were removed and stained with acid fuchsin (Hussey, 1985a), and the infecting nematodes were counted to confirm that adequate nematode infection had occurred.

At 2, 4 and 6 weeks after inoculation with *F. o. tracheiphilum*, 15 plants of each putative response group (one tub per water bath) were destructively sampled to determine the upward progress of the fungus. To reduce the risk of cross-contamination, each whole plant first was surface-disinfested in 1% NaOCl. Sections (2–5 mm long) were

cut by hand, in descending order, from the base (proximal end) of each petiole, middle of each stem internode, hypocotyl (middle of zone with chlorophyll), transition zone (middle of white zone between root and hypocotyl), and upper primary root. The scalpel was dipped in ethanol and flamed after cutting each section. Each section was surface-disinfested in 1% NaOCl for 1 min, then rinsed with an antibiotic solution by syringe. The antibiotic solution consisted of 1 l of sterile distilled water plus 50 mg chloramphenicol, 50 mg chlorotetracycline, 0·2 ml pimaricin, and 300 mg vancomycin. Each section was shaken dry, then plated onto PCNB agar in petri dishes and incubated at room temperature for 4 days to determine the presence or absence of *F. oxysporum*. Ends of each section also were rated for vascular discoloration on a scale of 0–5 (Harris & Ferris, 1991a). Representative fungal colonies were sub-cultured onto carnation leaf-piece agar (CLA) (Fisher *et al.*, 1982) or YDA medium and placed at 25 C under cool white fluorescent lights, with one near-ultraviolet tube, set on a 12-h photoperiod. After 3 days, fungal morphology was examined under a compound microscope to confirm that the isolated fungi were *F. oxysporum*.

For each plant, the most distal tissue section from which *F. o. tracheiphilum* was recovered was given a numerical position rating (*F.o.t.* distal position rating) as follows: 0 = no *F. o. tracheiphilum* recovered from any section; 1 = root; 1·5 = transition zone; 2 = hypocotyl; 3 = primary internode (between cotyledonary and first unifoliolate nodes); 3·5 = primary node (first unifoliolate node) petioles; 4 = secondary internode; 4·5 = secondary node petioles; 5 = tertiary internode; and so on.

Data on distal position reached by *F. o. tracheiphilum* in each plant were analysed by analysis of covariance, with time as the covariate, as described earlier. Fungal spread was compared in the three putative response groups by comparing slopes from linear contrasts with Bonferroni adjustments.

Proliferation of *F. oxysporum*

An experiment was conducted to test an alternative hypothesis that infection of roots by *M. javanica* facilitates proliferation of *F. o. tracheiphilum* in the roots and hypocotyl, but not in the lower stem. To compare proliferation of *F. oxysporum* in the lower parts of resistant and susceptible cowpeas, seeds of cultivars CB3 and CB5 were sown in 460-cm³ polystyrene cups filled with sand infested with *F. o. tracheiphilum*. Cups were nested in groups of three in sand in plastic tubs, which were replicated five times and immersed in five constant-temperature water baths (blocks). Ten thousand eggs of *M. javanica* (13·7% hatch) in 5 ml of sterile distilled water were pipetted immediately into each seed-planting hole in the appropriate treatment. The treatments were CB3 with *M. javanica*, CB3 without *M. javanica*, and CB5 without *M. javanica* as a susceptible control.

At 4, 6 and 8 weeks after inoculation, three plants (one tub) of each cultivar–nematode treatment were destructively sampled, washed, bulked, and assessed for root galling. The roots, hypocotyls, and first internodes were excised and separated. The five composite samples of each tissue from each treatment were separately surface-disinfested in 0·5% NaOCl for 1 min, rinsed in sterile distilled water for 1 min, blotted dry, chopped with sterile scissors, then weighed. Each sample then was homogenized for 1 min in sufficient sterile distilled water to make a 1:10 dilution. A dilution series was made in sterile distilled water, and 1 ml of each dilution was pipetted onto each of three replicate petri dishes of PCNB agar. Petri dishes were incubated at room temperature for 4 days, then colonies of *F. oxysporum* were counted. The number of c.f.u. of *F. oxysporum* per gram of tissue (wet weight) was calculated from the dishes with the lowest dilution that yielded about 50 colonies per dish.

Data for each tissue type were analysed separately by analysis of covariance, with time as the covariate, as described earlier. Fungal proliferation in each cultivar–nematode treatment was compared by linear contrasts with Bonferroni adjustments to compare the slopes over time.

RESULTS

Effect of nematode–fungus inoculation interval

The nematode–fungus inoculation interval affected ($P < 0·0001$) the vascular discoloration rating, which was best described by a quadratic model (Fig. 2). A linear contrast showed that treatments inoculated previously with *M. javanica* had higher vascular discoloration ratings ($P < 0·001$) than the control treatment with neither *M. javanica* nor root wounding. Vascular discoloration ratings were not different ($P < 0·05$) between either control (no *M. javanica* with or without root wounding) and the treatment in which *M. javanica* and *F. o. tracheiphilum* were

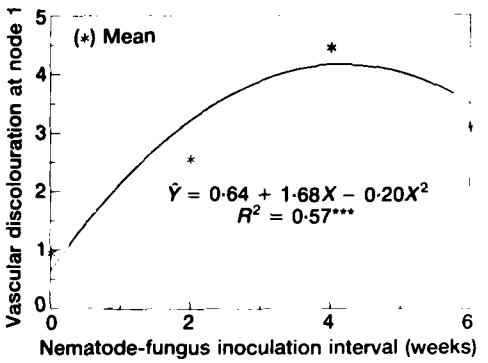

Fig. 2. Effect of nematode–fungus inoculation interval on vascular discoloration at the primary node in cowpea cultivar CB3. (***) Indicates a significant coefficient of determination at $P = 0.001$.

added simultaneously. The root-wounding treatment had a lower vascular discoloration rating ($P < 0.05$) than any other treatment, except that in which plants were inoculated simultaneously with nematodes and fungi. Foliar symptoms of Fusarium wilt were masked on some plants by natural senescence and mite damage, particularly of lower leaves. Therefore, disease rating was not considered a reliable measure of Fusarium wilt in this experiment.

Effects of root wounding

Root wounding had no effect ($P < 0.05$) on the severity or incidence of vascular discoloration at the primary node of cultivar CB3. In contrast, *M. javanica* increased the vascular discoloration rating ($P < 0.01$). The mean ratings were 1·45 for *M. javanica* alone and 1·33 for wounding plus *M. javanica*, and only three plants in each treatment were rated as susceptible (i.e. rating > 2). There was no interaction between wounding and *M. javanica* ($P < 0.05$).

Persistence and dispersion of effects of *M. javanica*

The CB3 treatments did not differ ($P < 0.05$) from each other in vascular discoloration ratings. All CB3 treatments, however, had lower ($P < 0.0001$) vascular discoloration ratings than the wilt-susceptible CB5 control. The mean rating for all CB3 treatments for vascular discoloration at the primary node was 0·63. In contrast, the mean vascular discoloration rating for the CB5 control treatment was 4·33, and all plants showed severe wilt symptoms (10 plants died).

Split-root experiments

In both split-root experiments, there were differences ($P < 0.05$) among treatments in vascular discoloration at the primary node. In the first experiment, the presence or absence of *M. javanica* did not affect vascular discoloration ($P < 0.05$) in the three treatments inoculated with *F. o. tracheiphilum* (Table 1).

In the second experiment, concurrent inoculation with both fungus and nematode resulted in more vascular discoloration at the primary node than the other treatments ($P < 0.05$), which did not differ from each other (Table 1). The treatment with *F. o. tracheiphilum* and *M. javanica* on separate halves of the root system had a lower disease rating than the treatment with both organisms together, and a higher disease rating than any of the other three treatments ($P < 0.05$). When groups of treatments were compared by linear contrasts with a Bonferroni adjustment, the three treatments inoculated with *M. javanica* had smaller stem length ($P < 0.01$), pod number, and seed number ($P < 0.001$) than the two treatments with no *M. javanica*. In contrast, the three treatments inoculated with *F. o. tracheiphilum* were not different ($P < 0.05$) in these plant growth parameters from the two control treatments.

Microscopic examination of the fungi isolated on PCNB agar confirmed that the inoculated plants were infected by *F. oxysporum*. Root galling indicated that inoculated parts of root systems were infected by root-knot nematodes, with very little cross-contamination of uninoculated roots.

Systemic spread of *F. oxysporum*

The slope of the fungus progress line for the 'nematode-predisposed' plants (CB3 + *M. javanica*) did not differ ($P < 0.05$) from the slope for CB3 plants without nematodes (Fig. 3a). Each of the CB3 treatments gave slopes that were different ($P < 0.001$) from the slope for CB5 (susceptible to *F. o. tracheiphilum*). *F. oxysporum* was recovered from near the tops of CB5 plants, especially 6 weeks after inoculation. In CB3 plants, *F. oxysporum* was found above the cotyledonary node in only 27 of 80 plants. Only CB5 plants showed typical Fusarium wilt symptoms.

Vascular discoloration was closely associated with the presence of *F. oxysporum* in both resistant and susceptible plants, except that the fungus was not isolated from over one-quarter of the root sections that showed vascular discoloration. *F. oxysporum* occasionally was recovered

Table 1. Mean vascular discoloration, disease ratings, and growth of cowpea cultivar CB3 10 weeks after inoculation with *Meloidogyne javanica* (*M.j.*) and/or *Fusarium oxysporum* f. sp. *tracheiphilum* (*F.o.t.*) on the same or opposite halves of split-root systems

Treatment	Experiment 1	Experiment 2				
	Vascular discoloration at node 1	Vascular discoloration at node 1	Disease rating	Stem length (cm)	Number of pods	Number of seeds
1. *F.o.t./M.j.* separate	2·33[a]	0·45	1·40	21·5	4·6	19·4
2. *F.o.t./M.j.* together	1·39	2·68	1·96	34·0	4·9	21·4
3. *F.o.t.*/no *M.j.*	1·33	0·05	0·14	54·2	5·7	30·7
4. *M.j.*/no *F.o.t.*	0·89	0·00	0·07	37·4	4·9	22·6
5. No *F.o.t.*/no *M.j.*	0·56	0·00	0·05	54·8	6·5	29·9
PLSD ($P<0·05$)		0·49	0·40	25·3	1·0	4·4

[a] In experiment 1 only, treatment 5 (control) was less than either treatments 1 ($P<0·01$) or 3 ($P<0·05$); treatment 4 was less ($P<0·05$) than treatment 1 (Kruskal-Wallis test).

from tissues that showed no vascular discoloration, particularly between the transition zone and the secondary internode. Approximately 20% of the added *M. javanica* infected the roots, despite the fact that infective juvenile nematodes had to survive up to 1 week from extraction until roots were available for infection.

Proliferation of *F. oxysporum*

In both the hypocotyl and first internode, the concentration of propagules of *F. oxysporum* increased over time in CB5 plants (Fig. 3b, c). The slope of the line for concentration of *Fusarium* was greater for CB5 than for CB3 ($P<0·05$), either with or without *M. javanica*, but the two CB3 treatments did not differ from each other. In the roots, however, there were no significant differences among treatments.

DISCUSSION

Fusarium wilt symptoms in wilt-resistant CB3 were more severe when *M. javanica* was injected into soil 4 (or 6) weeks before chlamydospores of *F. o. tracheiphilum* than when both organisms were added simultaneously. Addition of *M. javanica* at the same time as *F. o. tracheiphilum*, however, did increase disease incidence and severity. Similar results were reported for tobacco inoculated with other species of *Meloidogyne* (Porter & Powell, 1967). Plants in all our treatments probably suffered some root wounding during injection of inoculum into the soil, but additional severe root wounding did not increase the Fusarium wilt ratings. In the experiment on the effects of root wounding we confirmed that *M. javanica*, but not root wounding, increased Fusarium wilt symptoms. *M. javanica*, however, does not make most plants completely susceptible. These results lead us to support the hypothesis that *M. javanica* increases Fusarium wilt of cowpea by a mechanism(s) other than merely wounding roots and providing entry sites for the fungus. The nematode apparently must induce time-dependent changes in the cowpea before *Fusarium* can cause additional symptoms in a resistant plant. The optimum inoculation interval for Fusarium wilt development that we observed was consistent with the period required for root-knot nematodes to induce maximum physiological and histological changes in leguminous plants (Rushdi *et al.*, 1980). Whether the nematode–fungus inoculation interval affected symptom development time was not determined. Although the nematodes infected plants first, the nematode and fungus infections were concurrent from 6 weeks after planting until the end of our experiment.

Severe infection of CB3 roots by *M. javanica* for 5 weeks, followed by removal of the infected roots, did not affect the subsequent severity of Fusarium wilt symptoms after hypocotyl inoculation with *F. o. tracheiphilum*. Thus we cannot support the hypothesis that *M. javanica* induces persistent physiological changes that increase susceptibility to *F. o. tracheiphilum* in plant parts other than the galled roots. The split-root experiments also yielded no evidence that the nematode

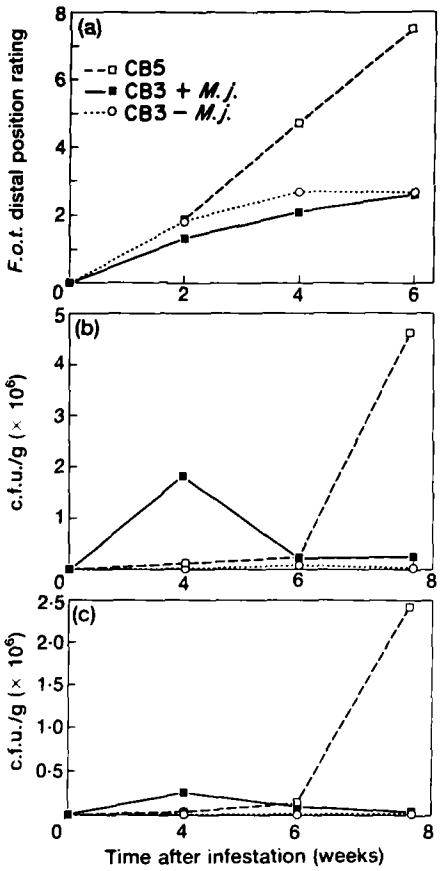

Fig. 3. Response of *Fusarium oxysporum* f. sp. *tracheiphilum* (*F.o.t.*) at various time intervals after infesting soil, in cowpea cultivars CB3 infected (+) or not infected (−) with *Meloidogyne javanica* (*M.j.*), and CB5 without *M. javanica*. Slopes for the two CB3 treatments did not differ ($P < 0.05$). (**a**) Distal position that *F.o.t.* reached. The slope for the CB5 treatment was greater than slopes for either CB3 treatment ($P < 0.001$). (**b**, **c**) Colony-forming units (c.f.u.) of *F.o.t.* recovered from hypocotyl tissue (**b**) and first internode tissue (**c**). The slopes for the CB5 treatments were greater than slopes for either CB3 treatment ($P < 0.05$).

induces a translocatable factor that suppresses resistance. Therefore, it is probable that resistant cowpea must be infected concurrently by the nematode and fungus, as occurs in nature, to increase susceptibility to Fusarium wilt. In contrast, wilt-resistant cotton plants that were stem-inoculated with *F. oxysporum* showed increased wilt severity when previously inoculated with *M. incognita* (Hillocks, 1986). If two pathogens infect a plant simultaneously, its resistance responses to each pathogen are likely to be reduced or delayed because of the increased demand for energy and defence metabolites. In cowpeas, *F. oxysporum* may appropriate the enriched substrate of giant cells of *M. javanica* (Hussey, 1985b) to increase its energy reserves, thereby depriving the plant. The increased energy available to the fungus may enable it to spread further or faster within xylem, thus avoiding the plant's resistance reponses in a few xylem vessels.

The two split-root experiments yielded no evidence that *M. javanica* reduced resistance to *F. o. tracheiphilum* in cowpea cultivar CB3 when inoculated to the opposite side of the root system from the fungus. Cotton inoculated with *M. incognita* at the same dose gave similar results (Hillocks, 1986). The experiment on persistence and dispersion of effects of *M. javanica* also yielded no evidence of a nematode-induced factor that is translocated upward and inhibits resistance to the fungus. Thus, we do not support the hypothesis that infection of cowpea by *Meloidogyne* produces a translocatable factor that suppresses resistance reactions in the stem (Swanson, 1984). A systemic effect of the nematode may be detectable only at artificially high nematode inoculum levels. Swanson inoculated 4.5×10^3 juveniles to each plant in 250-cm^3 cups. In tomato, there was a correlation between initial nematode inoculum level and Fusarium wilt severity in two wilt-resistant cultivars (Sidhu & Webster, 1981). Nematode inocula of up to 1000 per plant had little influence on leaf chlorosis or propagule count of *F. oxysporum*. In other experiments that suggested that species of *Meloidogyne* systemically reduced resistance to Fusarium wilt, each plant was inoculated with 5×10^3 to 9×10^3 nematodes (Bowman & Bloom, 1966; Porter & Powell, 1967; Sidhu & Webster, 1977). These inoculum levels are uncommon in the field (Harris, 1984), thus raising the question of the incidence of predisposition of Fusarium-resistant cowpea by *Meloidogyne* in the field. In our two experiments, few plants developed severe Fusarium wilt symptoms. *M. javanica* was more detrimental than *F. o. tracheiphilum* to growth and production of Fusarium wilt-resistant cultivar CB3.

Infection by *M. javanica* had no effect on upward spread of *F. oxysporum* in the wilt-resistant cultivar CB3, although the nematodes did not significantly reduce resistance to the fungus over 6 weeks. Therefore, no conclusions can be drawn about the mechanism of nematode-induced susceptibility. The failure to demonstrate an interaction may be a consequence of the deliberate choice of a realistic inoculum level of

nematodes, although the same levels caused interactions in eight other experiments (Harris, 1989; Harris & Ferris, 1991a, b). *F. oxysporum* moved much higher and faster in the wilt-susceptible cultivar CB5 than in cultivar CB3. In CB5, fungus spread kept up with plant growth, and, by the time the plants wilted and died, virtually all xylem tissue was invaded. In CB3 plants, *F. oxysporum* was found above the primary internode in only 12 of 80 (15%) plants. These results provided some evidence for us to support some, but not all, of the hypotheses of MacHardy & Beckman (1981). They suggested that multiplication and distribution of *F. oxysporum* in the upper roots and hypocotyl, which have many vascular cross-connections, is crucial to Fusarium wilt development. They further proposed that resistance is an active process in which fungal development and spread is limited by the rapid development of physical barriers in vessels and by the accumulation of antifungal compounds. In our experiment with the resistant cultivar, the fungus was inhibited or at least delayed in upward movement, even after nematodes infected roots. *F. oxysporum* did, however, reach above the cotyledonary node in approximately one-third of the plants. The lack of wilting may have been the result of the plants' resistance mechanisms confining the fungus to either a few vessels, or to the lower stem. We did not determine either the proportion of vessels invaded, or the potential migration of the fungus after 6 weeks. In any case, the plants' resistance mechanisms probably delayed or confined the fungus sufficiently for other resistance mechanisms to become effective. If xylem occlusion is a major resistance mechanism (Forrest, 1971), our experiment provided no evidence that *M. javanica* retards it, at least in the absence of visible nematode-induced susceptibility.

Propagule concentration of *F. o. tracheiphilum* increased in hypocotyls and lower stems of wilt-susceptible CB5 from 4 to 6 weeks after inoculation. In CB3, however, infection by *M. javanica* did not result in increased fungal growth over the same period. Thus, the resistance mechanism(s) in CB3 that suppresses fungal growth apparently is effective even in the presence of the nematode. Forrest (1971) observed disintegrating hyphae in roots of resistant, but not susceptible, cowpea 12 days after inoculation, suggesting toxic host products, accumulated fungal metabolic waste, or fungal starvation. In stems of susceptible cowpea, he observed hyphae and spores 6 days after root inoculation. After 12 days, hyphae had spread and some had reached petioles. However, he did not find hyphae or spores in stems of resistant cowpeas. Growth of *F. oxysporum* from stem sections and stem homogenates of resistant plants in our experiments indicates that viable propagules were present for at least 8 weeks after inoculation. These propagules, however, may not have been sufficiently numerous or mobile to cause the vascular occlusion that leads to wilt (Duniway, 1971). The colonization of only a few xylem vessels in resistant plants would explain the occasional chlorosis and abscission of some leaves and apparent recovery of plants seen in these experiments and in those of Wellman (1939).

Our research, with realistic inoculum densities of one isolate of *F. o. tracheiphilum* and one of *M. javanica* in the wilt-resistant cultivar CB3, generally did not support either of our models for the interaction. Further research is needed to determine the mechanisms of nematode-enhanced Fusarium wilt in CB3 and other cultivars of cowpea. The wilt resistance of CB3 is expressed internally as restricted proliferation and spread of *F. o. tracheiphilum*. In contrast, in wilt-susceptible CB5, the fungus proliferates and becomes systemic within 6 weeks.

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