Parasitism and Biological Control of *Verticillium dahliae* by *Pythium oligandrum*

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ABSTRACT

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Pythium oligandrum was a parasite of *Verticillium dahliae* in dual culture, impeding the latter's ability to grow and form microsclerotia. Variation occurred among isolates of *P. oligandrum* in their parasitism toward isolates of *V. dahliae*, and isolates of *V. dahliae* expressed a range of susceptibilities to parasitism by *P. oligandrum*. Temperature and matric potential significantly affected interactions between these fungi. Populations of *P. oligandrum* in the rhizosphere of pepper (*Capsicum annuum*) were similar in a potting soil infested and noninfested with *V. dahliae*, but the populations of *V. dahliae* in combined samples of rhizosphere and nonrhizosphere soil of pepper were significantly lower in the presence of *P. oligandrum* than in its absence. In greenhouse experiments with pepper grown in soil infested with *V. dahliae*, shoot and fruit weights were significantly higher in the presence of *P. oligandrum* than in its absence. However, when soil was infested only with *P. oligandrum*, fresh weights of shoots and fruits were 40 to 50% higher than when plants were grown in its absence, evidence that this fungus significantly promoted growth of this crop. Therefore, processes responsible for improved plant health associated with soil treatment with *P. oligandrum* could be the result of complex interactions between pathogen, host, and mycoparasite.

Additional keywords: biocontrol, plant growth promotion

The potential of *Pythium oligandrum* Drechs. as a biocontrol agent was demonstrated for damping-off (2,18,26,27,31,43,46) and root diseases (7,45). Because *P. oligandrum* grew in the rhizospheres of a wide range of agriculturally important crops and persisted in the rhizosphere for extended periods of time, it was anticipated that this fungus could be used as a protectant against a range of fungal root invaders (5).

Mycoparasitic interactions of *P. oligandrum* with a wide range of susceptible fungi have been demonstrated, but not with *V. dahliae* (8,11,13,14,22,23,26,44). Mycoparasitism by *P. oligandrum* involves gaining nutrients by enveloping, penetrating, and causing lysis of host hyphae. This fungus does not produce diffusible inhibition compounds but can produce volatile antibiotics on certain media that inhibit growth of certain fungi (9,14,22,23). Fungi have been evaluated for their susceptibility or resistance to attack by *P. oligandrum* by several methods. They include quantitative assessments of growth of *P. oligandrum* over host colonies in culture and hyphal coiling around host hyphae (8,11–13,23), estimates of the re-

duction in cellulolytic activity by host fungi grown on filter paper in the presence of *P. oligandrum* (3,9,11,14,22), determinations of the cytoplasmic disruption rates of susceptible host hyphae upon contact with *P. oligandrum* hyphae (23), and measurements of propagules of *P. oligandrum* produced in the interaction zone (8). All of these interactions were studied on agar, which limits the scope of the investigation and hinders measurements of the interactions. Interactions in a liquid medium could facilitate rapid in vitro screening of susceptible fungal hosts and serve as a means of identifying aggressive isolates of *P. oligandrum*. These mycoparasitic activities normally result initially in vegetative growth by the mycoparasite followed by the production of oogonia and oospores (13). Hence, objective measurements of the intensities of interactions may be assessed by counting propagules of the mycoparasite.

Verticillium wilt is a serious disease of a wide range of annual and perennial field and greenhouse crops with world-wide distribution (30,35). Control measures often include eradicant soil treatments, such as chemical fumigation, steam sterilization, soil solarization, and flooding for the protection of high-value greenhouse and horticultural crops. For many field and vegetable crops, cultural practices and use of resistant cultivars are alternatives to soil eradicants (6,10,19,20,24,35,38). Unfortunately, the nature of pathogenesis by *V. dahliae* makes the prospective use of biological control unattractive because con-

tinuous protection of feeder root apices is necessary to prevent vascular invasion of host plants over most of their vegetative growth period (16,39). However, successful control of Fusarium wilt diseases by use of suppressive soils and avirulent strains of *Fusarium oxysporum* has demonstrated that biologicals have potential for control of diseases with refractory systems of pathogenesis (1). Indeed, stimulation of systemic resistance with biologicals was demonstrated for both vascular wilt and local lesion diseases (32,36,47), and it was recently reported that *P. oligandrum* stimulated defense reactions of tomato roots to infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (7).

The objectives of this study were to investigate (i) the susceptibility of plant pathogenic isolates of *V. dahliae* to mycoparasitism by isolates of *P. oligandrum* in dual culture, (ii) the impacts of temperature and matric potential on these interactions, (iii) whether nutritional competitiveness or direct mycoparasitism governs the nature of these interactions, (iv) the effect of *P. oligandrum* on rhizosphere populations of *V. dahliae*, and (v) the ability of *P. oligandrum* to reduce severity of Verticillium wilt of pepper.

MATERIALS AND METHODS

Inoculum production. Fungal isolates used in this study are listed in Table 1. Stock cultures of *P. oligandrum* were maintained on wheat leaf-water cultures at room temperature ($24 \pm 2^\circ\text{C}$) (40). Stock cultures of *V. dahliae* were stored at 4°C as air-dried microsclerotia that were produced on potato dextrose agar (PDA) overlaid with sterile filter paper (33). After storage, cultures of *P. oligandrum* or *V. dahliae* were grown on water agar (WA, 1.75%), corn meal agar (CMA, 1.7%), or PDA (5.25%, Difco Laboratories, Detroit, MI).

Unless otherwise stated, inoculum of *P. oligandrum* consisted of 4-day-old WA cultures grown in 9-cm-diameter plastic petri plates. Test tubes or plates containing 10 ml of liquid or solid media were inoculated with a 10-mm-diameter disk of inoculum. *V. dahliae* inoculum was prepared by culturing the stored microsclerotia (MS) on PDA for 1 week before the plates were flooded with 10 ml of sterile distilled H_2O , and the conidial suspension was used to inoculate WA plates.

Large quantities of *P. oligandrum* oospores were obtained by growing each isolate in 10 ml of a synthetic liquid medium (MMPO) in petri dishes for 5 weeks

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at room temperature ($24 \pm 2^\circ\text{C}$) (4). At harvest, mycelial mats were aseptically transferred into sterile glass test tubes (25×200 mm) containing 10 ml of sterile distilled, deionized H_2O . Contents of tubes were sonicated for 80 s continuously in an ice bath at 50% duty cycle (Branson Sonifier, Cell Disruptor 185, Smith-Kline, Danbury, CT). This treatment lysed about 99% of the mycelium without causing obvious mechanical damage to oospores. Oospores were then washed with sterile distilled H_2O by centrifugation at $3,000 \times g$ for 10 min. The process was repeated three times or until all mycelial debris had been removed. Concentrated oospore suspensions of each isolate were stored separately at 4°C and then formulated into calcium alginate pellets (4). The dry pellets were stored in the dark at 4°C .

Large quantities of MS were obtained by growing *V. dahliae* on minimal agar medium (MAM) (37). Cellophane membranes that had been autoclaved for 15 min at 121°C and 0.13 MPa were laid on the surface of the agar and inoculated with conidial suspensions harvested from 1-week-old cultures grown on PDA. Inoculated plates were sealed in plastic bags and incubated in the dark at 24°C for 18 days. At harvest, the cellophane membranes were covered with MS, which were scraped off using a scalpel and suspended in sterile water. Each MS suspension was blended in a Waring blender for 1 min at high speed, and the densities of MS were determined with the aid of a hemacytometer.

Fungal interactions. Preliminary trials indicated that isolates of *V. dahliae* were susceptible to parasitism by *P. oligandrum* when they were co-cultured on PDA, potato carrot agar (PCA; 42), or MAM. This interaction was studied in 20% potato carrot broth (PCB) in test tubes. To minimize nutritional variability among experiments, the PCB was frozen and stored as concentrated filtrate at -20°C until use.

The interactions of five isolates of *V. dahliae* (Vd-1 to Vd-5; Table 1) were tested against five isolates of *P. oligandrum* (76-13 to 91-4; Table 1). The experiment was arranged in a 6×6 factorial design, testing all possible combinations including controls where isolates of both fungi were grown separately. For each treatment, four test tubes (replicates) were inoculated with agar disks as outlined above, and tubes were arranged in a completely randomized design in test tube racks. Racks of filled tubes were sealed in plastic bags to prevent evaporation and contamination and incubated at room temperature ($24 \pm 2^\circ\text{C}$) for 14 days. Tube contents were sonicated for 30 s as described previously. To inhibit further growth and interactions of both fungi, and to allow enough time to count oospores, sonicated materials were stored at 2°C . Oospore counts were achieved with the aid of a hemacytometer. To ensure an accurate

estimation of the number of oospores, tube contents were thoroughly vortexed prior to sample removal. When oospore counts exceeded 60 per field, dilutions were made by the addition of 10 ml of sterile water. Conidia formation by *V. dahliae* was negligible in this medium, and therefore conidia were not counted. Sporangial production by isolates of *P. oligandrum* was measured using a method similar to the one described for measuring oospore production. This experiment was performed three times.

Temperature. To assess the impact of incubation temperature on parasitism of *V. dahliae* by *P. oligandrum*, isolates of *V. dahliae* (Vd-1, Vd-7, and Vd-8) were grown in dual culture with *P. oligandrum* isolate 76-13 in PCB. Inoculated test tubes were incubated at 16, 25, or 33°C as four replicates per temperature, fungal interaction, or control treatment. Tubes within a

temperature treatment were arranged in a completely randomized block. After 14 days of incubation, tube contents were sonicated and oospores were counted as outlined above. The experiment was performed three times.

Matric potential. To evaluate the impact of matric potential on mycoparasitism of *V. dahliae* by *P. oligandrum*, PCB was amended with none, 0.133, 0.196, 0.286, or 0.412 g of polyethylene glycol 8000 (PEG) g^{-1} of H_2O , which corresponds to 0, -0.25, -0.5, -1.0, and -2.0 MPa, respectively, at 25°C according to data compiled by Michel (34). Verification of actual matric potential achieved from PEG amendments was determined at the end of experiments using a thermocouple psychrometer (Model SC-10A, Decagon Devices, Inc., Pullman, WA). Tubes were inoculated with isolate Vd-1 of *V. dahliae* and isolate 76-13 of *P. oligandrum* and incubated for 20 days

Table 1. Isolates of fungi used in this investigation and their origins

Fungi	Isolate	Substrate and origin	Source ^z
<i>Pythium oligandrum</i>	76-13	Bean roots, CA	1
<i>P. oligandrum</i>	86-21	Sugar beet, CA	ATCC
<i>P. oligandrum</i>	87-6	Unknown	2
<i>P. oligandrum</i>	87-7	Unknown	3
<i>P. oligandrum</i>	91-4	Tomato soil, CA	1
<i>P. oligandrum</i>	94-1	Tomato rhizosphere, CA	4
<i>P. oligandrum</i>	94-5	Cotton soil, CA	4
<i>P. oligandrum</i>	94-8	Chard soil, CA	4
<i>Verticillium dahliae</i>	Vd-1	Pepper plant, CA	5
<i>V. dahliae</i>	Vd-2	Cotton roots, CA	6
<i>V. dahliae</i>	Vd-3	Eggplant stem, CA	6
<i>V. dahliae</i>	Vd-4	Tomato plant, CA	7
<i>V. dahliae</i>	Vd-5	Watermelon stem, CA	7
<i>V. dahliae</i>	Vd-6	Cauliflower stem, CA	8
<i>V. dahliae</i>	VD-7	Tomato plant, CA	7
<i>V. dahliae</i>	Vd-8	Tomato plant, CA	7

^z 1, J. G. Hancock; 2, M. C. Middleton; 3, H. Shull; 4, A. Al-Rawahi and J. Hancock; 5, B. A. Holtz; 6, J. Liebman; 7, A. Sundwall-Byers and T. Gordon; 8 = F. Cook. All addresses: University of California, Berkeley. 86-21 = ATCC 38472.

Table 2. Interactions of *Pythium oligandrum* with isolates of *Verticillium dahliae* on minimal agar medium

Treatment ^x	Growth rating ^y		Reproduction rating ^z		
	<i>P. oligandrum</i>	<i>V. dahliae</i>	Conidia	Microsclerotia	Oospores
91-4	+				0
Vd-1		+++	NM	10	
91-4 + Vd-1	+++	+	NM	0	10
Vd-2		+++	10	1	
91-4 + Vd-2	+++	++	2	0	10
Vd-3		+++	5	5	
91-4 + Vd-3	+++	+	1	1	9
Vd-4		+++	10	1	
91-4 + Vd-4	+++	+	2	1	8
Vd-5		+++	2	9	
91-4 + Vd-5	+++	+	1	1	6
Vd-6		+++	0	10	
91-4 + Vd-6	+++	+	1	0	10

^x Tests were performed four times in minimal nutrient agar medium at 25°C , and readings were made at 14 days. Mean values are presented.

^y + = poor growth, sparse mycelial growth for *P. oligandrum* and reduced growth with minimal aerial mycelial mats for *V. dahliae*; +++ = normal growth for *V. dahliae* and enhanced growth for *P. oligandrum*.

^z In a linear scale of 0 to 10, 0 = no propagules produced and 10 = maximum number of propagules produced. Maximum number was greater than 300 or 100 per microscopic field at $\times 10$ for microsclerotia and oospores, respectively. NM = not measured.

at 25°C. Tubes were arranged in a completely randomized design using four replicates per treatment in test tube racks. This experiment was performed twice.

Interactions on minimal agar medium. Ten-ml of MAM were dispensed into each petri plate (60 × 15 mm). Six isolates of *V. dahliae* were grown in dual cultures with *P. oligandrum* isolate 91-4, or the fungi were grown separately (Table 2). For each treatment, three plates were inoculated. They were sealed in a plastic bag and incubated at 25°C for 14 days in the dark. At the end of the incubation period, plates were examined microscopically at ×10 and assessed for growth and reproduction. Growth was rated as poor, normal, or enhanced. Poor growth refers to sparse mycelial growth for *P. oligandrum* and reduced growth with minimal aerial mycelial mats for *V. dahliae*. Reproduction was rated on a scale of 0 to 10, where 0 = no propagules produced and 10 = maximum numbers were produced. Maximum number was greater than 300 or 100 per microscopic field at ×10 for microsclerotia and oospores, respectively. This experiment was performed four times.

Interactions on fresh and precolonized minimal agar medium. To determine whether the interactions between *P. oligandrum* and *V. dahliae* were competitive or parasitic, fungi were grown on sterile polycarbonate membranes (PCM; 0.2 µm, 50 mm diameter) placed on the surface of MAM in plates. The membranes were used to permit mycelial growth of the fungi without their establishment on or within the agar medium and to allow removal of the entire colony prior to reinoculation of the same plate. Membrane disks were cut from 0.2-µm membrane Nuclepore (Costar

Corp., Cambridge, MA) sheets and autoclaved. PCM was resistant to biodegradation by these fungi, and its small pores allowed diffusion of metabolites and nutrients. The test involved inoculating fresh MAM plates with either *P. oligandrum* isolate 76-13 or *V. dahliae* isolate Vd-1 or inoculating with both fungi simultaneously. These treatments were designated the fresh medium test.

For the second set of treatments, *P. oligandrum* was inoculated on PCM overlaying MAM, and plates were incubated for 24 h at 25°C in the dark. Then the colony of *P. oligandrum* on PCM was aseptically removed, and these plates were designated as *P. oligandrum* precolonized medium. These plates subsequently were inoculated with *V. dahliae*, *P. oligandrum*, or both fungi and incubated as in the above treatments. In a similar fashion, *V. dahliae* precolonized medium was prepared by allowing *V. dahliae* to grow and form MS on the PCM overlaying MAM for 9 days before the colony was aseptically removed. These plates were then inoculated with *P. oligandrum*, *V. dahliae*, or both fungi and incubated as in the first set of treatments. Upon termination of the test, plates were examined microscopically under ×10, and growth rates and types of propagules were noted. Each test was based on three plates per treatment, and each experiment was performed four times.

Growing conditions. The soilless potting mix used in this study was UC mix (29). Autoclaved UC mix refers to batches that were autoclaved at 121°C and 0.055 MPa for 10 h, cooled for 24 h, and re-autoclaved. Plastic pots (1.5 liter, 15.5 cm diameter, 15 cm deep, containing 1.2 kg of air-dried potting soil, or 10 liter, 32 cm

diameter, 36 cm deep, containing 8.4 kg of air-dried potting soil) were used throughout the study and will be referred to by their volumes.

Pepper (*Capsicum annuum* L. cv. California Wonder) seeds were treated with trisodium phosphate (TSP) solution for 30 min, washed several times with water, and allowed to air-dry. TSP consisted of 10% Na₃PO₄·12H₂O and 0.05% Triton X100 and was used to inactivate seedborne tobacco mosaic virus (TMV). Transplants were grown in vermiculite in Styrofoam trays (20 × 60 cm) divided into 96 cones (3 × 3 × 7 cm). Vermiculite was sterilized by autoclaving. Seeds were sown into cones and thinned after germination to one seedling per cone. When *P. oligandrum* inoculum was delivered with transplants, 1 ml of an oospore suspension (10⁴ oospores ml⁻¹) was added to each cone at sowing time as a mixture of five isolates (76-13, 86-21, 87-6, 87-7, 91-4) in equal proportions. Seedlings were grown under greenhouse conditions for 21 days after germination before they were transplanted into UC mix in plastic pots. Plants were fertilized daily with half-strength Hoagland's solution (15) and were watered twice a day. Greenhouse temperatures ranged between 24 and 28°C, and relative humidities ranged between 75 and 85%.

Biocontrol efficacy trials. Experiments were organized as a split-plot in a randomized complete block design with five replicates per treatment. The main plot treatment was sampling time, and the subplot treatments were a 2 × 2 factorial with and without *P. oligandrum* and with and without *V. dahliae*. Plants were sampled destructively at designated times. The experiment was conducted from November 1993 to March 1994 (trial I) and was repeated from April to July 1994 (trial II) and from October 1994 to January 1995 (trial III). Sampling intervals for dry weight measurements after transplanting were: trial I: 1, 3, 5, 6, and 16 weeks; trial II: 1, 3, 5, 6, and 16 weeks, and trial III: 1, 3, 6, and 13 weeks. Other variables measured in these trials included nonrhizosphere (trial I) or rhizosphere densities (trial II and III) of *P. oligandrum* and densities of *V. dahliae* (trials I, II, and III).

Microsclerotia of *V. dahliae* were prepared as outlined above and mixed with the UC mix prior to transplanting. The UC mix for each pot was infested separately by shaking the MS in a plastic bag with a small volume of UC mix, and this mixture was mixed again in a larger plastic bag with the remaining volume of UC mix. The initial inoculum densities were approximately 60, 40, and 20 MS g⁻¹ in trials I, II, and III, respectively, as measured by the method of Huisman and Ashworth (17). The inoculum of *P. oligandrum* was added either as outlined above or mixed in the top 0.5-cm layer of the potting mix as 0.2 g of oospore-alginate pellets per pot (10 liters

Fig. 1. Oospore production by isolates of *Pythium oligandrum* grown alone (control) or in dual culture with isolates of *Verticillium dahliae* in 20% potato carrot broth for 2 weeks at 25°C. LSD_{0.01} = 0.56 × 10⁴ oospores ml⁻¹ according to the Student-Newman-Keuls test. Vertical bars equal 1 standard error.

of UC mix) during transplanting (4). In trial III, *P. oligandrum* inoculum was added at transplanting and at the end of the third and sixth week after transplanting.

Assays. In trial I, the nonrhizosphere soil (NRS) was assayed for *P. oligandrum*. Three cores (2 cm diameter and 10 cm deep) of rootless soil were collected from each pot and combined after plants were uprooted. The soil sample was pooled for each treatment and assayed. For each treatment, 25 g of soil was suspended in 175 ml of H₂O, allowed to stand for 30 min, and mixed well on a magnetic stirrer for 3 min. Aliquots of 1 ml were plated on the surface of five plates of the *P. oligandrum* semi-selective medium (POSSM) (5). Equivalent soil samples were oven dried at 110°C for 72 h, and soil dry weights were determined.

Rhizosphere populations of *P. oligandrum* were monitored during trials II and III because it was found, in a related investigation, to persist in the rhizospheres of several crop plants (5). The root systems of all plants in each treatment were cut into 3-cm-long root segments. All root segments with adhering soil particles were thoroughly mixed, weighed, and suspended in distilled H₂O to give 1:8 dilution (wt/vol⁻¹) in test tubes and allowed to stand for 30 min. Tubes were vortexed at high speed for 1 min, and 1-ml aliquots of the rhizosphere soil (RS) were plated on the surface of five POSSM plates. Additionally, two 1-ml aliquots of the RS were placed on predried, preweighed filter paper and oven dried at 110°C for 48 h. Average weights of the two were used to determine colony-forming units per g (CFU g⁻¹) of dry rhizosphere soil. All plates were incubated at 33°C for 24 h. Soil residues were washed off media

surfaces, and colonies of *P. oligandrum* were counted.

For estimates of *V. dahliae* populations, the RS and NRS samples were pooled, and 25 g was assayed. Soil samples were wet-sieved serially through 125- and 38-μm pore diameter sieves. The soil residues retained on the 38-μm sieve were treated twice with 0.5% NaOCl for 5 s and rinsed with water according to the method of Huisman and Ashworth (17). This soil residue was suspended in distilled H₂O and distributed evenly on the surfaces of ten plates containing sodium polygalacturonic acid agar (28) and subsequently incubated in the dark at 24°C for 17 days. Soil residues were rinsed off, and colonies of *V. dahliae* were counted with the aid of a dissecting microscope. Equivalent soil samples were oven-dried at 110°C for 72 h, and soil dry weights were determined and used to calculate population densities as CFU g⁻¹.

Plant shoots for each time interval and treatment were dried separately at 60°C for 1 week, and shoot dry weights were determined. At the last sampling in each trial, 12 replicates were used. Fruits from each pepper plant were harvested, and fresh weight was determined.

Isolation of *V. dahliae* was attempted from each plant at the termination of each trial. A 0.5-cm-long section of the basal stem cut 10 cm above the soil line and three 1-cm-long petiole pieces were used for isolation. These pieces were disinfected in 1% NaOCl for 3 min, rinsed three times in sterile water, blotted dry on sterile filter papers, and plated on sodium polygalacturonic acid agar. Plates were incubated in the dark for 14 days at 24°C and examined for colonies of *V. dahliae*.

Statistical analyses. Data from each experiment were tested for homogeneity of variance, and data from repetitive trials were analyzed for variance using the Co-Stat statistical program (CoHort Inc., Berkeley, CA.). The Student-Newman-Keuls test was used for the least significant difference (LSD) determination (41). Unless indicated otherwise, data presented are from combined experiments.

RESULTS

Isolate interactions. Isolates of *V. dahliae* grew well and produced abundant conidia ranging between 0.5 and 2.0 × 10⁶ conidia per tube (10 ml of 20% PCB) when grown alone, but they did not form microsclerotia (MS) in this medium. *P. oligandrum* isolates grew sparsely and produced sporangia and limited numbers of oogonia and oospores when grown alone. However, in the dual culture system, the numbers of oospores were increased 6- to 18-fold over the number observed when *P. oligandrum* isolates were inoculated alone (Fig. 1). Isolates 76-13, 91-4, and 87-6 were significantly ($P < 0.001$) more aggressive than isolates 87-7 and 86-21 in their attack of all isolates of *V. dahliae* based on oospores produced. Among isolates of *V. dahliae*, the tomato isolate (Vd-4) was the least susceptible to *P. oligandrum* attack, while the remaining isolates showed similar degrees of susceptibility ($P < 0.001$). *P. oligandrum* isolates 91-4 and 87-6 were the most aggressive against the pepper isolate (Vd-1) of *V. dahliae*.

Effect of temperature. Temperature significantly affected parasitism of the three isolates of *V. dahliae* by *P. oligandrum* (Fig. 2). When cultured alone, *P. oligandrum* formed oospores at 25 and 33°C but not at 16°C. However, at 16°C, sporangia, zoospores, and their cysts were formed. The pepper isolate (Vd-1) of *V. dahliae* was most susceptible at 25°C, while two tomato isolates (Vd-7 and Vd-8) were equally susceptible at 25 and 33°C. The results also indicated significant variability among the isolates of *V. dahliae* in their susceptibility to *P. oligandrum* (Fig. 2). The two tomato isolates varied significantly from each other and from the pepper isolate (Vd-1) ($P < 0.05$).

Effect of matric potential. When cultured alone, *P. oligandrum* was stimulated to produce more oospores when matric potentials ranged from -0.2 to -0.6 MPa (Fig. 3). Below -0.6 MPa, oospore production declined sharply, with nearly complete inhibition at -1.0 MPa. When dual cultured with Vd-1, the optimum interaction with *P. oligandrum* was in pure water. At lower matric potentials, the interaction was reduced in a linear fashion, with total inhibition at -1.0 MPa (Fig. 3).

Interactions on MAM. Dual culture of the six *V. dahliae* isolates with *P. oligandrum* isolate 91-4 on MAM indicated a significant effect on *V. dahliae* growth and

Fig. 2. Effect of temperature on oospore production by *Pythium oligandrum* (*P.o.*) isolate 76-13 when grown alone or in dual culture with isolates of *Verticillium dahliae* on 20% potato carrot broth. LSD_{0.01} = 0.38 × 10⁴ oospores ml⁻¹ according to the Student-Newman-Keuls test. Vertical bars equal 1 standard error.

Fig. 3. Effect of matric potential on oospore production by *Pythium oligandrum* (*P.o.*) isolate 76-13 when cultured alone or in dual culture with *Verticillium dahliae* (Vd-1, pepper isolate) on 20% potato carrot broth at 25°C for 20 days. Vertical bars equal 1 standard error.

reproduction (Table 2). In every case, *V. dahliae* growth was reduced, while that of *P. oligandrum* was stimulated when compared with growth in pure culture. MS formation also was reduced significantly by the presence of *P. oligandrum*, being totally inhibited in the case of Vd-1 and Vd-6. Although conidia formation by *V. dahliae* usually was greatly reduced in dual culture, all isolates formed at least some conidia (Table 2). The cotton (Vd-2) and tomato (Vd-4) isolates, which formed few MS and abundant conidia when cultured alone, produced fewer conidia under dual culture conditions. In each test, *P. oligandrum* formed oospores extensively on MAM when cultured with *V. dahliae* isolates but not when grown alone (Table 2).

Interactions on fresh and precolonized MAM. *P. oligandrum* growth was poor when cultured on either fresh MAM or *P. oligandrum* precolonized MAM, but increased on *V. dahliae* precolonized MAM (Table 3). However, growth of *P. oligandrum* was greater under dual culture conditions regardless of the medium. Haustorial threads (HT) and intertwining hyphae (IH) were observed in dual cultures and when *P. oligandrum* was grown alone on the *V. dahliae* precolonized MAM. Oospores were produced abundantly by *P. oligandrum* in dual culture irrespective of the medium. However, no oogonia or oospores were formed when *P. oligandrum* was inoculated alone on fresh or *V. dahliae* or *P. oligandrum* precolonized MAM. On the other hand, *V. dahliae* failed to form MS in dual culture, while abundant MS were formed when it was inoculated alone on fresh or *V. dahliae* or *P. oligandrum* precolonized MAM. Its vegetative growth was similar when grown alone in fresh or *V. dahliae* and *P. oligandrum* precolonized MAM, but growth was reduced significantly in dual culture.

Incidence of vascular infection. Pepper plants inoculated with *V. dahliae* were stunted in all trials (Fig. 4). Five weeks after transplanting, wilting was observed, and by the end of the experiment, 6 (trial

I), 7 (trial II), and 3 (trial III) plants out of 12 in each trial were infected by *V. dahliae* based on isolation from petioles and basal stems. In the presence of *P. oligandrum*, plant growth was enhanced (Fig. 4), and by the end of the experiment, 5 (trial I), 4 (trial III), and 2 (trial III) plants out of 12 in each trial were infected with *V. dahliae*. Differences in incidences of vascular infection between treatments involving *V. dahliae* alone or in combination with *P. oligandrum* were insignificant.

Biocontrol efficiency. Although *P. oligandrum* did not affect incidences of vascular infection of pepper by *V. dahliae*, it did provide significant reductions in some of the effects of disease on pepper yield components (Table 4 and Fig. 4). Shoot dry weights were consistently higher when *P. oligandrum* was added to *V. dahliae*-infested potting soils (Table 4 and Fig. 4). On a fresh weight basis, the pathogen reduced fruit yield to 35% of control, but when *P. oligandrum* was added to *V. dahliae*-infested potting mix, fruit yield was 63% of the control (Table 4). This represented a 78% increase in yield over the pathogen treatment alone. However, when *P. oligandrum* was added alone to autoclaved UC mix, it stimulated vegetative growth by 42% and enhanced fruit yield by 49% over noninfested controls (Table 4).

Population dynamics of *P. oligandrum*. In trial II, rhizosphere populations of *P. oligandrum* were not significantly higher in the presence of *V. dahliae* than when it was applied singly. However, in trial III, where successive applications of *P. oligandrum* were made, populations of *P. oligandrum* remained higher in the rhizospheres in the *V. dahliae*-infested treatment than in the treatment where it was used alone (Fig. 5). In each trial, the germinable propagules in the rhizosphere declined sharply in the presence and absence of *V. dahliae* after 3 weeks. Multiple times of infestation resulted in higher populations than a single infestation throughout trial III, but they did not pre-

vent population decline. In trial I, populations of *P. oligandrum* remained relatively stable in the nonrhizosphere soil (Fig. 5).

In trial II, the control treatment was contaminated with *P. oligandrum* by a fungus gnat (*Bradysia* spp.) infestation (Fig. 5). After 2 weeks, the growth dynamics of the fungus in the rhizospheres of peppers in the inadvertently infested pots were similar to those in the experimental treatments.

Population dynamics of *V. dahliae*. The population of *V. dahliae* in the rhizosphere and nonrhizosphere of pepper was significantly reduced in the presence of *P. oligandrum* (Fig. 6). Multiple infestations with *P. oligandrum* during trial III

Fig. 4. Gain in shoot dry weight of pepper plants (*Capsicum annuum* cv. California Wonder) over time. Plants grown in autoclaved UC mix were infested with *Verticillium dahliae* (V.d.), *Pythium oligandrum* (P.o.), or a combination of the two fungi (P.o. + V.d.), or were noninfested (control). Vertical bars equal 1 standard error.

Table 3. Interactions between *Pythium oligandrum* and *Verticillium dahliae* on fresh and precolonized minimal agar medium

Medium status (prior to inoculation) ^v	Stage	Monoculture <i>P. oligandrum</i> ^w	Monoculture <i>V. dahliae</i>	Dual culture	
				<i>P. oligandrum</i>	<i>V. dahliae</i>
Fresh ^x	Vegetative	PG, no HT or IH	NG	EG, +HT, +IH	PG
	Reproduction	No Sp, Oog, or Oosp	MS ++	Abundant Oog and Oosp	No MS, few C
<i>P. oligandrum</i> precolonized ^y	Growth	PG, no HT or IH	NG	EG, +HT, +IH	PG
	Reproduction	No Sp, Oog, or Oosp	MS ++, few C	Abundant Oog and Oosp	No MS, few C
<i>V. dahliae</i> precolonized ^z	Growth	NG, + HT, +IH	NG	EG, +HT, +IH	PG
	Reproduction	No Oog or Oosp	MS ++, few C	Abundant Oog and Oosp	No MS, few C

^v Medium conditions prior to inoculation with *P. oligandrum* isolate 76-13 or *V. dahliae* pepper isolate (Vd-1). Assessments were based on three plates per treatment, and the experiment was performed four times.

^w Abbreviations signify poor growth (PG), normal growth (NG), extensive growth (EG), haustorial threads (HT), intertwining hyphae (IH), sporangia (Sp), oogonia (Oog), oospores (Oosp), microsclerotia (MS), and conidia (C).

^x Fresh minimal agar medium (MAM) overlaid with polycarbonate membrane (PCM, 0.2 µm) in which fungi were inoculated with 10-mm-diameter disks.

^y Fresh MAM was overlaid with PCM and inoculated with *P. oligandrum*. After 24 h, the *P. oligandrum* colony on PCM was removed (*P. oligandrum* precolonized), and the medium reinoculated with either *P. oligandrum*, *V. dahliae*, or both.

^z Fresh MAM was overlaid with PCM and inoculated with *V. dahliae*. After 9 days, the *V. dahliae* colony on PCM was removed (*V. dahliae* precolonized), and the medium reinoculated with either *P. oligandrum*, *V. dahliae*, or both.

were effective in reducing and maintaining populations of *V. dahliae* at low densities.

DISCUSSION

This investigation demonstrated that *P. oligandrum* was able to parasitize *V. dahliae* and to negatively impact the latter's ability to grow and produce microsclerotia in dual culture. Results indicated that these effects were caused by mycoparasitism and not chemical inhibition or nutritional competition. Based on production of oospores by *P. oligandrum* in dual culture, it was found that there was variation among isolates of *P. oligandrum* in their parasitism of *V. dahliae* and in the susceptibility of different isolates of *V. dahliae* to parasitism by *P. oligandrum*. However, oospore production in dual culture should be used as an indication of aggressiveness of *P. oligandrum* toward *V. dahliae* rather than as a quantitative measure of mycoparasitism.

It also was found that environmental influences, such as temperature and matric potential, significantly affected the interactions of these fungi. In considering applications of a mycoparasite for biological control, the role of the physical environment must be taken as seriously as the behavioral traits of the biological agent, such as rhizosphere competence. Better knowledge of how these factors affect mycoparasitism could assist in selection of isolates and strain improvement.

Interactions among various isolates of both fungi indicate that all tested isolates of *V. dahliae* were susceptible to *P. oligandrum* attack in both liquid and solid media. While isolates of *V. dahliae* varied in their susceptibility, the tomato isolates were found to be the most resistant. These results suggest that *V. dahliae* possesses a mechanism(s) of resistance to parasitism by *P. oligandrum*, which shows the necessity of testing the effectiveness of a variety of isolates of the biocontrol agent against a variety of isolates of the targeted pathogen.

Isolates of *P. oligandrum* produced large quantities of oospores during their digestion of hyphae of *V. dahliae* in dual cultures. Additionally, microsclerotial formation was affected to various degrees depending on combinations of isolates.

Reproduction of these fungi in dual culture could have been affected reciprocally by secondary metabolites produced by either fungus. Growth and reproduction by *V. dahliae* was not affected by metabolites produced by *P. oligandrum*. The ability of *V. dahliae* to form abundant microsclerotia on *P. oligandrum* precolonized MAM but not when co-cultured with *P. oligandrum* supports this conclusion. Likewise, *P. oligandrum* failed to form oogonia or oospores on fresh MAM as well as MAM precolonized by *V. dahliae*. However, metabolites apparently produced by *V. dahliae* in culture were observed to enhance the vegetative growth of *P. oligandrum* and to stimulate the formation of its mycoparasitic organs (haustorial threads and intertwining hyphae) when *P. oligandrum* was grown on *V. dahliae* precolonized MAM. These structures were reported to be involved in its mycoparasitic interactions with susceptible fungi (8,14,23,25,44). The production of these infection structures on media precolonized by *V. dahliae* suggests that *P. oligandrum* responded to chemical cues of the host. However, it appears that oospore production by *P. oligandrum* was the result of direct parasitism of *V. dahliae*. Similar observations were quantified in other systems (8).

Disease losses caused by *V. dahliae* on pepper were reduced significantly by *P. oligandrum*. However, some of these effects apparently were due to growth promotion of pepper by *P. oligandrum* rather than direct protection against disease. Growth promotion of several crops was attributed to *P. oligandrum* in earlier reports (4,21). This phenomenon could confound disease assessment, especially if measurements of yield components are used to assess disease severity. Processes responsible for growth promotion also may be factors in induced systemic resistance (ISR) and may have a role in ISR. Meera et al. (32) showed recently that inoculating plant roots with plant growth promoting fungi induced systemic resistance against a foliar pathogen, and Benhamou et al. (7) reported that *P. oligandrum* induced resistance in tomato toward *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Similar effects could be responsible for sup-

pression of Verticillium wilt of pepper by *P. oligandrum*.

Mycoparasitism also may have a significant role in biological control of Verticillium wilt of pepper. Although the reduction in the incidence of infection of pepper by *V. dahliae* was not significant in the presence of *P. oligandrum*, inoculum reduction of the pathogen was significant, as shown

Fig. 5. Population dynamics of *Pythium oligandrum* (*P.o.*) in nonrhizosphere (trial I) and rhizosphere (trials II and III) soils of pepper (*Capsicum annuum* cv. California Wonder) grown in autoclaved UC mix in the presence or absence of *Verticillium dahliae* (*V.d.*). Arrows indicate multiple dates of *P. oligandrum* infestation during trial III. Vertical bars equal 1 standard error.

Fig. 6. Population dynamics of *Verticillium dahliae* (*V.d.*) in the combined rhizosphere and nonrhizosphere soil of pepper plants (*Capsicum annuum* cv. California Wonder) grown in autoclaved UC mix in the presence or absence of *Pythium oligandrum* (*P.o.*). Vertical bars equal 1 standard error.

Table 4. Effect of *Verticillium dahliae* and *Pythium oligandrum* on shoot and fruit growth of pepper (*Capsicum annuum* cv. California Wonder)^a

Treatment	Shoot		Fruit	
	Fresh wt. (g) ^z	Controls (%)	Dry wt. (g) ^z	Controls (%)
Control	19.6 b	100	207.6 b	100
<i>P. oligandrum</i>	27.8 a	142	308.7 a	149
<i>V. dahliae</i>	11.2 d	57	73.5 d	35
<i>P. oligandrum</i> + <i>V. dahliae</i>	15.6 c	78	131.0 c	63
LSD _{0.05}	4.1	...	54.1	...

^a Data are based on two independent trials each with 12 replicates per treatment.

^z Values within columns followed by the same letter are statistically nonsignificant from each other based on the Student-Newman-Keuls test at $P = 0.05$.

in the effect of *P. oligandrum* treatments on the population dynamics of *V. dahliae*. This gives reason to believe that the mycoparasitic interactions observed in vitro could also occur in the more complex plant-soil system. Indeed, future studies should focus on the different possible mechanisms of biological control by *P. oligandrum*.

This study provided additional support for the potential application of *P. oligandrum* as a biocontrol agent of soilborne root pathogens. While further testing of *P. oligandrum* is needed on Verticillium wilt in field settings, intensive greenhouse cropping systems lend themselves to inundative applications of biologicals such as *P. oligandrum*.

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