Colonization of *Pythium oligandrum* in the Tomato Rhizosphere for Biological Control of Bacterial Wilt Disease Analyzed by Real-Time PCR and Confocal Laser-Scanning Microscopy

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ABSTRACT

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It recently has been reported that the non-plant-pathogenic oomycete *Pythium oligandrum* suppresses bacterial wilt caused by *Ralstonia solanacearum* in tomato. As one approach to determine disease-suppressive mechanisms of action, we analyzed the colonization of *P. oligandrum* in rhizospheres of tomato using real-time polymerase chain reaction (PCR) and confocal laser-scanning microscopy. The real-time PCR specifically quantified *P. oligandrum* in the tomato rhizosphere that is reliable over a range of 0.1 pg to 1 ng of *P. oligandrum* DNA from 25 mg dry weight of soil. Rhizosphere populations of *P. oligandrum* from to-

mato grown for 3 weeks in both unsterilized and sterilized field soils similarly increased with the initial application of at least 5×10^5 oospores per plant. Confocal microscopic observation also showed that hyphal development was frequent on the root surface and some hyphae penetrated into root epidermis. However, rhizosphere population dynamics after transplanting into sterilized soil showed that the *P. oligandrum* population decreased with time after transplanting, particularly at the root tips, indicating that this biocontrol fungus is rhizosphere competent but does not actively spread along roots. Protection over the long term from root-infecting pathogens does not seem to involve direct competition. However, sparse rhizosphere colonization of *P. oligandrum* reduced the bacterial wilt as well as more extensive colonization, which did not reduce the rhizosphere population of *R. solanacearum*. These results suggest that competition for infection sites and nutrients in rhizosphere is not the primary biocontrol mechanism of bacterial wilt by *P. oligandrum*.

The non-plant-pathogenic oomycete *Pythium oligandrum* Drechsler is a common soil fungus with worldwide distribution (38) that has received considerable attention as a potential biocontrol agent because of its ability to parasitize a wide range of fungal and oomycete plant pathogens (4,5). It also has the advantages of producing cell wall hydrolytic enzymes and antibiotics (4,20,28), competing for nutrients (22), promoting plant growth (2,17), and inducing plant resistance (3). Previous studies have shown that *P. oligandrum* is an effective biocontrol agent of damping-off and root diseases caused by several soilborne plant fungal pathogens (2,7,22,24). Recent investigations have focused on the forms of induced resistance exerted by this fungus. Biochemical analyses have provided evidence of the activation of phenylpropanoid and terpenoid pathways during the early events of tomato root colonization by *P. oligandrum* (16), and light and electron microscope studies have shown that the occurrence of limited and local host reactions in response to *P. oligandrum* colonization of tomato, such as the plugging of invaded cells with heterogeneous osmiophilic or electron-dense materials, probably were related to the enhancement of host resistance (16,29). Ethylene levels in *P. oligandrum* oospore-treated tomato roots were transiently elevated 11-fold from 6 to 8 h after treatment, followed by high expression of three ethylene-inducible defense-related genes (12). Picard et al. (27) isolated a soluble elicitor

protein, oligandrin, from culture filtrates, and our group purified two elicitor proteins, POD-1 and POD-2, from cell wall fractions of *P. oligandrum* (35,36).

It is likely that *P. oligandrum* must colonize the rhizosphere before it can trigger a defense response and it also is likely that this colonization causes *P. oligandrum* to compete directly with pathogens and other nonpathogenic rhizosphere organisms for space and nutrients. Many studies have shown a relationship between increased colonization of the rhizosphere by a nonpathogen and disease suppression (43). Martin and Hancock (22) showed that *P. oligandrum* actively colonized the endosperms and emerging radicles of sugar beet seedlings, but was a poor colonizer of secondary roots when seed were treated with oospores. McQuilken et al. (24) also reported that *P. oligandrum* did not appear to be rhizosphere competent on cress and sugar beet. On the contrary, Al-Rawahi and Hancock (1) found that *P. oligandrum* colonized the rhizosphere of 11 crop species grown in *P. oligandrum*-infested sterilized potting mix or an unsterilized mineral soil, even though the density of colonization in the former was higher than the latter. Le Floch et al. (18) reported that *P. oligandrum* was a strong colonizer of the tomato rhizosphere in hydroponic systems. These findings raise some doubt about whether *P. oligandrum* really has the ability to aggressively colonize the rhizosphere in field soils to act as an effective biocontrol agent. This is an important consideration because the complex interactions that take place in the rhizosphere between biocontrol agents and indigenous microbiota need to be considered during development of commercial microbial products (43). Thus, further studies detailing rhizosphere colonization by

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P. oligandrum are required, and can be much aided with the application of modern monitoring techniques such as real-time polymerase chain reaction (PCR) and confocal laser-scanning microscopy.

Previous studies (1,18) have used direct isolation of *P. oligandrum* by selective media to quantify populations on roots or in soils (i.e., CFU or percent root colonization), but these methods are time consuming and may not have sufficient sensitivity or specificity to give an accurate count or location of fungal propagules or somatic structures. Real-time PCR recently has been used to determine the levels of soilborne plant pathogen DNA in infected plant tissue and soil and in microbe interactions with high reproducibility and sensitivity (6,9,13,15). Real-time PCR provides a means of detecting and quantifying specific target DNA by monitoring PCR product accumulation, measured as increased fluorescence during cycling. There are three main strategies to generate the fluorescence signals. The first uses sequence-independent dyes that fluoresce when intercalated with double-stranded DNA (e.g., SYBR green I; Molecular Probe Inc., Eugene, OR, USA), and the second uses a single probe that enzymatically releases fluorophores (e.g., TaqMan; Roche Molecular Systems Inc., Alameda, CA, USA). The third uses two adjacent hybridization probes in which the fluorescence is generated by resonance energy transfer from a donor fluor on one probe to an acceptor fluor on the other (e.g., hybridization probes; Roche Applied Science, Mannheim, Germany). In this study, we developed a quantification method for *P. oligandrum* in the rhizosphere of tomato plants using a hybridization probe assay based on the high specificity of detection found in a comparative study of the methods (26). Furthermore, we examined an immuno-enzymatic staining procedure with confocal laser-scanning microscopy to visualize *P. oligandrum* on the roots of tomato. Using these complementary methods, we determined the ability of this fungus to colonize the tomato rhizosphere. Recently, our group found that *P. oligandrum* was able to suppress bacterial wilt caused by *Ralstonia solanacearum* in tomato (12). This soilborne bacterium colonizes on the root surface, infects roots through root tips and lateral root cracks, and rapidly develops within intercellular

spaces of the inner cortex. Subsequently, it progresses toward xylem vessels and, finally, causes wilting of the tomato plants (39). Determination of the colony population density of *P. oligandrum* in the tomato rhizosphere and the level of bacterial wilt over the course of disease suppression would serve as a useful tool for the evaluation of one of the suspected primary mechanisms of *P. oligandrum* biocontrol.

MATERIALS AND METHODS

Fungal cultures. Five isolates of *P. oligandrum*, nine other *Pythium* spp., and four non-oomycete fungal species were used to test specificity (Table 1). Our previous study (36) divides *P. oligandrum* isolates into two groups based on the number of major proteins in their cell wall protein fractions (CWPs). Isolates with two major proteins (D-type) and isolates with one major protein (S-type) were used. Genomic DNA from isolates with either two major proteins (D-type) or one major protein (S-type) was tested for PCR cross-amplification. *Pythium* isolates were grown in liquid B medium amended with penicillin at 100 µg/ml (23) or in V8 broth (100 ml of Campbell's V8 juice, 1.5 g of CaCO₃ per liter) at 25°C for 3 to 14 days. *Aphanomyces cochlioides*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* were grown in Difco potato dextrose broth at 20 or 25°C for 3 to 15 days. Mycelial mats were collected by filtration, washed with distilled water, and blotted dry with a paper towel for the extraction of genomic DNA.

For the production of large quantities of *P. oligandrum* oospores, *P. oligandrum* isolate MMR10 was grown in containing 0.1% wheat germ oil at 25°C for 28 days. At harvest, mycelia were washed with sterile distilled water several times, and then transferred aseptically to a Waring blender cup. After homogenization at 14,000 rpm for 5 min to disrupt hyphae without causing any obvious physical damage to the oospores, oospore concentration was adjusted to 1×10^6 oospores/ml with sterile distilled water and stored at 4°C. Although this suspension mainly consisted of oospores, it was considered to be a mycelial homogenate because it contained some hyphal debris.

TABLE 1. Fungal isolates tested with hybridization probe assay^a

Species name	Isolate number	Habitat	Origin	GenBank accession no.	C _T
<i>Pythium oligandrum</i>					
D-type	MMR2 ^v	Soybean soil	Hokkaido	AB302881	23.34 ± 0.40
D-type	MMR10 ^v	Wheat soil	Hokkaido	AB302884	23.08 ± 0.23
D-type	MAFF240291 ^w	Vegetable soil	Osaka	AB355600	22.27 ± 0.10
S-type	MMR3 ^v	Wheat soil	Hokkaido	AB302882	22.41 ± 0.27
S-type	MMR8 ^v	Wheat soil	Hokkaido	AB302883	23.67 ± 0.34
Other <i>Pythium</i> spp.					
<i>P. acanthicum</i>	MAFF240293 ^w	Spinach soil	Saga	AB302889	42.97 ± 1.76
<i>P. acanthophoron</i>	A-1-2-4 ^x	Wheat	Hokkaido	AB355644	45.00 ± 0.00
<i>P. vanterpoolii</i>	NBRC31923 ^y	Barley	Fukui	...	39.85 ± 1.79
<i>P. volutum</i>	NBRC31927 ^y	Barley	Fukui	...	45.00 ± 0.00
<i>P. torulosum</i>	NBRC32168 ^y	Maschrengrass	Kagawa	...	41.89 ± 1.80
<i>P. irregulare</i>	NBRC32072 ^y	Tulip	Kyoto	...	45.00 ± 0.00
<i>P. ultimum</i>	Py71-1 ^v	Sugar beet soil	Hokkaido	AB355596	40.89 ± 2.38
<i>P. deliense</i>	MAFF240292 ^w	Vegetable soil	Osaka	...	45.00 ± 0.00
<i>P. aphanidermatum</i>	MAFF240290 ^w	Vegetable soil	Osaka	...	41.02 ± 2.31
Other fungal species					
<i>Aphanomyces cochlioides</i>	MMRA1 ^v	Sugar beet	Hokkaido	...	43.52 ± 1.48
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>	MAFF103047 ^w	Tomato	Koch	...	43.70 ± 1.30
<i>Rhizoctonia solani</i> AG2-2	Pf-28 ^v	Sugar beet	Hokkaido	...	45.00 ± 0.00
<i>Sclerotinia sclerotiorum</i>	Sm5f-1 ^z	Kidney bean	Hokkaido	...	45.00 ± 0.00

^a Threshold number of polymerase chain reaction cycles (C_T) is indicated. Data represent the means ± standard error of four assays, and no signal is indicated as C_T = 45.00.

^v Maintained in our laboratory.

^w Provided by National Institute of Agrobiological Sciences, Japan.

^x Provided by N. Kondo, Hokkaido University, Japan.

^y Provided by National Institute of Technology and Evaluation, Japan.

^z Provided by I. Saito, Hokkaido University, Japan.

Plant material. Laboratory-grown miniature tomato cv. Micro-Tom, which is valuable as a functional genomic tool for plant pathology (31,33), was seeded in unsterilized or sterilized Brown Andosol, which is a well-drained, fine volcanic ash soil, in plastic trays (28 by 54.5 cm) divided into 128 cones (3 by 3 by 5 cm). The sterilized soil was kept in an autoclave bag (Fisher Scientific, Pittsburgh, PA, USA) at room temperature for ≈ 1 month after being autoclaved at 121°C for 2 h until use. Plants were grown for 3 weeks in a growth chamber at 25 and 20°C (day and night) with a 16-h photoperiod (66.5 W/m²). Each plant was fertilized with 5 ml of a 500-fold-diluted Hyponex solution (Hyponex Japan, Osaka, Japan) at 1 week intervals.

Rhizosphere colonization of *P. oligandrum*. To evaluate the efficiency of rhizosphere colonization of *P. oligandrum*, each soil cone was watered with 5 ml of mycelial homogenate containing different oospore concentrations (0 , 5×10^2 , 5×10^3 , 5×10^4 , 5×10^5 , or 5×10^6) just before seeding. Three weeks after growth under the above conditions, four to six plants from each treatment were used for determination of *P. oligandrum* rhizosphere populations with real-time PCR in both unsterilized and sterilized soil conditions and confocal laser-scanning microscopic observation in only unsterilized soil condition. Plants were uprooted gently from each cone to preserve the small feeder roots and were shaken to remove clumps of soil around roots. For the determination of rhizosphere populations, these plants additionally were shaken in a plastic bag to collect as many soil particles adhering to root surfaces as possible. To observe root surfaces, whole roots then were fixed in FAA solution (formalin, acetic acid, 95% ethanol, and water, 135:45:450:315) (42) for microscopic observation. Soil dry weights were determined after incubation at 70°C for 48 h, and these data were used to calculate the concentration of *P. oligandrum* DNA per gram dry weight of rhizosphere soil.

Dynamics of *P. oligandrum* in the rhizosphere after transplanting. To assess changes of *P. oligandrum* populations in the rhizosphere after transplanting into *P. oligandrum*-free soil, seedlings were prepared by sowing seed into sterilized soil cones containing different oospore concentrations (5×10^4 , 5×10^5 , or 5×10^6). Four weeks after growth under the above conditions, each seedling was transplanted into sterilized field soil in a plastic pot (9 cm in diameter by 9 cm deep). Rhizosphere soils from two to three plants of each treatment were collected as above 0, 2, 4, and 6 weeks after transplanting. The root systems from harvested plants were divided into root bases and root tips. The experiment was repeated three times.

Inoculation of *Ralstonia solanacearum*. To evaluate the relationship between the efficiency of rhizosphere colonization of *P. oligandrum* and the level of bacterial wilt disease suppression, seedlings grown in sterilized soil cones containing different oospore concentrations (0 , 5×10^2 , 5×10^3 , 5×10^4 , 5×10^5 , or 5×10^6) for 3 weeks under the above conditions were inoculated with *Ralstonia solanacearum* strain 8242 (race 1, biovar 4). The bacterial suspension was adjusted to 1×10^7 CFU/ml as described previously (25). Roots of the *P. oligandrum*-inoculated tomato plants were injured by directly piercing each soil cone in four places ≈ 1 cm part from a base of hypocotyl with a skewer and watered with 5 ml of the bacterial suspension. Inoculated plants were incubated in a growth chamber at 30°C with a 16-h photoperiod. Six plants in each treatment were used to assay disease severity, and the experiment was repeated five times. Disease severity was measured 10 days after inoculation and categorized on a 0-to-4 scale, where 0 = no wilting, 1 = 1 to 25% wilting, 2 = 26 to 50% wilting, 3 = 51 to 75% wilting, and 4 = 76 to 100% wilting or dead (30). To determine whether rhizosphere colonization of *R. solanacearum* is affected by the precolonization of *P. oligandrum*, rhizosphere soils were collected 4 days after inoculation as described above, and rhizosphere populations of *R. solanacearum* were determined by plating diluted soil onto selective medium (11). Two plants from each treatment were used for

determination of *R. solanacearum* rhizosphere population, and the experiment was repeated three times. The effect of preinoculation with *P. oligandrum* oospores on suppression of bacterial wilt disease and bacterial rhizosphere population was determined by an analysis of variance (ANOVA) with a randomized block design. Dunnett's one-sided *t* test was used to determine significant differences in disease severity between mycelial homogenates and distilled water treatments ($P < 0.05$). Bacterial counts were transformed using $\log_{10}$ before performing the ANOVA, and a comparison of bacterial rhizosphere population between treatments was done by Tukey's studentized range test ($P < 0.05$).

DNA extraction. Fungal genomic DNA was extracted from 50 to 100 mg (wet weight) of ground fungal cultures using the DNeasy plant DNA extraction kit as described by the manufacturer (Qiagen Inc., Mississauga, ON, Canada). DNA was eluted in 100 μ l of buffer and quantified by measuring its absorbance at 260 nm. Total soil DNA was extracted from 0.2 to 0.3 g of soil (expressed as dry weight) using a Bio 101 FastDNA SPIN kit as described by the manufacturer (Qbiogene, Inc., Irvine, CA) with some modifications. To increase DNA extraction efficiency from soil, a fecal dry bead tube containing quartz (MoBio Laboratories, Inc., Solana Beach, CA) was used instead of the original FastDNA SPIN kit tube, and 100 μ l of a 20% skim milk solution was added to the tube along with 978 μ l of sodium phosphate buffer and 122 μ l of MT buffer to avoid degradation and adsorption of DNA to soil particles (40). The tube was processed three times in a FastPrep apparatus (Thermo Savant, Holbrook, NY) at speed level 6.5 for 45 s with 5-min cooling periods between each process. After elution from the SPIN Filter with DES (Qbiogene, Inc.), the DNA solution was mixed with an equal volume of chloroform, vortexed thoroughly, and centrifuged for 5 min at $14,000 \times g$. The DNA was precipitated with 100% ethanol and washed with 80% ethanol. The purified DNA was redissolved in 56 μ l of sterile Milli Q water and quantified as above.

PCR primers and hybridization probes. A species-specific primer set and two adjacent fluorescent-labeled hybridization probes for fluorescence resonance energy transfer were used to amplify *P. oligandrum* DNA. The *P. oligandrum*-specific primers P.OLG.F1 and P.OLIG.R04 designed by Godfrey et al. (10) were used to produce a 384-bp amplicon within the internal transcribed spacer (ITS) region of ribosomal (r)DNA. The PCR product was cloned into pGEM-T Easy Vector (Promega Corp., Madison, WI) and transformed into *Escherichia coli* JM109 (Promega Corp.). Plasmid DNA was purified with a GFX Micro Plasmid Prep Kit (Amersham Biosciences, Piscataway, NJ), and sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems Inc.) according to the manufacturer's instructions.

To design two *P. oligandrum*-specific adjacent fluorescent-labeled hybridization probes, HybriOLI-1 and HybriOLI-2, multiple alignment of the ITS region from 25 *Pythium* spp. (*P. oligandrum*, accession number AB302881; *P. aphanidermatum*, AY598622; *P. deliense*, AY598674; *P. myriophyllum*, AY598678; *P. torulosum*, AY598624; *P. graminicola*, AY598625; *P. arrhenomanes*, AY598628; *P. vanterpoolii*, AY598685; *P. volutum*, AY598686; *P. dissotocum*, AY598634; *P. insidiosum*, AY598637; *P. acanthicum*, AF330196; *P. rostratum*, AY598696; *P. pleroticum*, AY598642; *P. spinosum*, AY598701; *P. irregulare*, AY598702; *P. sylvaticum*, AY598645; *P. iwayamai*, AY598648; *P. paddicum*, AY598707; *P. helicanthum*, AY598653; *P. ultimum* var. *ultimum*, AY598657; *P. acanthophoron*, AY598711; and *P. vexans*, AY598713) was conducted using CLUSTAL-X (37). These species were selected as representative of each of 11 phylogenetic clades from A to K based on the rDNA ITS region (19). The 3' end of HybriOLI-1 and the 5' end of HybriOLI-2 were labeled with Fluorescein and Red640, respectively. Primers and

probes used in this study were synthesized by Hokkaido System Science (Hokkaido, Japan) and Nihon Gene Research Laboratory (Miyagi, Japan), respectively.

Real-time quantitative PCR assay. Real-time PCR amplifications were carried out in glass capillaries (Roche Diagnostics, Tokyo) in a total volume of 20 µl using a LightCycler (Roche

Diagnostics). The reaction mixture consisted of 2 µl of Light-Cycler FastStart DNA Master HybProbe (Roche Diagnostics), 4 mM MgCl₂, 300 nM each primer, 200 nM HybriOLI-1 probe, and 400 nM HybriOLI-2 probe. Extracted fungal DNA was diluted to 100 pg/µl and 1 µl of each sample was used in the 20-µl real-time PCR mixtures. Reactions containing sterile Milli Q

Fig. 1. Comparison of internal transcribed spacer (ITS) region sequences of *Pythium oligandrum* and other *Pythium* spp. **A**, ClustalX multiple alignment of nucleotide sequence from HybriOLI-1 and HybriOLI-2 designed from ITS region 1 of *P. oligandrum* with corresponding regions of 22 selected *Pythium* spp. **B**, ClustalX multiple alignment of ITS region sequences of *P. oligandrum* (accession number AY598618) and *P. acanthicum* (AF330196). P.OLIG.F1 and P.OLIG.R04 are specific primers designed by Goodfrey et al. (10). Gaps introduced for alignment are indicated by hyphens.

water without genomic DNA served as negative controls for each PCR. The thermal cycling conditions for PCR were as follows: after initial denaturation at 95°C for 10 min, samples were amplified for 45 cycles by touchdown PCR (8) with denaturation at 95°C for 10 s, annealing on a decline ramp of 1°C from 70 to 55°C every second cycle over the first 16 cycles and then at 55°C for the last 29 cycles for 20 s, and primer extension at 72°C for 15 s. Fluorescence was monitored during the annealing step at 640 nm. Fluorescence intensity is correlated with the number of accumulated PCR amplicons. The PCR cycle at which the increase in fluorescence of a sample exceeded the background noise of 0.04 was defined as the threshold cycle (C_T).

Quantification of *P. oligandrum* DNA in soil samples. Soil DNA sample concentrations were adjusted to correspond to the DNA amount extracted from 25 mg of soil expressed as dry weight. To test whether PCR inhibitors were present in soil DNA samples, two standard curves were made by diluting extracted *P. oligandrum* DNA from 100 pg/μl to 1 fg/μl with sterile Milli Q water or soil DNA solution extracted from 25 mg of sterilized soil, and comparing their reactivity in real-time PCR. A standard curve was generated by the Fit Points analysis method in the LightCycler Data Analysis software (version 3.5; Roche Diagnostics), with the fit point number set at 2. Using this method, the log of the concentration of the *P. oligandrum* DNA dilution series was plotted versus the C_T value. The concentrations of *P. oligandrum* DNA in soil samples then could be calculated by comparing C_T values to the crossing-point values of the linear regression line of the standard curve. Real-time PCR was conducted at least three times for each sample.

Confocal laser-scanning microscopy analysis. Whole roots of tomato plants from the *P. oligandrum* colonization test were immunocytochemically stained. Root tissues fixed with FAA as described above were washed briefly with TBST (20 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN₃, 0.05% Tween 20, pH 7.5) and incubated for 45 min in TBST containing 3.0% bovine serum albumin (BSA) to block nonspecific antibody binding sites. The blocked root tissues then were incubated for 1 h with antiserum against the deglycosylated 32-kDa protein of *P. iwayamai* (34), which recognizes *Pythium* cell wall proteins, at a 1:500 dilution with TBST containing 0.2% BSA. After washing three times for 5 min per wash with excess TBST, the tissues were incubated with the Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit immunoglobulin G, (Invitrogen Life Technologies, Carlsbad, CA) for 1 h at a 1:500 dilution with TBST containing 0.2% BSA. After washing three times for 5 min per wash with excess TBST, stained samples were placed on glass slides in drops of 2.5% of DABCO (Sigma-Aldrich, St. Louis) in Mowiol (Calbiochem, La

Jolla, CA) as an anti-fading mounting medium, and covered with a coverslip. Microscopy analysis was performed with a FluoView FV1000 confocal laser scanning unit (Olympus, Tokyo) with an argon ion laser attached to an Olympus BX61 microscope equipped with UplanSApo 20×/0.75, 40×/0.90, and 60×/1.20 w objectives (Olympus). The excitation source was an argon ion laser for Alexa Fluor 488 visualization (excitation dichroic mirror DM405/488, barrier filter BA505 IF). Z-stacks were collected with Z-steps of 1.24, 0.62, and 0.31 μm for 20×/0.75, 40×/0.90, and 60×/1.20 w objectives, respectively.

RESULTS

Specificity of real-time PCR analysis. A real-time PCR hybridization probe assay using ITS rDNA oligonucleotides was developed for detection and quantification of *P. oligandrum*. The ITS rDNA nucleotide sequences amplified with *P. oligandrum*-specific primers P.OLIG.F1 and P.OLIG.R04 designed by Godfrey (10), 384-bp amplicons, were compared among five *P. oligandrum* isolates (MMR2, -3, -8, and -10 and MAFF240291) in this study and one New Zealand isolate (NZWM07) in the GenBank database. There was only a two-nucleotide difference among MMR2 (accession number AB302881), MMR3 (AB302882), MMR8 (AB302883), MMR10 (AB302884), MAFF240291 (AB355600), and NZWM07 (AF364536), showing that the selected ITS region is highly conserved within this species. The *P. oligandrum*-specific hybridization probes HybriOLI-1 and HybriOLI-2, which are separated by one base, were designed from an ITS-1 region exhibiting variability from another 24 *Pythium* spp., except for *P. acanthicum* (Fig. 1A). Although *P. acanthicum* and *P. oligandrum* have very similar ITS sequences because both species belong to the same phylogenetic clade based on ITS region of rDNA (19), their annealing sites for the primers P.OLIG.F1 and P.OLIG.R04 have different sequences (Fig. 1B). The DNA from five isolates of *P. oligandrum*, nine *Pythium* spp., and four non-*Pythium* fungal species was checked for cross reactivity of primers and probes (Table 1). The five *P. oligandrum* isolates, including D- and S-types, had strong fluorescence signals and similar C_T values. On the other hand, very weak or no signals were obtained from the other nine *Pythium* spp., including *P. acanthicum* isolate MAFF240293, or from *A. cochlioides*, *F. oxysporum* f. sp. *radicis-lycopersici*, *R. solani* AG2-2, and *S. sclerotiorum* (Table 1). These results indicate that, at least for the fungal isolates tested, the real-time PCR assay is specific to *P. oligandrum*.

Quantification of *P. oligandrum* in soil. Genomic DNA of *P. oligandrum* isolate MMR10 was serially diluted from 100 pg/μl

Fig. 2. Standard curves of *Pythium oligandrum* DNA in a real-time polymerase chain reaction hybridization probe assay. The threshold cycle (C_T) values are plotted against the concentration of diluted DNA with Milli Q water or soil DNA solution (log scale). Error bars represent standard deviation of the means of three independent experiments.

Fig. 3. Rhizosphere soil populations of *Pythium oligandrum* of tomato plants grown for 3 weeks in unsterilized or sterilized field soils initially treated with different concentrations of oospores. The *P. oligandrum* population was expressed as the DNA concentration determined by real-time polymerase chain reaction hybridization probe assay. Error bars represent standard error of the means of three independent experiments.

to 1 fg/ μ l with sterile Milli Q water or DNA solution isolated from 25 mg dry weight (dw) of sterilized soil to test the sensitivity of the real-time PCR assay. There was a strong linear ($R^2 = 0.999$) inverse relationship between C_T and $\log_{10}$ (*P. oligandrum* DNA concentration) over five orders of magnitude from 100 pg to 10 fg, with little variation between triplicate samples in either dilution series (Fig. 2). Two calibration curves were compared to test for the potential presence of PCR inhibitors in soil DNA samples. There was no significant difference ($P < 0.05$) in the slopes and intercepts for dilution series with Milli Q water and soil DNA solution, indicating that there were no detectable soil PCR inhibitors to interfere with the quantification of *P. oligandrum* in soil.

Rhizosphere population of *P. oligandrum* in tomato. The amount of *P. oligandrum* genomic DNA in the rhizosphere of tomato grown for 3 weeks in unsterilized or sterilized field soils with initial applications of different concentrations of oospores was determined by real-time PCR hybridization probe assay (Fig. 3). No amplification product was obtained from sterilized soil without the addition of oospores, whereas 9.0 pg of *P. oligandrum* DNA/g of dry soil were detected from unsterilized soil without

the addition of oospores, indicating that indigenous *P. oligandrum*, or at least its DNA, was in the field soil used in this study. Soil assayed with differential medium (21) contained viable *P. oligandrum* (data not shown). Although there was some native *P. oligandrum* in unsterilized soil, populations of *P. oligandrum* in the tomato rhizosphere increased with the application of at least 5×10^5 oospores per plant. A similar trend was observed in sterilized soil, but the amounts of *P. oligandrum* DNA detected from the rhizosphere of tomato plants grown in 5×10^6 oospores/plant treatment in sterilized soils always were higher than in unsterilized soil.

Tomato root surface colonization by *P. oligandrum*. Confocal laser-scanning microscopic observation with immunocytochemical staining provided a tool for visualization of *P. oligandrum* on tomato roots after 3 weeks of plant growth in sterilized soils with initial applications of oospores (5×10^4 , 5×10^5 , or 5×10^6). Hyphal development was observed more frequently on the root surface than oogonium development. *P. oligandrum* colonized the tomato root by attaching to root hairs (Fig. 4A) and by growing along the surface of the root (Fig. 4B). Some fungal biomass colonized tightly on the root surface and could not be removed by

Fig. 4. Confocal laser-scanning microscopy of tomato root colonization by *Pythium oligandrum*. Tomato plants were grown for 3 weeks in sterilized field soil initially treated with 5×10^6 oospores of *P. oligandrum* per plant. **A**, Oospore and hyphae attaching to root hairs. **B**, Hyphae growing over the root surface at high density. **C**, Strong root surface colonization by hyphae not removed by vigorous washing. **D**, Hyphae growing along the epidermal cells and penetrating one of them (white arrow). The size bar represents 50 μ m in all panels.

vigorously washing with tap of water (Fig. 4C). Hyphal penetration into root epidermal cells was observed at these tightly adherent colonization sites (Fig. 4D). Additionally, tomato root colonization was quantified by determining colonization frequency as the percentage of visual fields with *P. oligandrum* colonization (Table 2). Colonization frequency in the 5×10^5 and 5×10^6 oospore treatments was significantly higher than treatment with 5×10^4 oospores. Root colonization was most frequently and abundantly observed in tomato seedlings grown in sterilized soil treated with 5×10^6 oospores.

Rhizosphere population dynamics of *P. oligandrum* after transplanting. The changes of *P. oligandrum* population in the rhizosphere of plants grown for 4 weeks in sterilized soil initially treated with 5×10^4 , 5×10^5 , or 5×10^6 oospores, followed by transplanting into sterilized soil, were determined by real-time PCR hybridization probe assay (Fig. 5). Rhizosphere populations decreased with time after transplanting, but the rate of decrease was different between the root base and the root tip. At the root tip, the population sharply declined to a minimum level by 2 weeks after transplanting (6 weeks after seeding), whereas a gradual population decrease occurred by 6 weeks after transplanting (10 weeks after seeding) at the root base. The amount of *P. oligandrum* DNA 6 weeks after transplanting (10 weeks after seeding) was 1.8 pg/g of dry rhizosphere soil at the root tips from plants initially treated with 5×10^6 oospores, but the amounts were below the detection limit for real-time PCR in the 5×10^4 and 5×10^5 oospore treatments.

Bacterial wilt suppression. To determine the minimum inoculum concentration of *P. oligandrum* required for suppression of bacterial wilt disease, tomato seedlings were grown for 3 weeks in sterilized soils initially treated with 5×10^2 to 5×10^6 /plant.

TABLE 2. Root colonization frequency of *Pythium oligandrum* in tomato plants initially treated with different concentrations of its oospores after 3 weeks of growth^y

Treatment	Colonization frequency (%) ^z
5×10^4 oospores/plant	14.8 ± 5.2 a
5×10^5 oospores/plant	52.4 ± 10.3 b
5×10^6 oospores/plant	76.2 ± 2.9 b

^y Colonization was determined as the percentage of visual fields in which *P. oligandrum* was detectable, with 44 to 50 fields per experiment in three replicates, by confocal laser-scanning microscopy.

^z Values are the means $\pm$ standard error of three replicates and values followed by a different letter within columns are significantly different using Tukey's studentized range test ($P < 0.05$) after angular transformation of percentage data.

Plants then were challenged with 5×10^7 CFU/plant of *Ralstonia solanacearum*. Ten days after inoculation with *R. solanacearum*, disease severity was compared with plants grown in soil without the addition of oospores. At an inoculum concentration of $>5 \times 10^4$ oospores/plant, *P. oligandrum* significantly reduced disease severity compared with the control (Table 3). Although there was an inverse correlation between bacterial wilt disease severity and *P. oligandrum* inoculum concentration, there was no significant difference among the 5×10^4 , 5×10^5 , and 5×10^6 oospores/plant treatments according to a least significant difference test ($P < 0.05$), indicating that an inoculum concentration of 5×10^4 oospores/plant was sufficient to give maximum protection from bacterial wilt disease. Additionally, *R. solanacearum* rhizosphere populations 4 days after inoculation were compared. Inoculum concentrations of $>5 \times 10^4$ oospores/plant reduced pathogen populations slightly but not significantly compared with the control (Table 4). Furthermore, there was no significant difference in pathogen populations in the rhizosphere among the 5×10^4 , 5×10^5 , and 5×10^6 oospores/plant treatments, although the rhizosphere populations of *P. oligandrum* was quite different among them (Fig. 3, sterilized soil).

DISCUSSION

In this study, we developed a real-time PCR hybridization probe assay for the quantification of *P. oligandrum* in soil and, using this method, examined the colonization of this fungus in the tomato rhizosphere. The primary advantages of the use of hybridization probes in real-time PCR is that the binding of two additional probes results in increased specificity compared with

TABLE 3. Disease severities caused by *Ralstonia solanacearum* in tomato initially treated with different concentrations of oospores of *Pythium oligandrum*^y

Treatment	Disease severity ^z
Control (0 oospore/plant)	3.40 ± 0.25
5×10^2 oospores/plant	3.07 ± 0.25
5×10^3 oospores/plant	2.68 ± 0.41
5×10^4 oospores/plant	$2.45 \pm 0.40^*$
5×10^5 oospores/plant	$2.27 \pm 0.35^*$
5×10^6 oospores/plant	$2.27 \pm 0.40^*$

^y Values are the means $\pm$ standard error of five independent experiments and asterisks indicate significant difference ($P < 0.05$) compared with control according to Dunnett's test.

^z Disease severity was estimated on a 0-to-4 scale, where 0 = no wilting, 1 = 1 to 25% wilting, 2 = 26 to 50% wilting, 3 = 51 to 75% wilting, and 4 = 76 to 100% wilting or dead.

Fig. 5. Population dynamics of *Pythium oligandrum* in rhizosphere soil from **A**, root bases or **B**, root tips of tomato grown for 4 weeks in sterilized field soil initially treated with 5×10^4 , 5×10^5 , or 5×10^6 oospores of *P. oligandrum* per plant, followed by transplanting into sterilized soil. Error bars represent standard error of the means of three independent experiments.

other real-time PCR assays such as SYBR Green I and TaqMan (26). We used *P. oligandrum*-specific primers (10) and hybridization probes (Fig. 1A) designed from the ITS region because ITS-1 and -2 are ideal for the production of specific primers that have potential for differentiating *Pythium* species (19,41). The primer and probe sets of *P. oligandrum* used in this assay were checked against the corresponding nucleotide sequences of other representative *Pythium* spp. belonging to each of 11 phylogenetic clades from A to K, into which Lévesque and de Cock divided 116 species and varieties of *Pythium* based on the ITS region of rDNA. *P. acanthicum* has an ITS sequence very similar to its fellow clade species *P. oligandrum* (19) and has identical HybriOLI-1 and HybriOLI-2 probe hybridization sites (Fig. 1B), but different annealing sites for the primers P.OLIG.F1 and P.OLIG.R04. Thus, the combination of the primers and probes was found to be specific for *P. oligandrum* within the context of the isolates tested in this work. This was borne out by the experimental results, which indicated detection and quantification with high fidelity to *P. oligandrum* among the nine *Pythium* spp., including a *P. acanthicum* isolate, and four non-*Pythium* fungal species. There was no difference in reactivity by any of the D-type or S-type *P. oligandrum* isolates (Table 1). The reaction conditions of the primer and probe sets have been optimized such that they provide an almost linear response and can quantify the DNA concentration of this fungus from 0.1 pg to 1 ng from 25 mg dw of soil. These results suggest that real-time PCR with the above primer and probe sets can be used to quantify the biomass of *P. oligandrum* with highly specificity from soil.

We examined *P. oligandrum* colonization of the tomato rhizosphere using the complementary approaches of nucleotide quantification by real-time PCR and direct observation by confocal laser-scanning microscopy. Complex interactions can take place in the rhizosphere between biocontrol agents and indigenous microbes (43). Although *P. oligandrum* populations from the rhizospheres of tomato grown in the 5×10^6 oospores/plant treatment was lower in unsterilized soil than in sterilized soil, its rhizosphere population increased with the application of at least 5×10^5 oospores/plant (Fig. 3), suggesting that *P. oligandrum* is rhizosphere competent for space (i.e., root zone niches or attachment) and nutrients with native soil microflora when its oospores are directly applied to soil. To confirm this trait in *P. oligandrum*, further studies currently are being undertaken by differentiating the introduced *P. oligandrum* isolate from indigenous isolates in native soils and by determining the rhizosphere populations of this fungus in tomato grown in other soil types under different microfloral regimes. Confocal microscopic observation also showed strong rhizosphere colonization by *P. oligandrum*, in which this fungus developed over the tomato root surface and some hyphae strongly colonized and penetrated into root epidermal cells (Fig. 4). The penetration into tomato root tissues by *P. oligandrum* had been observed with light and transmission electron microscopy of root tissue sections (16,29). The frequency

of *P. oligandrum* colonization observed on the roots obviously was higher when the initial application of oospore concentration was high (Table 2), which corresponds with the result obtained by real-time PCR assays. Martin and Hancock (21) and McQuilken et al. (24) reported that *P. oligandrum* was not rhizosphere competent on sugar beet and cress when their seed were coated with its oospores, which may be due to the differences in oospore treatments (seed coating versus direct soil application). We also determined changes in rhizosphere population after transplanting into sterilized soil, and found that *P. oligandrum* population decreased with time after transplanting, particularly at the root tips (Fig. 5). This result indicates that *P. oligandrum* does not appear to actively spread its population along roots and does not protect over the longer term from root-infecting pathogens with direct competition for infection sites and nutrients.

Larkin and Fravel (14) determined the primary mechanisms of action of three nonpathogenic *Fusarium* isolates (CS-1, CS-20, and Fo47) for the biocontrol of *Fusarium* wilt of tomato by varying the inoculum levels of the biocontrol agent and pathogen in soil and with subsequent mathematical modeling. Isolate CS-20 controlled disease effectively with only 100 chlamydospores/g of soil (c/g) with pathogen concentrations of up to 10^5 c/g, suggesting that this control was largely due to induced resistance. In contrast, Fo47 was effective only at 10^4 to 10^5 c/g, regardless of pathogen density, suggesting that this isolate was active primarily through competition for nutrients (14). It is likely that increasing the propagule density of *P. oligandrum* in the rhizosphere protects plants from disease by increasing the competition for space and nutrients, thereby decreasing the activity of the pathogen. However, in the interactions between *P. oligandrum* and *R. solanacearum* in the tomato rhizosphere, increasing the inoculum concentration of *P. oligandrum* from 5×10^4 oospores/plant to 5×10^6 oospores/plant did not further reduce disease severity (Table 3). The rhizosphere population of *P. oligandrum* of tomato initially treated with 5×10^6 oospores/plant was represented by ≈ 130 pg of *P. oligandrum* DNA/g of dry soil, which is 25 and 130 times higher than those initially treated with 5×10^5 and 5×10^4 oospores/plant, respectively (sterilized soil in Fig. 3). Microscopic observations found that the colonization frequency of the 5×10^4 oospore treatment was only 15% (Table 2), which means that 85% of visual fields on tomato root surface were free of *P. oligandrum*. These *P. oligandrum*-free root areas included the elongation zones and the axils of emerging or developed secondary roots, where *R. solanacearum* preferably colonizes (39). Furthermore, reduction of the pathogen population in the rhizosphere was not significant in the 5×10^4 , 5×10^5 , or 5×10^6 oospore treatments (Table 4). These results indicate that direct competition for infection sites and nutrients is unlikely to be main motive force in the reduction of bacterial wilt by *P. oligandrum*.

Phenolic precursors accumulate within 3 h of inoculation with *P. oligandrum*, followed by rishitin synthesis by 14 h after inoculation (16). Recently, our group found that ethylene- and jasmonic acid-dependent signaling pathways were significantly accelerated in tomato treated with a mycelial homogenate of *P. oligandrum* (12; S. Hase, K. Nakaho, T. Arie, S. Seo, Y. Ohashi, S. Takenaka, and H. Takahashi, *unpublished data*). Furthermore, macroarray analysis indicated that 144 genes, including defense-related genes such as pathogenesis-related protein P14 and class II chitinase genes, were upregulated in tomato roots treated with a cell wall protein fraction of *P. oligandrum* containing two elicitor proteins (32). These results suggest that the main mechanism by which *P. oligandrum* controls tomato bacterial wilt is induced resistance.

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TABLE 4. Rhizosphere soil populations of *Ralstonia solanacearum* of tomato plants initially treated with different concentrations of oospores of *Pythium oligandrum* at 4 days after inoculation^y

Treatment with oospores	<i>R. solanacearum</i> at CFU $\times 10^7$ g ⁻¹ z
Control (0 oospore/plant)	5.64 $\pm$ 4.21 a
5×10^2 oospores/plant	5.31 $\pm$ 3.51 a
5×10^3 oospores/plant	6.02 $\pm$ 4.42 a
5×10^4 oospores/plant	4.03 $\pm$ 2.23 a
5×10^5 oospores/plant	3.29 $\pm$ 1.79 a
5×10^6 oospores/plant	4.18 $\pm$ 2.87 a

^y Tomato plants were grown for 3 weeks and the inoculum concentration of *R. solanacearum* was 5×10^7 cfu per plant.

^z Values are the means $\pm$ standard error of three independent experiments and values followed by a different letter within columns are significantly different using Tukey's studentized range test ($P < 0.05$).

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