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Interactions in the Tomato Rhizosphere of Two *Pseudomonas* Biocontrol Strains with the Phytopathogenic Fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*

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The fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* causes foot and root rot of tomato plants, which can be controlled by the bacteria *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391. Induced systemic resistance is thought to be involved in biocontrol by *P. fluorescens* WCS365. The antifungal metabolite phenazine-1-carboxamide (PCN), as well as efficient root colonization, are essential in the mechanism of biocontrol by *P. chlororaphis* PCL1391. To understand the effects of bacterial strains WCS365 and PCL1391 on the fungus in the tomato rhizosphere, microscopic analyses were performed using different autofluorescent proteins as markers. Tomato seedlings were inoculated with biocontrol bacteria and planted in an *F. oxysporum* f. sp. *radicis-lycopersici*-infested gnotobiotic sand system. Confocal laser scanning microscope analyses of the interactions in the tomato rhizosphere revealed that i) the microbes effectively compete for the same niche, and presumably also for root exudate nutrients; ii) the presence of either of the two bacteria negatively affects infection of the tomato root by the fungus; iii) both biocontrol bacteria colonize the hyphae extensively, which may represent a new mechanism in biocontrol by these pseudomonads; and iv) the production of PCN by *P. chlororaphis* PCL1391 negatively affects hyphal growth and branching, which presumably affects the colonization and infecting ability of the fungus.

The fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* causes tomato foot and root rot (TFRR), which is a serious problem for field and greenhouse crops (Jarvis 1988). Chemical pesticides do not efficiently suppress TFRR (Benhamou et al. 1994). In contrast, the biopesticides *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391 efficiently suppress TFRR (Chin-A-Woeng et al. 1998; Dekkers et al. 2000). The mechanism of biocontrol of TFRR by *P. fluorescens* WCS365 has not been elucidated; however, because it was shown that strain WCS365 triggers induced systemic resistance (ISR) in *Arabidopsis thaliana* (Gerrits and Weisbeek 1996), it is likely that ISR also is the underlying mechanism of biocontrol of TFRR by *P. fluorescens* WCS365 (Dekkers et al. 2000). Strain PCL1119, a mutant derivative of *P. chlororaphis* PCL1391 impaired in the production of the antifungal metabolite phenazine-1-carboxamide (PCN), was not able to suppress TFRR in potting soil, showing that the production of PCN is required for biocontrol (Chin-A-Woeng et al. 1998). Testing of three different competitive root tip colonization mutants of strain PCL1391 showed that efficient colonization of the tomato root system is also essential for suppression of the disease by this strain (Chin-A-Woeng et al. 2000).

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Many genes and traits involved in biocontrol have been identified to explain biocontrol at the molecular level (Bloemberg and Lugtenberg 2001; Lugtenberg et al. 2002; Thomashow and Weller 1996; Whipps 2001). Reports describing interactions between pathogens and control agents at the cellular level are limited (Benhamou and Chet 1993; Benhamou et al. 1997, 1999; Chet et al. 1981; Etchebar et al. 1998; Hogan and Kolter 2002) and reports on the spatiotemporal analysis at the cellular level of the interactions between the biocontrol agent and the phytopathogenic fungus in the rhizosphere are scarce (Bao and Lazarovits 2001; Benhamou et al. 1996). In previous work, we have analyzed, using confocal laser scanning microscope analysis (CLSM), the colonization process of the tomato rhizosphere by *Pseudomonas* biocontrol strains (Bloemberg et al. 1997; Chin-A-Woeng et al. 1997; Dekkers et al. 2000) and by *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi et al. 2002). In the present article, we report, using microbes differentially labeled with autofluorescent proteins, an analysis of the interactions between *F. oxysporum* f. sp. *radicis-lycopersici* and the *Pseudomonas* biocontrol strains WCS365 and PCL1391 in the tomato rhizosphere, with the purpose of obtaining a better understanding of the biocontrol process.

RESULTS

Biocontrol of TFRR by *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 in a gnotobiotic sand–nutrient solution system.

To test the biocontrol ability of *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 (Table 1), tomato seedlings were inoculated with cells of either strain and grown in a gnotobiotic system containing sand infested with *F. oxysporum* f. sp. *radicis-lycopersici* spores. After 4 days, disease symptoms were visible in 40% of the plants. After 7 days of incubation,

the plants were analyzed for disease symptoms and statistical analysis was performed. The presence of the pathogenic fungus caused disease symptoms in 70 to 90% of the plants (Tables 2 and 3) after 7 days. The presence of *P. fluorescens* WCS365 on tomato seedlings reduced the percentage of sick plants considerably, to 0 to 15% (Table 2). Using a χ^2 goodness-of-fit statistical test, two different treatments were compared. Comparison of plants grown in sand containing *F. oxysporum* f. sp. *radicis-lycopersici* spores with and without *P. fluorescens* WCS365 showed that strain WCS365 significantly suppressed TFRR in the gnotobiotic system (Table 2). Inoculation with *P. fluorescens* WCS365 improved the health condition of plants grown in the presence of *F. oxysporum* f. sp. *radicis-lycopersici* up to the level of the seedlings grown only in the presence of *P. fluorescens* WCS365, which were all healthy (Table 2). Inoculation of tomato seedlings with *P. chlororaphis* PCL1391 reduced the percentage of sick plants significantly, to 6 to 15% (Table 3). In addition, PCL1119, a derivative of *P. chlororaphis* PCL1391 in which a promoterless Tn5*luxAB* has been inserted in *phzB* and which, therefore, does not produce PCN, was tested to study the role of PCN production in the biocontrol ability of *P. chlororaphis* PCL1391 in the gnotobiotic system. PCL1119 re-

Table 1. Microorganisms and plasmids

Strains	Relevant characteristics	Reference or source
Bacteria		
WCS365	*Pseudomonas fluorescens;* efficient competitive root colonizer; biocontrol strain of tomato foot and root rot (TFRR) caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*	Dekkers et al. 2000; Simons et al. 1996
PCL1391	*P. chlororaphis;* efficient competitive root colonizer; biocontrol strain of TFRR; produces phenazine-1-carboxamide (PCN)	Chin-A-Woeng et al. 1998, 2000
PCL1119	PCL1391 (phzB::Tn5luxAB); does not produce PCN	Chin-A-Woeng et al. 1998
Fungi		
ZUM 2407	*F. oxysporum* f. sp. *radicis-lycopersici* causing TFRR	IPO-DLO, Wageningen, The Netherlands
FCL14	*F. oxysporum* f. sp. *radicis-lycopersici* ZUM 2407 containing *gfp* under control of the constitutive *gpdA* promoter	Lagopodi et al. 2002
Plasmids		
pMP4662	pME6010 containing *rfp* under the control of the *lac* promoter; is maintained in *Pseudomonas* spp. without antibiotic pressure; Tc^R	Bloemberg et al. 2000

Table 2. Biocontrol of tomato foot and root rot by *Pseudomonas fluorescens* WCS365 in a gnotobiotic sand–nutrient solution system

	Experiment 1			Experiment 2		
Microorganisms present[a]	Healthy	Sick	χ^2 values[b]	Healthy	Sick	χ^2 values[b]
(i) None	20	0	…	20	0	…
(ii) *F. oxysporum*	6	14	…	2	18	…
(iii) *F. oxysporum* + *P. fluorescens*	20	0	…	17	3	…
(iv) *P. fluorescens*	20	0	…	20	0	…
Compared treatments	…	…	…	…	…	…
(ii) and (iii)	…	…	21.54[c]	…	…	22.56[c]
(iv) and (iii)	…	…	0[d]	…	…	3.24[d]

[a] Twenty plants were grown (i) in the absence of microbes, (ii) in the presence of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, (iii) in the presence of both *F. oxysporum* f. sp. *radicis-lycopersici* and *P. fluorescens* WCS365, or (iv) in the presence *P. fluorescens* WCS365. Seven days after plant inoculation, the disease status of the plants was scored.
[b] Statistical analysis of the biocontrol experiment was performed using a χ2 goodness-of-fit test (Heath 1995). Critical χ^2 value = 3.841.
[c] The two compared treatments are significantly different.
[d] The two compared treatments are not significantly different.

Table 3. Biocontrol of tomato foot and root rot by *Pseudomonas chlororaphis* PCL1391 in a gnotobiotic sand–nutrient solution system

	Experiment 1			Experiment 2		
Microorganisms present[a]	Healthy	Sick	χ^2 values[b]	Healthy	Sick	χ^2 values[b]
(i) None	16	0	…	20	0	…
(ii) *F. oxysporum*	4	12	…	2	18	…
(iii) *F. oxysporum* + PCL1391	15	1	…	17	3	…
(iv) *F. oxysporum* + PCL1119	10	6	…	8	12	…
(v) PCL1391	16	0	…	20	0	…
PCL1391 compared						
(ii) and (iii)	…	…	15.81[c]	…	…	22.56[c]
(v) and (iii)	…	…	1.03[d]	…	…	3.24[d]
PCL1119 compared						
(ii) and (iv)	…	…	4.57[c]	…	…	4.8[c]
(iii) and (iv)	…	…	4.57[c]	…	…	8.64[c]
(v) and (iv)	…	…	7.38[c]	…	…	17.14[c]

[a] Plants (n = 16 or 20 in experiment 1 or 2, respectively) were grown (i) in the absence of microbes, (ii) in the presence of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, (iii) in the presence of both *F. oxysporum* f. sp. *radicis-lycopersici* and *P. chlororaphis* PCL1391, (iv) in the presence of both *F. oxysporum* f. sp. *radicis-lycopersici* and *P. chlororaphis* PCL1119, or (v) in the presence *P. chlororaphis* PCL1391. Seven days after plant inoculation, the disease status of the plants was scored.
[b] Statistical analysis of the biocontrol experiment was performed using a chi-squared goodness-of-fit test (Heath, 1995). Critical χ^2 value = 3.841.
[c] The two compared treatments are significantly different.
[d] The two compared treatments are not significantly different.

duced the percentage of sick plants significantly, to 38 to 60% (Table 3). The disease reduction caused by *P. chlororaphis* PCL1119 was significantly less than the reduction caused by *P. chlororaphis* PCL1391 (Table 3).

Spatiotemporal analysis of interactions between the biocontrol bacteria *P. fluorescens* WCS365, *P. chlororaphis* PCL1391, and *F. oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere.

Using CLSM, we visualized *F. oxysporum* f. sp. *radicis-lycopersici* and the *Pseudomonas* biocontrol bacteria simultaneously in the tomato rhizosphere. To distinguish the bacteria and the fungus, green fluorescent protein (GFP)-labeled *F. oxysporum* f. sp. *radicis-lycopersici* derivative FCL14 (Lagopodi et al. 2002) and DsRed-labeled (encoded by *rfp*) *P. fluorescens* WCS365 or *P. chlororaphis* PCL1391 harboring the plasmid pMP4662 (Bloemberg et al. 2000) were used.

After inoculation of tomato seedlings with either *P. fluorescens* WCS365 or with *P. chlororaphis* PCL1391 and subsequent plant growth in the gnotobiotic sand system, bacterial cells were detected on the main root and on root hairs. After 3 days of plant growth, single bacterial cells were observed on

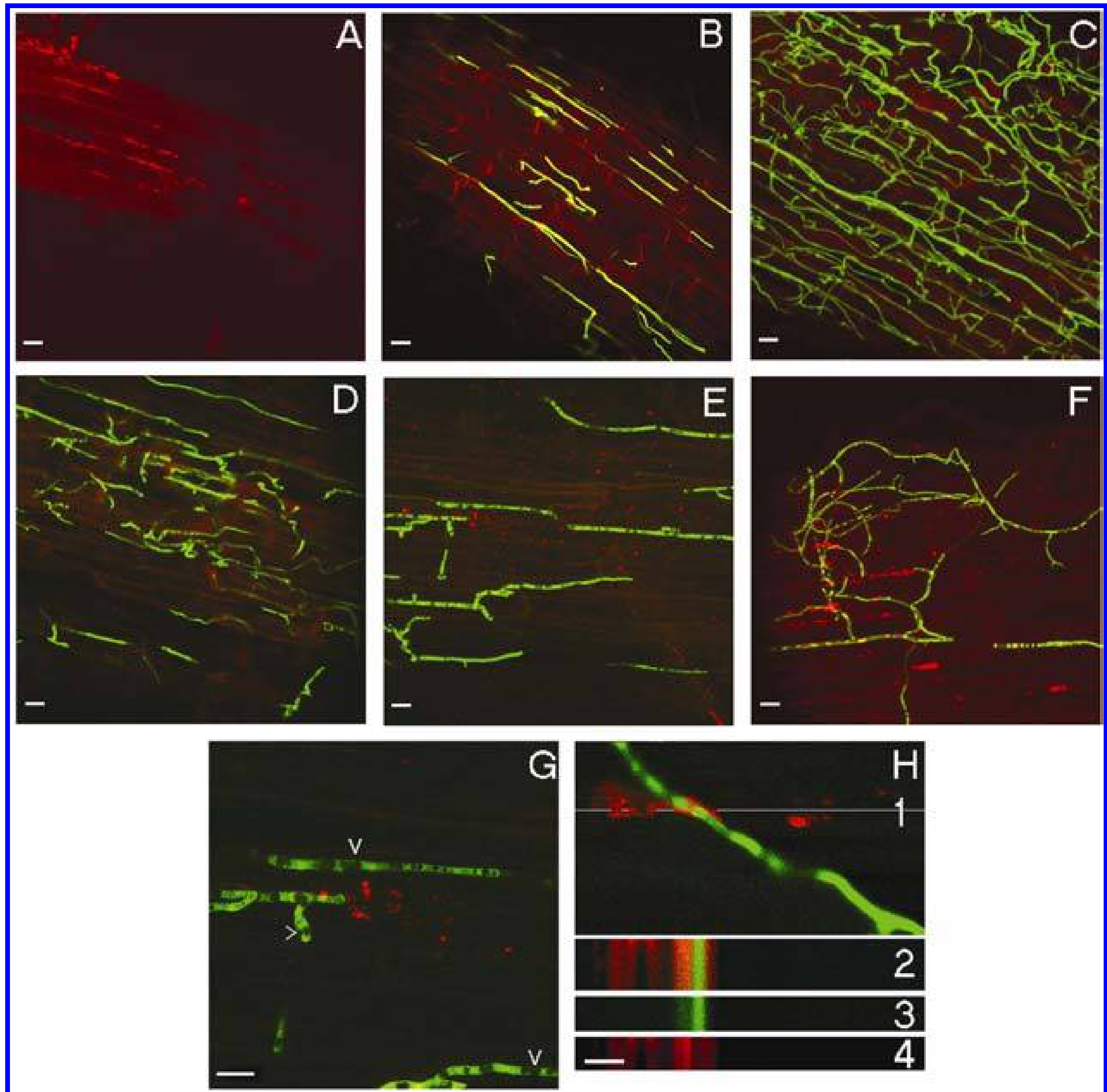

Fig. 1. Confocal laser scanning microscope analysis of tomato root colonization by the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* and by *Pseudomonas* biocontrol bacteria. Two-day-old tomato seedlings were inoculated at time zero with cells of either *Pseudomonas fluorescens* WCS365 or *P. chlororaphis* PCL1391 harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F. oxysporum* f. sp. *radicis-lycopersici* harboring a constitutively expressed *gfp* gene. Walls of tomato root cells appear as red due to autofluorescence. **A,** *P. fluorescens* WCS365 colonizing the intercellular junctions of root cells of an inoculated seedling planted in sterile sand 3 days after planting. **B,** *F. oxysporum* f. sp. *radicis-lycopersici* hyphae growing along the intercellular junctions of root cells of a sterile seedling 3 days after planting in sand containing fungal spores. **C,** Hyphal network present in the rhizosphere of a sterile seedling planted in sand containing *F. oxysporum* f. sp. *radicis-lycopersici* spores 7 days after planting in the absence of biocontrol bacteria; **D,** in the presence of *P. fluorescens* WCS365; **E,** in the presence of *P. chlororaphis* PCL1391; and **F,** in the presence of *P. chlororaphis* PCL1119. **G,** Vacuoles (indicated by arrowheads) abundantly present in hyphae in the rhizosphere of seedlings inoculated with *P. chlororaphis* PCL1391 3 days after planting. **H,** *P. chlororaphis* PCL1391 attached to fungal hyphae 3 days after inoculation. The lower part of the panel (2–4) is a cross section in the Z direction at the white line in the upper part (1) showing the attachment. 2, Both the fungus and the bacteria. 3, The green fluorescent protein signal of the fungus. 4, The DsRed signal of the bacteria. The size bar represents 10 μm in all panels.

the 7-cm-long roots from the crown to 2.5 cm above the root tip; the cells were present predominantly along the junctions of the epidermal cells (Fig. 1A). Small microcolonies were formed on the upper half of the root. After 6 days of growth, microcolonies on the upper half of the root were estimated to be increased up to four times in both number and length.

Seedlings coated with either *P. fluorescens* WCS365 or with *P. chlororaphis* PCL1391 grown in *F. oxysporum* f. sp. *radicis-lycopersici*-infested sand were examined four times (three seedlings per condition). Focusing on regions of the main root where either the fungus was most abundantly present or where both the fungus and the bacteria were present and interactions were visible, showed the following. i) The colonization pattern of the *Pseudomonas* biocontrol bacteria was unaffected by the presence of the phytopathogenic fungus over 14 days. ii) The bacteria reached the root surface earlier and proliferated faster than the fungus. The bacteria were already visible within 24 h, whereas the fungus could be observed only after 48 to 72 h. iii) The bacteria occupied the same niches as the fungus (compare Fig. 1A and B). iv) During the first 3 days, the attachment and initial growth of the fungus along the cellular junctions of the tomato root were not affected by the presence of the biocontrol bacteria. v) In contrast, after 7 days, the density of the hyphal network, expressed as the number of fluorescent pixels per square centimeter of root, was strongly reduced by the presence of the biocontrol bacteria (Table 4). *P. Fluorescens* WCS365 and *P. chlororaphis* PCL1391 reduced the hyphal network up to five times (compare Fig. 1C with D and E, respectively), whereas the reduction by *P. chlororaphis* PCL1119 was less strong, three times (compare Fig. 1C with F). In two other experiments, the density of the hyphae also was reduced, although to a lesser extent (Table 4). vi) In the close vicinity of the *Pseudomonas* biocontrol bacteria, penetration of the tomato root by the fungus was not observed. vii) At 3 to 4 days after planting, the *Pseudomonas* biocontrol bacteria caused an increase of the number of vacuoles within the fungal hyphae (Fig. 1G). viii) After 3 days, the bacteria attached to and subsequently colonized the hyphae, as was shown by a three-dimensional analyses making Z-sections (Fig. 1H). ix) At 10 days after inoculation, the bacteria were found predominantly around the hyphae and they had further colonized the hyphae (compare Fig. 2A with B).

Spatiotemporal analysis of interactions between *P. chlororaphis* PCL1391 and *F. oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere.

The observations described below were observed for *P. chlororaphis* PCL1391 but were not seen in the presence of *P. fluorescens* WCS365. i) After 7 days, *P. chlororaphis* PCL1391 caused an increase of the thickness of part of the hyphae (Fig. 2C). The strain also caused a disturbance of hyphal growth directionality, resulting in ii) curly growth of hyphae growing along the intercellular junctions of the plant root after 9 days (Fig. 2D) and iii) abrupt changes in the growth direction after 10 days (Fig. 2E). iv) An increase of the frequency of hyphal branching was found after 10 days. v) After 13 days, fork-like branching structures of some hyphae were observed (Fig. 2F).

Analysis of *P. chlororaphis* PCL1119, which does not produce PCN, revealed that hyphal growth, morphology, and branching was altered. Compared with its wild type, the hyphal network was less strongly reduced (Table 4). The increase in the number of vacuoles within the fungal hyphae was observed 1 day later compared with its wild type (Table 5). The increase in hyphal thickness, the abrupt changes in the growth direction, and the increase of the frequency of hyphal branching were observed 3 days later (Table 5). Curly growth of hyphae was not observed along the cellular junctions (Fig. 2G) and the fork-like branching structures consisted of two branched hyphae; whereas, in the presence of its wild type, the structures consisted of three branched hyphae (Fig. 2H).

Analysis of *F. oxysporum* f. sp. *radicis-lycopersici* growth in the tomato rhizosphere in presence of purified PCN.

Tomato plants were grown in the gnotobiotic system containing sand infested with *Fusarium* spores. After 3 days of growth, the plants were gently taken out of the gnotobiotic system, without removing the sand adhering to the root, and transferred to plant nutrient solution (PNS)-agar plates. At this time, the fungus had attached to the tomato root and started to grow along the cellular junctions of the tomato

Table 4. Reduction of the hyphal network by *Pseudomonas* biocontrol bacteria in a gnotobiotic sand–nutrient solution system

	Fluorescent pixels[a]					
	Experiment 1		Experiment 2		Experiment 3	
Microorganisms present	**No.**	**%**	**No.**	**%**	**No.**	**%**
(i) *F. oxysporum*	11.016	100	113.846	100	25.337	100
(ii) *F. oxysporum* + *P. fluorescens* WCS365	2.370	22	55.793	49	…	…
(iii) *F. oxysporum* + *P. chlororaphis* PCL1391	2.213	20	…	…	7.749	31
(iv) *F. oxysporum* + *P. chlororaphis* PCL1119	3.551	32	…	…	13.737	54

[a] Density of the hyphal network in the presence of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and in the (i) absence of *Pseudomonas* bacteria, (ii) presence of *P. fluorescens* WCS365 (iii), presence of *P. chlororaphis* PCL1391, and (iv) presence of *P. chlororaphis* PCL1119. Density is expressed as the number of fluorescent pixels per square centimeter of root and as a percentage of the network in the absence of *Pseudomonas* bacteria (i).

Table 5. Effects of the *Pseudomonas* bacteria on fungal growth in the gnotobiotic sand–nutrient solution system

	Day observed		
Effects in tomato rhizosphere	***P. fluorescens* WCS365**	***P. chlororaphis* PCL1391**	***P. chlororaphis* PCL1119**
Reduction of hyphal network	Day 7 (51–78%)	Day 7 (69–80%)	Day 7 (46–68%)
Vacuole formation	Day 4	Day 3	Day 4
Attachment to fungal hyphae	Day 3	Day 3	Day 3
Increase in hyphal diameter	…	Day 7	Day 10
Curly growth along cellular junction	…	Day 9	…
Abrupt changes in growth direction	…	Day 10	Day 13
Increased branching frequency	…	Day 10	Day 13
Altered branching structures	…	Day 13, three branches	Day 13, two branches

root. A solution of purified PCN in ethyl acetate was spotted onto the root. As a control, ethyl acetate was applied. This had no effect on the growth. CLSM studies of fungal hyphae in the tomato rhizosphere showed that, in the direct presence of purified PCN, alterations in hyphal growth took place similar to those in the presence of *P. chlororaphis* PCL1391 (Table 5). i) The presence of PCN caused an increase in the number of vacuoles after 4 h (compare the growth in the absence of PCN in Figure 3A and B with that in the presence of PCN in Figure 3C). ii) An increase in the hyphal diameter (Fig. 3D), iii) abrupt changes in the growth direction (Fig. 3E), iv) increased branching frequencies (Fig. 3F), and v) altered branching structures (Fig. 3G) were observed after 1 day. vi) Curly growth was observed after 3 days (Fig. 3H). Analysis of the tomato rhizosphere 2 cm (or more) from the PCN-inoculation spot showed that hyphal growth was not altered at these sites.

In vitro analysis of interactions between biocontrol strains and *F. oxysporum* f. sp. *radicis- lycopersici*.

To facilitate the interpretation of the effects on fungal growth by the *Pseudomonas* biocontrol strains in the rhizosphere as described above, in vitro experiments were performed in which *F. oxysporum* f. sp. *radicis-lycopersici*

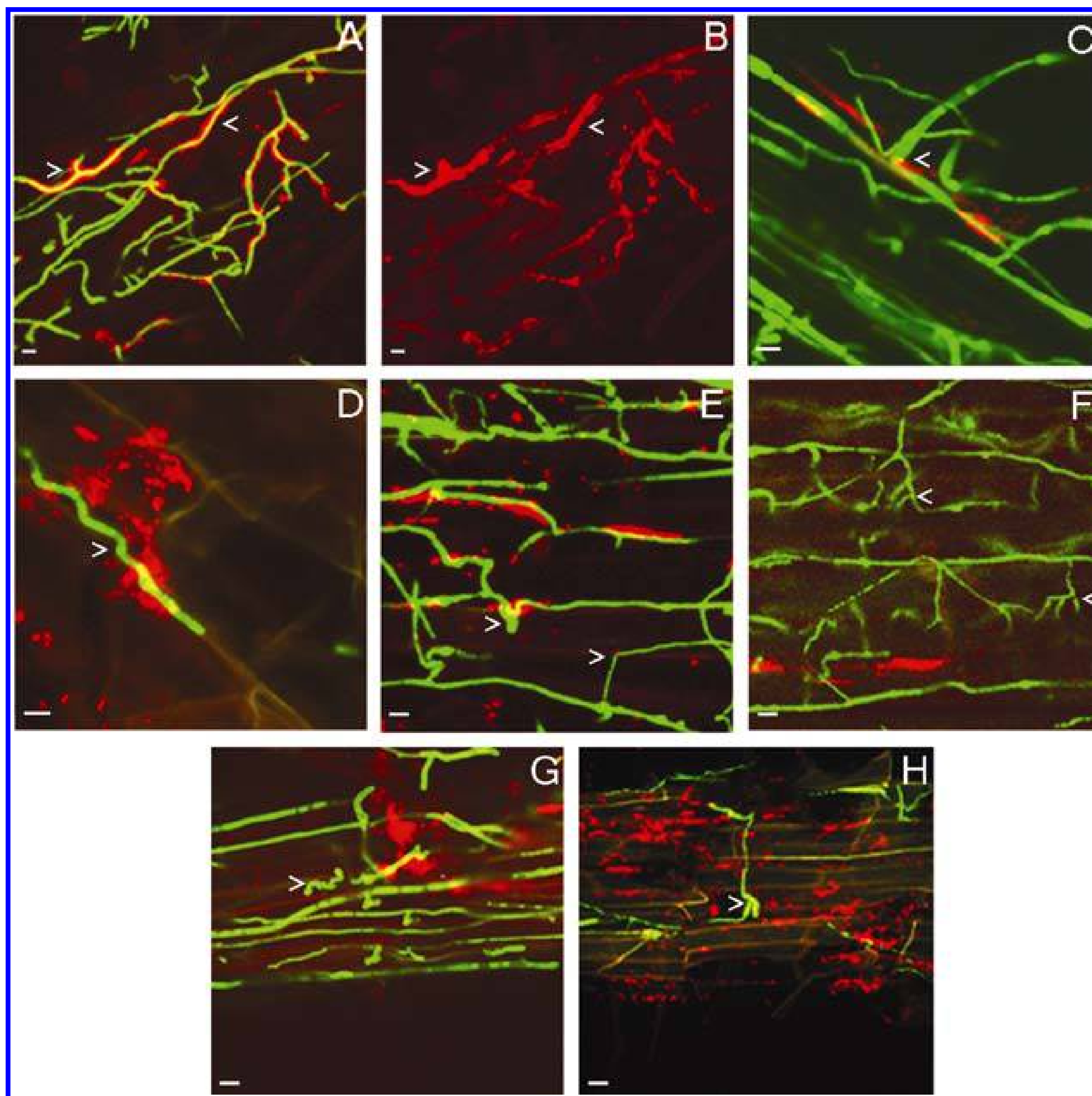

Fig. 2. Confocal laser scanning microscope analysis of effects of the presence of *Pseudomonas chlororaphis* PCL1391 and PCL1119 cells on growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere. Two-day-old tomato seedlings were inoculated at time zero with *P. chlororaphis* PCL1391 cells harboring a reporter plasmid expressing the *rfp* gene, which here appear as red cells. Plants were grown in a gnotobiotic sand system containing spores of *F. oxysporum* f. sp. *radicis-lycopersici* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A,** *P. chlororaphis* PCL1391 cells concentrating around the hyphae and colonizing *F. oxysporum* f. sp. *radicis-lycopersici* hyphae 10 days after inoculation. **B,** Same picture as A without the green fluorescent protein signal showing that all bacterial cells are attached to the fungal hyphae. **C,** In the presence of strain PCL1391, an increase of the diameter of hyphae (indicated by arrowheads) was observed after 7 days. **D,** Curly growth of hyphae along the cellular junction of the tomato root was observed in close vicinity of PCL1391 cells, 9 days after planting. **E,** In the presence of strain PCL1391, abrupt changes in the growth direction of hyphae (indicated by arrowheads) were observed after 10 days. **F,** Branching of *F. oxysporum* f. sp. *radicis-lycopersici* hyphae resembles fork-like structures (indicated by arrowheads) in the presence of strain PCL1391 13 days after inoculation. **G,** Hyphal growth in the presence of strain PCL1119 in the rhizosphere. **H,** Branching of *F. oxysporum* f. sp. *radicis-lycopersici* hyphae resembles fork-like structures at lower frequency in the presence of strain PCL1119 13 days after inoculation. The size bar represents 10 μm in all panels.

was grown on Luria Bertani (LB) agar in the vicinity of *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391. The fungus and the bacteria were spotted next to each other on agar and subsequently allowed to grow. Differential interference contrast (DIC) microscopy studies of *F. oxysporum* f. sp. *radicis-lycopersici* growing in the absence of *Pseudomonas* spp. showed straight, radially orientated hyphae (Fig. 4A). The presence of *P. fluorescens* WCS365 cells had no visible effects on growth, branching, and morphology of fungal hyphae, which finally grew over the bacterial clump (*data not shown*). The presence of *P. chlororaphis* PCL1391 caused a strong inhibition zone of fungal growth. Microscopy studies focused on the inhibition region near the hyphal tips growing toward the *P. chlororaphis* PCL1391 cells (Fig. 4B). The following effects on *F. oxysporum* f. sp. *radicis-lycopersici* hyphae growing toward *P. chlororaphis* PCL1391 were observed. i) The hyphae lost the radial growth orientation and grew in different directions (compare Fig. 4A with B and C). ii) Approximately 1% of the hyphae showed looping growth (Fig. 4D). iii) Branching of the hyphae was observed approximately 10 times more frequently and closer to the hyphal tip than when bacteria were not present (compare Fig. 4A with E). iv) Structures very similar to chlamydospores were observed (Fig. 4F); however, their size is bigger. Therefore, these chlamydospores also could be swollen bodies. To analyze the role of PCN in the above-described effects on hyphal growth, branching, and morphology, we studied growth of fungal hyphae on agar in the vicinity of a PCN-negative mutant *P. chlororaphis* PCL1119. Hyphae growing toward strain PCL1119 were straight and radially orientated, as in the absence of bacteria (Fig. 4G). To further analyze the role of PCN, hyphae were allowed to grow toward purified

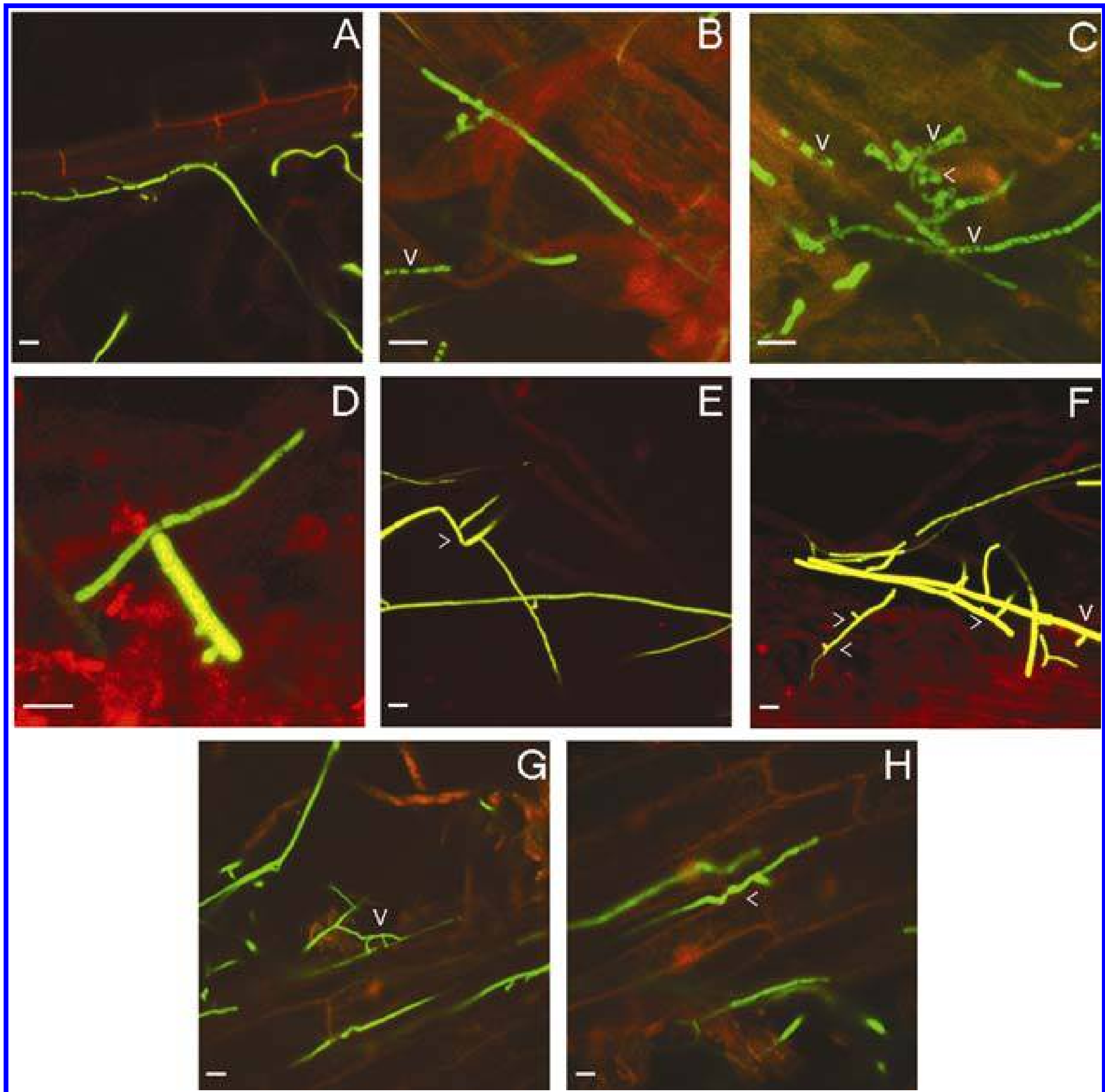

Fig. 3. Confocal laser scanning microscope analysis of effects of the presence of purified phenazine-1-carboxamide (PCN) on growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in the tomato rhizosphere. Plants were grown in a gnotobiotic sand system containing spores of *F. oxysporum* f. sp. *radicis-lycopersici* harboring a constitutively expressed *gfp* gene. Cell walls of the tomato root appear as red due to autofluorescence. **A** and **B,** Hyphal growth in the presence of ethyl acetate. **C,** An increase in the number of vacuoles was observed after 4 h in the presence of PCN. **D,** An increase in the hyphal diameter was observed after 1 day. **E,** Abrupt changes in the growth direction was observed after 1 day of incubation. **F,** Increased branching frequencies were observed after 1 day. **G,** Altered branching structures were observed after 1 day. **H,** Curly growth was observed after 3 days. The size bar represents 10 μm in all panels.

PCN. Purified PCN (0.2 mg) spot inoculated on the agar surface caused an inhibition zone of fungal growth and had effects on hyphal growth, branching, and morphology similar to those of the presence of cells of *P. chlororaphis* PCL1391. The hyphae i) lost the radial growth orientation (Fig. 4H and I) and showed ii) looping growth (Fig. 4J) and iii) an increased branching frequency (Fig. 4K), whereas iv) chlamydospore-like structures were observed (Fig. 4L).

DISCUSSION

Visualization of biocontrol of TFRR by *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 in a gnotobiotic sand–nutrient solution system.

TFRR is an important disease caused by the soilborne pathogen *F. oxysporum* f. sp. *radicis-lycopersici*. The process of colonization and infection of the tomato root has been studied

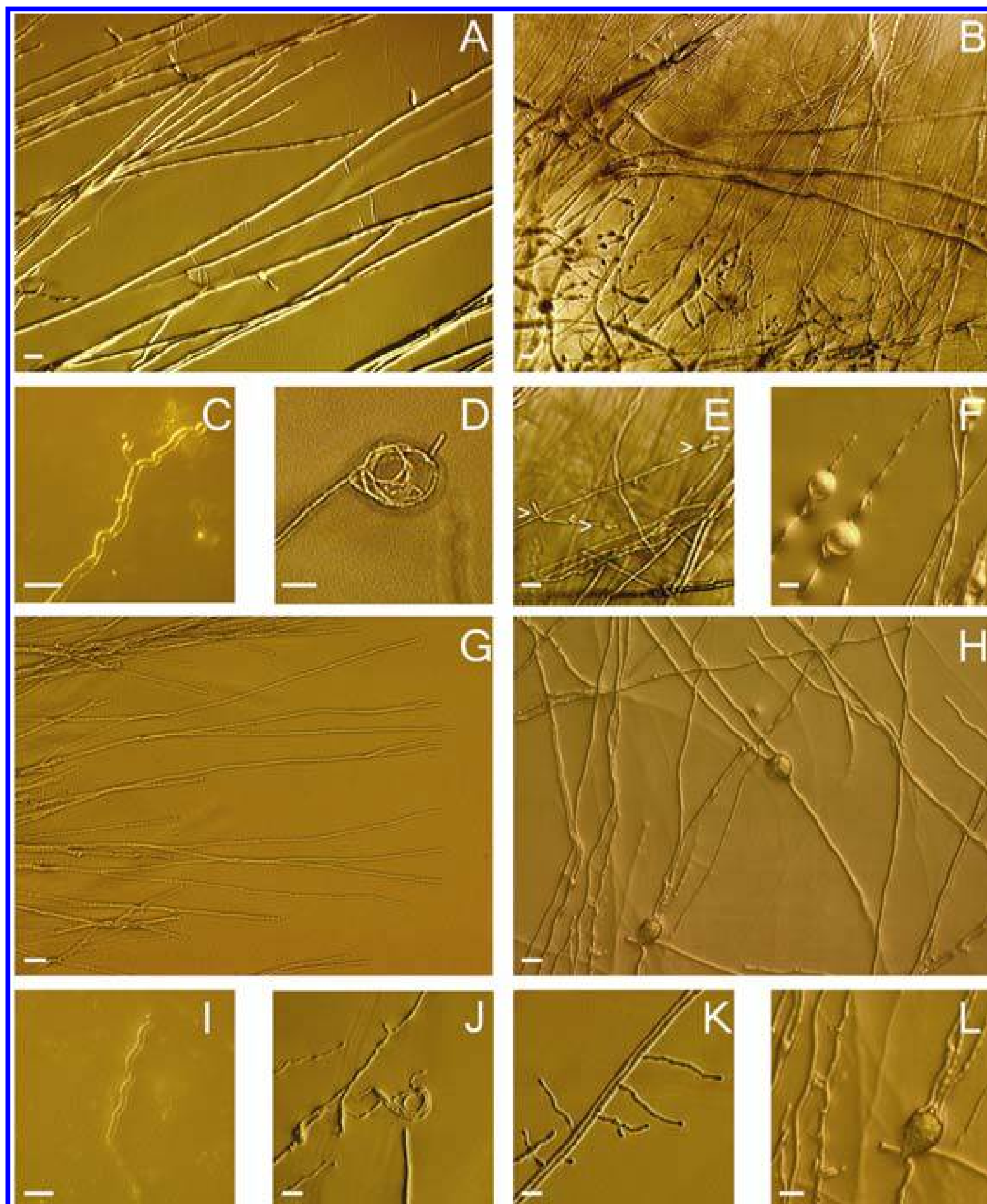

Fig. 4. Differential interference contrast microscopy analysis of in vitro effects of *Pseudomonas chlororaphis* PCL1391 on hyphal growth and spore formation by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *F. oxysporum* f. sp. *radicis-lycopersici* was grown in the vicinity of *P. chlororaphis* PCL1391, *P. chlororaphis* PCL1119, or purified phenazine-1-carboxamide (PCN), on microscopy glass slides covered with a thin layer of Luria Bertani agar. Three days after growth, *F. oxysporum* f. sp. *radicis-lycopersici* hyphae were examined for effects on growth and spore formation. **A,** Growth of *F. oxysporum* f. sp. *radicis-lycopersici* in the absence of bacteria. **B–F,** Growth of *F. oxysporum* f. sp. *radicis-lycopersici* towards *P. chlororaphis* PCL1391, which is located (outside the picture) in the upper right corner. **B,** Overview of the region close to the inhibition zone caused by PCL1391. **B–D,** Disturbance of hyphal growth directionality. **E,** Frequent branching close to the hyphal tip. **F,** Chlamydospores, observed within the hyphae. **G,** Overview of the region close to PCL1119, which is located (outside the picture) on the right. **H–L,** Growth of *F. oxysporum* f. sp. *radicis-lycopersici* toward purified PCN, which is located (outside the picture) in the upper right corner. **H,** Overview of the region close to the inhibition zone caused by PCN. **H–J,** Disturbance of hyphal growth directionality. **K,** Frequent branching close to the hyphal tip. **L,** Chlamydospres, observed within the hyphae. The size bar represents 10 µm in all panels.

(Brammall and Higgins 1988; Charest et al. 1984). Recently, more details were revealed using GFP-labeled *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi et al. 2002). Coating of seed or seedlings with the bacteria *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 can efficiently control TFRR in soil infested with *F. oxysporum* f. sp. *radicis-lycopersici* spores (Chin-A-Woeng et al. 1998; Dekkers et al. 2000).

In the present work, we visualized the interactions between the fungus and the bacteria in the tomato rhizosphere in order to get a better understanding of the biocontrol process. The microbes were differentially labeled with autofluorescent proteins to clearly visualize and distinguish them simultaneously. The GFP labeling does not affect the pathogenicity of the fungus (Lagopodi et al. 2002) and DsRed labeling does not cause a genetic or metabolic burden on the bacteria (Bloemberg et al. 2000). Quartz sand was used because it has the advantage that it can be removed easily from the tomato roots by gentle washing, after which the roots can be examined. In contrast, potting soil cannot easily be removed from the roots and subsequent CLSM studies are hampered by autofluorescence of small soil particles (Bloemberg et al. 1997; Chin-A-Woeng et al. 1997; Lagopodi et al. 2002). In addition, the quartz sand system enables us to focus better on the interactions between the two microbes directly involved in biocontrol due to the absence of other rhizosphere microorganisms, which are present in nonsterile soil systems. It should be noted that, presumably due to the absence of competing indigenous bacteria, the use of quartz sand results in a very high disease pressure (70 to 90%) and very efficient biocontrol (Tables 2 and 3).

In potting soil, it was it was found that, in contrast to wild-type strain PCL1391, mutant PCL1119 did not cause significant biocontrol (Chin-A-Woeng et al. 1998). In the gnotobiotic system, we saw again a strong effect of PCN production on biocontrol (Table 3) as well as a stronger reduction of the hyphal network (Table 5) and an acceleration of the stress responses in the fungus (Table 5). However, in contrast to in potting soil, there is a significant effect of the PCN-negative mutant PCL1119 on biocontrol in the gnotobiotic system (Table 3). This likely is due to the result of the absence of indigenous bacteria which allows higher levels of PCL1119, which is a derivative of the efficient root colonizing strain PCL1391. Therefore, we attribute the significant control activity of strain PCL1119 in the gnotobiotic system to its competition for niches and nutrients.

Colonization of the tomato root by the *Pseudomonas* biocontrol bacteria and the pathogenic fungus.

There are several major effects of inoculation of seedlings with either of the two biocontrol bacteria grown in quartz sand infested with *F. oxysporum* f. sp. *radicis-lycopersici* spores. i) The root colonization behavior of the bacteria was hardly influenced by the presence of the fungus. ii) The bacteria reached the root surface earlier and multiplied faster than the fungus. Chemotaxis toward root exudate compounds (de Weert et al. 2002) are likely to play a role in the former process. iii) Bacteria and hyphae colonize the same niches on the tomato root; namely, the intercellular junctions (compare Fig. 1A and B). This may be due to chemotaxis toward, and utilization of, exudate compounds that are supposed to be exuded preferentially at those niches (Campbell and Greaves 1990). iv) After 3 days, no effect of the bacteria on fungal growth was observed; however, after 7 days, the density of the hyphae in the rhizosphere was reduced approximately fivefold by the presence of the biocontrol bacteria (compare Fig. 1C with D and E). The fact that this effect only was observed in later stages can be explained in a number of ways. i) In earlier stages, the bacterial numbers are lower and, therefore, have less competitive capacity. ii) After 4 days, 40% of the plants (in absence of bacteria) show disease symptoms such as small brown lesions on the root. It is likely that cell fluids are leaking at these sites and, consequently, more nutrients become available for the microbes at the root surface, resulting in the fast increasing density of bacteria and hyphae. In the presence of bacteria, these sites with high densities of hyphae are absent (Fig. 1D, E, and F), suggesting that the presence of bacteria prevents the formation of severe lesions by the fungus. iii) Possibly, the sites where exudates are leaking from the tomato root are vulnerable and can be penetrated easily by the fungus. By colonizing these sites and utilizing the exudate nutrients, the bacteria prevent colonization and, therefore, penetration of the fungus. iv) Another possible factor could be that the bacteria have utilized or degraded a signal required for colonization of the epidermis by hyphae. v) Hyphae were colonized by the bacterial strains from day three on (Figs. 1H and 2A). vi) All three bacteria cause an increase in the number of vacuoles in the hyphae (Fig. 1G), which is indicative for acceleration of the aging process of the hyphae (Moore-Landecker 1996). Possibly this reduces the aggressive action of the fungus toward the plant as well. vii) Finally, strain PCL1391 caused a number of specific morphological alterations (Table 5), of which many can be explained by the fact that PCL1391 produces the antifungal metabolite PCN (Fig. 2C–F), because the purified PCN compound altered the growth and morphology of hyphae both in vitro (Fig. 4H–L) and in vivo (Fig. 3C–H). The lack of PCN production in strain PCL1119 caused a delay in the appearance of the morphological alterations of hyphae (Table 5). We speculate that the production of extracellular enzymes, such as chitinase, protease, lipase, and HCN (Chin-A-Woeng et al. 1998), affect growth and morphology of the fungus as well.

Mechanism of action of PCN.

Knowledge of the basic principles of hyphal growth and the possible mechanism by which PCN acts may contribute to our understanding of how PCN causes these stress responses. Wessels (1986) reported that polarized growth (i.e., endogenous electrical currents) is the basis of hyphal elongation and branching. When these electrical currents are altered, hyphal growth and branching will be influenced. The exact growth-inhibiting mechanism of PCN is unknown. However, Hernandez and Newman (2001) reported that phenazine compounds can function as electron shuttles. Such an activity of PCN could affect the endogenous electrical currents in the hyphae and could, thereby, affect hyphal growth and branching. As a result, the fungus could be affected in its colonization ability and, consequently, be reduced in its pathogenicity. Disruption of polarized growth also is described by Gadd and associates (2001). In their study, they showed that cadmium reduces the hyphal length and increases branching frequency. They postulate that cadmium affects the mechanisms which maintain the electrochemical gradients across the apex, which may be involved in polarized growth. Such an action of PCN would explain the following observed in vivo effects (Table 5): hyphal swelling (Fig. 2C), disturbance in hyphal growth directionality (Fig. 2D and 2E), increased frequency of branching, and altered branching structures (Fig. 2F). Of these, the latter also could be a response of the fungus to the presence of the bacteria, which occupy penetration sites, to search for free penetration sites.

Mechanisms of biocontrol.

The present results contribute to our insight in the mechanism of action of the two biocontrol strains. i) Strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 are the best

competitive tomato root tip colonizers (Chin-A-Woeng et al. 1998, 2000; Simons et al. 1996) we have tested so far. Consistent with this is the observation that both strains colonize the root surface quickly in comparison with *F. oxysporum* f. sp. *radicis-lycopersici* (discussed above). In the case of strain PCL1391, colonization is a prerequisite for biocontrol because three (competitive) root tip colonization mutant derivatives tested had no biocontrol activity (Chin-A-Woeng et al. 1998). Similar mutant studies showed that colonization is not or is less important for strain WCS365 (Dekkers et al. 2000). The observed fast colonization of the tomato root by *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 explains the observed decreased occupation of the plant root by *F. oxysporum* f. sp. *radicis-lycopersici* (Fig. 1D and E) due to competition with the fungus for niches and nutrients. Considering the results of Dekkers and associates (2000), it is doubtful whether this contributes substantially to biocontrol by strain WCS365. ii) ISR is supposed to play a major role in the mechanism used by *P. fluorescens* WCS365 for biocontrol (Dekkers et al. 2000; Gerrits and Weisbeek 1996). ISR plays no major role in biocontrol by *P. chlororaphis* PCL1391, because its colonization mutants did not show biocontrol anymore. No differences between the effects on the fungus by strains WCS365 and PCL1391 were observed that could be related to ISR. It cannot be excluded that the ISR effects of strain WCS365 are more or less compensated for by the antibiosis effect of strain PCL1391. iii) Starting at day three, colonization of *F. oxysporum* f. sp. *radicis-lycopersici* hyphae by cells of both biocontrol bacteria in the tomato rhizosphere was observed. Although experimental evidence is lacking, it seems likely to us that colonization of hyphae by biocontrol bacteria (Fig. 2A, B, D, and E) must negatively affect their pathogenic abilities. Therefore, colonization of hyphae may be a new mechanism contributing to biocontrol. *P. fluorescens* WCS365 shows a chemotactic response toward the culture supernatant of the *F. oxysporum* f. sp. *radicis-lycopersici* (S. de Weert, *personal communication*); therefore, it is likely that attraction of the bacteria by fungal secondary metabolites is involved in colonization of the fungus. Colonization of hyphae by biocontrol bacteria is likely to enhance biocontrol in case bacteria produce molecules toxic for the fungus, such as PCN, chitinase, and protease produced by strain PCL1391 (Chin-A-Woeng et al. 1998). Strikingly, a correlation between colonization of *Candida albicans* hyphae by *P. aeruginosa* and the subsequent killing of the fungus was described recently by Hogan and Kolter (2002). Such an attack would result in the generation of nutrients; therefore, this could explain the high bacterial numbers on the hyphae (Fig. 1B). To analyze the contribution of hyphal colonization to biocontrol, the molecular interactions between the bacteria and fungal hyphae will be studied in the near future. iv) No indication exists that *P. fluorescens* WCS365 produces antifungal metabolites. In contrast, the production of PCN is essential for the biocontrol ability of *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998). In vitro studies showed that both strain PCL1391 and purified PCN caused disturbance of hyphal growth directionality (Fig. 4B–D and H–J) and increased hyphal branching (Fig. 4E and K). This strongly suggests that PCN is the causal agent for the stress responses observed in vitro.

Overall, the results suggest that, in the case of strain WCS365 in addition to ISR (Gerrits and Weisbeek 1996), the colonization of hyphae and, possibly, to a lesser extent, competition for niches and nutrients may play a role in the biocontrol of TFRR. In the case of strain PCL1391, competition for niches and nutrients, the production of PCN, and, possibly, the production of extracellular enzymes and the colonization of hyphae play a role in its mechanism of biocontrol of TFRR.

MATERIALS AND METHODS

Microorganisms and growth conditions.

The microorganisms used are listed in Table 1. *Pseudomonas* spp. were routinely cultured in King's medium B (King et al. 1954) at 28°C. When appropriate, tetracycline was added to a final concentration of 80 µg/ml. *F. oxysporum* f. sp. *radicis-lycopersici* was cultured on potato dextrose agar (Difco Laboratories, Detroit) or shaken at 130 to 160 rpm in Armstrong medium (Singleton et al. 1992) for 2 days at 28°C.

Purification of phenazine-1-carboxamide.

Phenazine-1-carboxamide produced by *P. chlororaphis* PCL1391 was purified as described by Chin-A-Woeng and associates (1998) with minor modifications. *P. chlororaphis* PCL1391 was grown for 3 days in King's medium B at 28°C with shaking at 150 rpm. After removal of cells by centrifugation for 20 min at 6,000 rpm, the cell-free supernatant was extracted using an equal volume of toluene. The extracted material was concentrated by evaporation in vacuo and dissolved in acetonitrile. The dissolved extracted material was fractionated by high-performance liquid chromatography, using an Alltech Hypersil ODS 5-µm, 250-by-4.6-mm column (Alltech Associates, Deerfield, IL, U.S.A.) and a linear 18 to 80% (vol/vol) gradient of acetonetrile in water, with 0.1% (vol/vol) trifluoracetic acid and a flow rate of 1 ml/min (Fernandez and Pizarro 1997; Watson et al. 1986). UV detection was performed with a Pharmacia RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wavelength scanning from 190 to 400 nm. The peak corresponding to PCN was collected, dried in vacuo, and dissolved in ethyl-acetate to a concentration of 22 mg/ml.

Biocontrol.

F. oxysporum f. sp. *radicis-lycopersici* spores were isolated and mixed with quartz sand as described by Lagopodi and associates (2002). *Pseudomonas* spp. were grown overnight in King's medium B (King et al. 1954) at 28°C under vigorous shaking. Bacterial cells (1 ml) of overnight cultures culture were washed and resuspended in 1 ml of phosphate-buffered saline (PBS) (Sambrook et al. 1989). The cell suspension was diluted with PBS to an optical density of 620 nm of 0.1 and used for inoculating tomato (*Lycopersicon esculentum* Mill cv. Carmello) seedlings as described by Simons and associates (1996).

Tomato seed (kindly provided by Dr. R. Scheffer, Syntenga, Enkhuizen, The Netherlands) were sterilized (Simons et al. 1996) and incubated at 4°C for 5 days on PNS (Hoffland 1989), solidified with 1.8% agar. The seed were incubated for 2 days at 28°C to allow germination. The seedlings were coated with bacteria by incubating the seedlings for 15 min in the bacterial suspension prepared as described above.

The spatiotemporal analyses as well as the biocontrol experiments were performed in a gnotobiotic quartz sand system (Simons et al. 1996). The sterile glass tubes were filled with sand moisturized with PNS (10% vol/wt) and infested with *F. oxysporum* f. sp. *radicis-lycopersici* (5×10^3 spores/kg of sand). Tomato seedlings were placed 5 mm below the surface of the sand. The plants were grown in climate-controlled growth chambers at 21°C, 70% relative humidity, and 16 h of light per day. After 7 days of growth, the plants were scored by eye as healthy (no disease symptoms) or sick (ranging from plants with pin-point size brown spots on the main root or the crown to dead plants). In all, 16 or 20 seedlings were grown per treatment. The difference in health condition (healthy or sick) of plants between two different treatments was statistically analyzed using the χ^2 goodness-of-fit test (Heath 1995).

The degree of freedom was 1 (degree of freedom = [2 conditions tested – 1] × [2 classes of plants – 1]) resulting in the critical χ^2 value of 3.841 ($P \leq 0.05$). The null-hypothesis was defined as the lack of significant difference between two conditions tested. To test the null-hypothesis, the χ^2 value was calculated for the two conditions using the χ^2 goodness-of-fit test. In case the calculated χ^2 value was lower than the critical χ^2 value, the null-hypothesis was accepted (e.g., the two treatments were not significantly different). When the calculated χ^2 value was higher than the critical value, the null-hypothesis was rejected (e.g., the treatments differ significantly).

CLSM of tomato roots.

After growth in the gnotobiotic system, tomato roots were carefully taken out of the sand and gently swirled a few times in water in order to wash away the sand particles. Whole roots were placed directly on glass slides in drops of water and examined using a Zeiss Axioplan epifluorescence microscope (Zeiss, Mannheim, Germany) coupled to a Biorad 1024 confocal system (Biorad, Hemel Hempstedd, U.K.). Images were obtained with a Kr/Ar laser with excitation 488, emission 522/35 nm for EGFP and with excitation 568, long pass emission 585 nm for DsRed. The projections of the individual channels were merged in Photoshop 7.0 software (Adobe, San Jose, CA, U.S.A.). The density of the hyphal network was determined using Image J (NIH image Bethesda). First, the Biorad image (512 × 512 pixels) was loaded in Image J and, subsequently, the threshold for the fluorescent signal was set at a level at which the background signal was negligible. Analysis of the fluorescent pixels resulted in a total number of fluorescent areas counted and in a mean area size (expressed in the number of fluorescent pixels). By multiplying these factors, the total number of fluorescent pixels was calculated. The relative differences in the density of the hyphal network were determined by comparing the total number of fluorescent pixels between different Biorad images per square centimeter of root surface.

CLSM of tomato roots incubated in the presence of purified PCN.

After growth in the gnotobiotic system for 3 days in the presence of *Fusarium* spores, tomato roots were carefully taken out of the sand and placed on 10% PNS agar plates. The root was spot-inoculated with PCN by applying 5 µl of a phenazine-1-carboxamide solution (22 mg PCN/ml of ethyl-acetate) or ethyl-acetate at one spot on the root. The solution was allowed to diffuse through the sand layer surrounding the root and was incubated in climate-controlled growth chambers at 21°C, 70% relative humidity, and 16 h of light per day. A wet filter disc was placed in the lid and the plate was sealed with parafilm (American National Can, Chicago) to prevent drying of the tomato root. After 4 h to 3 days, the roots were examined for hyphal growth.

DIC microscopic analysis of *F. oxysporum* f. sp. *radicis-lycopersici* hyphae grown in vitro.

Microscopy glass slides were covered with a thin layer (2 to 3 mm) of LB medium (Sambrook et al. 1989) solidified with 1.8% agar and placed in a plastic petri dish. A 2-day-old *F. oxysporum* f. sp. *radicis-lycopersici* culture (10 µl) was placed in the center of the glass slide at a distance of 2 to 3 cm from a spot on which 10 µl of an overnight culture of *Pseudomonas* spp. or of a phenazine-1-carboxamide solution (22 mg of PCN/ml of ethyl-acetate) had been placed. The plates were incubated for 3 days at 28°C before DIC microscopy using a Zeiss Axioplan 2. Images were processed using Photoshop 7.0 (Adobe).

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