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Visualization of Interactions Between a Pathogenic and a Beneficial *Fusarium* Strain During Biocontrol of Tomato Foot and Root Rot

Annouschka Bolwerk, Anastasia L. Lagopodi, Ben J. J. Lugtenberg, and Guido V. Bloemberg

Leiden University, Institute of Biology Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

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The soilborne fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* causes tomato foot and root rot (TFRR), which can be controlled by the addition of the nonpathogenic fungus *F. oxysporum* Fo47 to the soil. To improve our understanding of the interactions between the two *Fusarium* strains on tomato roots during biocontrol, the fungi were labeled using different autofluorescent proteins as markers and subsequently visualized using confocal laser scanning microscopy. The results were as follows. i) An at least 50-fold excess of Fo47over *F. oxysporum* f. sp. *radicis-lycopersici* was required to obtain control of TFRR. ii) When seedlings were planted in sand infested with spores of a single fungus, Fo47 hyphae attached to the root earlier than those of *F. oxysporum* f. sp. *radicis-lycopersici*. iii) Subsequent root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* was faster and to a larger extent than that by Fo47. iv) Under disease-controlling conditions, colonization of tomato roots by the pathogenic fungus was significantly reduced. v) When the inoculum concentration of Fo47 was increased, root colonization by the pathogen was arrested at the stage of initial attachment to the root. vi) The percentage of spores of Fo47 that germinates in tomato root exudate in vitro is higher than that of the pathogen *F. oxysporum* f. sp. *radicis-lycopersici*. Based on these results, the mechanisms by which Fo47 controls TFRR are discussed in terms of i) rate of spore germination and competition for nutrients before the two fungi reach the rhizoplane; ii) competition for initial sites of attachment, intercellular junctions, and nutrients on the tomato root surface; and iii) inducing systemic resistance.

Fusarium oxysporum f. sp. *radicis-lycopersici* is the causal agent of tomato foot and root rot (TFRR), which is a serious problem in commercial tomato production (Brayford 1996; Jarvis 1988). Biological control of TFRR by *F. oxysporum* strain Fo47 has been described by Alabouvette and coworkers. To be effective, Fo47 should be introduced at concentrations 10 to 100 times higher than those of the pathogen (Alabouvette and Couteaudier 1992; Alabouvette et al. 1993; Fravel et al. 2003; Paulitz et al. 1987; Roberts and Lohrke 2003).

In previous work, we have analyzed the colonization process of the tomato rhizosphere by *F. oxysporum* f. sp. *radicis-lycopersici* using confocal laser scanning microscopy (CLSM) (Lagopodi et al. 2002) and the interactions between *F. oxysporum* f. sp. *radicis-lycopersici* and biocontrol *Pseudomonas* bacteria in the rhizosphere (Bolwerk et al. 2003). These results provided us with new insights into the mechanisms of tomato root infection by *F. oxysporum* f. sp. *radicis-lycopersici* and of biocontrol of TFRR, respectively. To our knowledge, reports on simultaneous colonization by both a pathogenic and a nonpathogenic biocontrol *Fusarium* strain are limited (Bao and Lazarovits 2001; Mandeel and Baker 1991) and reports on simultaneous visualization of root colonization by both a pathogenic and a nonpathogenic biocontrol *Fusarium* strain are scarce (Bao and Lazarovits 2001). In this article, we report the labeling of strains Fo47 and *F. oxysporum* f. sp. *radicis-lycopersici* with different autofluorescent proteins followed by an analysis of the tomato root colonization by both fungi simultaneously in relation to disease control. This allowed us to obtain a better understanding of the biocontrol process.

Corresponding author: G. V. Bloemberg; Telephone: +31-71-5275056; Fax: +31-71-5275088; E-mail: Bloemberg@rulbim.leidenuniv.nl

Current address of A. L. Lagopodi: Aristotelian University of Thessaloniki, Faculty of agriculture, Plant Pathology Laboratory, P. O. box 269, 54006 Thessaloniki, Greece.

RESULTS

Cloning of the *ecfp* and *eyfp* in pGPDGFP and its expression in *Fusarium* spp.

Construction of the enhanced green fluorescent-protein (EGFP)-labeled *F. oxysporum* f. sp. *radicis-lycopersici* derivative FCL14, which was used in CLSM studies, has been described previously (Lagopodi et al. 2002). To be able to distinguish the pathogenic and nonpathogenic *F. oxysporum* strains (Table 1) when visualizing them simultaneously, we constructed derivatives labeled with the enhanced cyan fluorescent protein (ECFP) and the enhanced yellow fluorescent protein (EYFP).

In order to express *ecfp* in both *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* strain Fo47, the *ecfp* gene was introduced between the *Aspergillus nidulans gpdA* promoter (Punt et al. 1988) and the *trpC* terminator (Mullaney et al. 1985) sequences as follows. Plasmid pGDPGFP (Lagopodi et al. 2002), which contains the *sgfp* gene between the *gpdA* promoter and the *trpC* terminator, was digested with *Nco*I and *Hin*dIII in order to isolate the *sgfp* gene (Fig. 1). The *sgfp* gene was cloned into an *Nco*I-*Hin*dIII-digested pUC21, which resulted in plasmid pMP4642. Subsequently, pMP4642 was digested with *Nco*I and *Bsr*gI in order to remove the *sgfp* gene. The *ecfp* gene was isolated from pMP4516 (Bloemberg et al. 2000) by *Nco*I-*Bsr*gI digestion and cloned into the *Nco*I-*Bsr*gI-digested pMP4642, which resulted in plasmid pMP4650. The pMP4650 plasmid was digested with *Nco*I and *Hin*dIII to isolate the *ecfp* gene. The *Nco*I-*Hin*dIII *cfp* gene fragment was ligated into the *Nco*I-

*Hin*dIII-digested pGDPGFP vector to yield pMP4653 (Fig. 1). The same strategy was used to express *eyfp* in *F. oxysporum* f. sp. *radicis-lycopersici*. The *eyfp* gene was isolated from pMP4518 (Bloemberg et al. 2000) by *Nco*I-*Bsr*gI. Cloning steps identical to those used for the *ecfp* cloning resulted in the pUC21 derivative pMP4651 and the pGPDGFP derivative pMP4654 (Fig. 1). *Fusarium* strains were cotransformed as described previously (Lagopodi et al. 2002) using pMP4653 or pMP4654 together with pAN7-1 (Punt et al. 1987). pAN7-1 carries the *Escherichia coli* hygromycin-B (Hm-B) resistance gene *hph*, between the *gpdA* promoter and the *trpC* terminator, which allows selection of transformants on media containing Hm-B. Transformants subsequently were selected as described for transformants expressing *sgfp* by Lagopodi and associates (2002) for i) high levels of *ecfp* or *eyfp* expression (10 of 20 Hm-B resistant transformants), ii) stable *ecfp* or *eyfp* expression (9 of 10 fluorescent transformants), iii) unaffected growth, and iv) unaffected pathogenicity for *F. oxysporum* f. sp. *radicis-lycopersici* and disease control for Fo47. This resulted in FCL55 (*F. oxysporum* f. sp. *radicis-lycopersici* expressing *eyfp*), FCL64 (*F. oxysporum* f. sp. *radicis-lycopersici* expressing *ecfp*), and FCL31 (Fo47 expressing *ecfp*).

Control of TFRR by the nonpathogenic strain Fo47 in the gnotobiotic sand system.

Plate confrontation assays were performed to test the antagonistic ability of the nonpathogenic Fo47 against the pathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici*. Both fungi were inoculated next to each other on an agar plate and subsequently allowed to grow. In another experiment, the pathogenic fungus was grown on agar plates containing the supernatant fluid of strain Fo47. Growth inhibition of *F. oxysporum* f. sp. *radicis-lycopersici* was not observed in these experiments (data not shown). In addition to growth, inhibition of spore germination was analyzed in relation to the antagonistic ability of strain Fo47. Spores of *F. oxysporum* f. sp. *radicis-lycopersici* were allowed to germinate in the presence of the culture supernatant of *F. oxysporum* f. sp. *radicis-lycopersici* or of strain Fo47 grown under nutrient-poor (Armstrong medium, Singelton et al. 1992; synthetic medium, Lorito et al. 1994) and nutrient-rich conditions (potato-dextrose broth). Neither the rate of spore germination nor the total percentage of germinated spores was affected by the supernatant fluid of strain Fo47 (data not shown).

To test whether strain Fo47 could protect tomato plants against TFRR in the gnotobiotic sand system (Simons et al. 1996), tomato seedlings were coated with spores of Fo47. This treatment resulted in a decrease of diseased plants from 100 to 75%. Visualization studies showed that Fo47 colonized only the upper two centimeters, close to the inoculation site, whereas further distribution over the rest of the root was not detected.

In a second strategy to test whether Fo47 can control TFRR in the gnotobiotic system, tomato seedlings were grown in sand infested with spores of *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47. This strategy was similar to that used by Alabouvette and colleagues (Alabouvette et al. 1992; Alabouvette et al. 1993; Couteaudier 1992; Lemanceau and Alabouvette 1990) for biocontrol. The inoculum concentration of *F. oxysporum* f. sp. *radicis-lycopersici* was the same in all further experiments (5×10^4 spores/kg of sand), whereas the inoculum concentration of strain Fo47 varied between 1×10^5 and 2×10^9 spores/kg sand; therefore, the inoculum size will be indicated further in this article as (inoculum) ratio. Different ratios of the pathogenic over the nonpathogenic *Fusarium* strains were analyzed to determine the minimum inoculum concentration of the nonpathogenic strain Fo47 required for significant biocontrol of TFRR in the gnotobiotic system. After 7 days of incubation, the plants were analyzed for disease symptoms. Healthy plants were scored in disease index (d.i.) 0 and sick plants, with increasing disease severity, were scored in d.i. 1 through 4 (details discussed below).

The presence of Fo47 alone did not affect the health condition of the plants (Table 2). At inoculum ratios *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47 of 1:2 and 1:10, a decrease in disease severity was observed as is illustrated by a shift from d.i. 3 to d.i. 2 and d.i. 3 to d.i. 1 and 2, respectively (Table 2). Although disease severity was decreased, healthy plants were not observed. Therefore, the inoculum concentration was increased in subsequent experiments and the plants were scored as either healthy or sick.

At an inoculum ratio of 1:50, strain Fo47 reduced the percentage of sick plants from 100 to 58 to 63% (Table 3). Com-

Table 1. Microorganisms and plasmids

Strains	Relevant characteristics[y]	Reference or source
Fungi		
ZUM 2407	*Fusarium oxysporum* f. sp. *radicis-lycopersici* causing tomato foot and root rot	IPO-DLO[z]
Fo47	Nonpathogenic *F. oxysporum,* biocontrol agent, isolated from a Fusarium wilt-suppressive soil in France	Alabouvette et al. 1993
FCL14	ZUM 2407 containing *sgfp* under control of the constitutive *gpdA* promoter	Lagopodi et al. 2002
FCL55	ZUM 2407 containing *eyfp* under control of the constitutive *gpdA* promoter	This work
FCL64	ZUM 2407 containing *ecfp* under control of the constitutive *gpdA* promoter	This work
FCL31	*F. oxysporum* Fo47 containing *ecfp* under control of the constitutive *gpdA* promoter	This work
Plasmids		
pUC21	Cloning vector	Promega/Stratagene
pGDPGFP	pAN52-10-S65TGFPn/n derivative containing *sgfp* under the control of the *gpdA* promoter; integrates into the chromosome	Lagopodi et al. 2002
pAN 7-1	*Escherichia coli* hygromycin-B (Hm-B) resistance gene *hph*, cloned between the *gpdA* promoter and the *trpC* from *Aspergillus nidulans*	Punt et al. 1987
pMP4516	pME6010 derivative containing the *ecfp* gene	Bloemberg et al. 2000
pMP4642	pUC21 derivative containing the *sgfp* gene	This work
pMP4650	pUC21 derivative containing the *ecfp* gene	This work
pMP4651	pUC21 derivative containing the *eyfp* gene	This work
pMP4653	pAN52-10-S65TGFPn/n derivative containing *ecfp* under the control of the *gpdA* promoter; integrates into the chromosome	This work
pMP4654	pAN52-10-S65TGFPn/n derivative containing *eyfp* under the control of the *gpdA* promoter; integrates into the chromosome	This work

[y] IPO-DLO, Wageningen, The Netherlands.
[z] *sgfp* = green fluorescent protein, *eyfp* = enhanced yellow fluorescent protein, *ecfp* = enhanced cyan fluorescent protein.

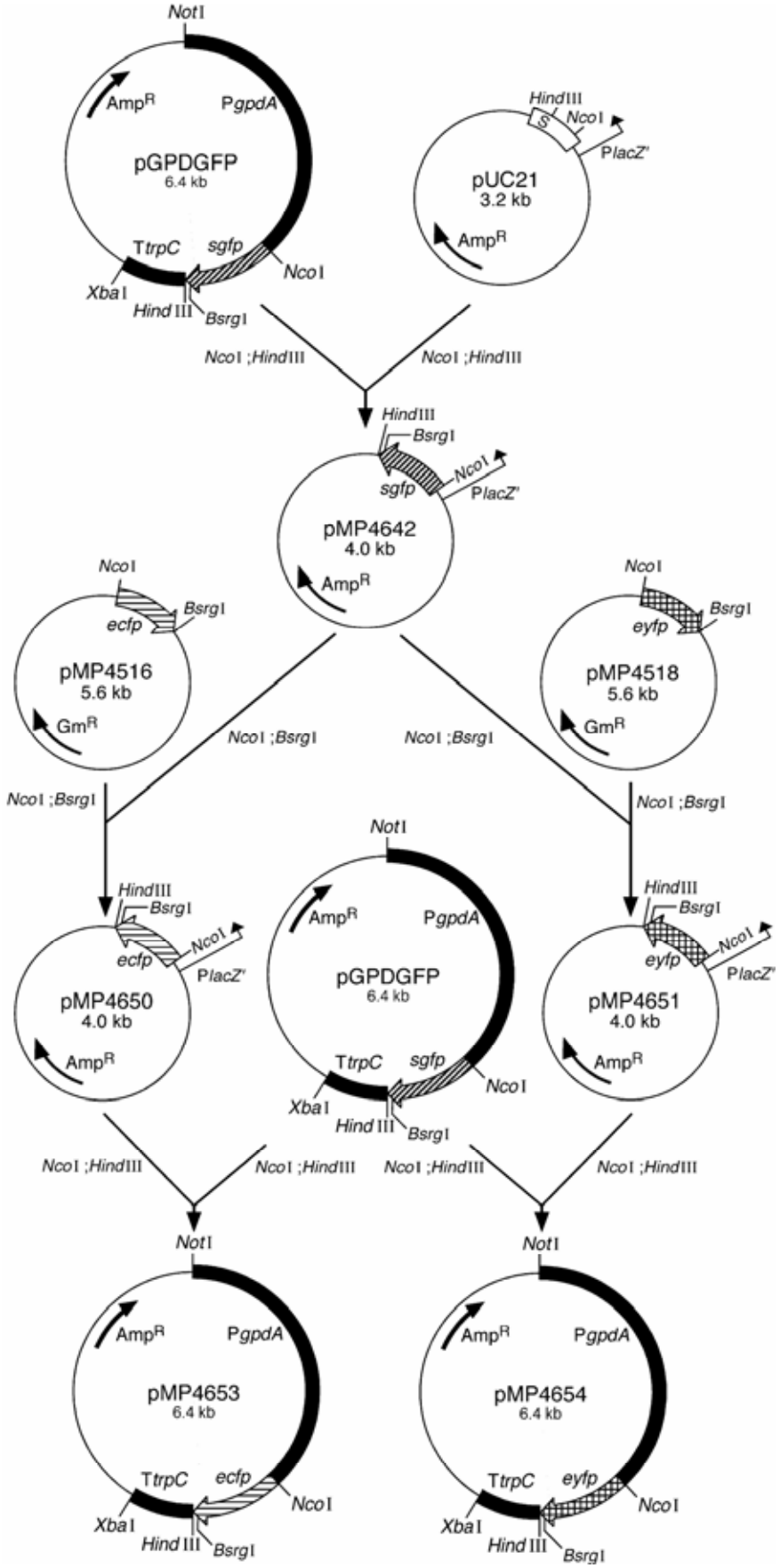

Fig. 1. Construction of reporter plasmids to express enhanced cyan fluorescent protein and enhanced yellow fluorescent protein genes (*ecfp* and *eyfp*, respectively) in *Fusarium* spp. Abbreviations: Amp = ampicillin, Gm = gentamicin, P*gpdA* = *gpdA* promoter, P*lacZ′* = *lacZ′* promoter, T*rpC* = *trpC* terminator, *sgfp* = green fluorescent protein.

parison of plants grown in sand containing *F. oxysporum* f. sp. *radicis-lycopersici* spores with and without the Fo47 spores, using a χ^2 goodness-of-fit statistical test, showed that strain Fo47 significantly suppressed TFRR in the gnotobiotic system (Table 3).

Increasing the inoculum concentration of strain Fo47 to 100-fold that of the pathogen did not improve the reduction of TFRR (Table 3; compare ii and iii with ii and iv). Increasing the pathogen/biocontrol *Fusarium* ratio to $1:4 \times 10^4$, as described by Lemanceau and Alabouvette (1990) for biocontrol in rockwool, resulted in a stronger reduction of diseased plants, from 100 to 42 to 50% (Table 3, v).

Quantitative and statistical analysis of root surface colonization by *F. oxysporum* f. sp. *radicis-lycopersici* in the presence Fo47.

CLSM allows us to differentially and simultaneously visualize *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 in the tomato rhizosphere under disease-reducing and -controlling conditions. To distinguish the two fungi, differentially labeled fungi expressing *sgfp*, *ecfp,* or *eyfp* were used. With regard to the emission spectra of the green-, cyan-, and yellow-fluorescent protein, the GFP-CFP and YFP-CFP combinations are most useful for distinguishing the two fungi. Initial CSLM studies indicated that the intensity of fluorescence was stronger for GFP than for YFP. Therefore, the GFP-CFP combination was chosen for subsequent CLSM studies. The GFP-labeled *F. oxysporum* f. sp. *radicis-lycopersici* derivative FCL14 (Lagopodi et al. 2002) and the CFP-labeled *F. oxysporum* Fo47 derivative FCL31 (Table 1) were used.

Tomato seedlings were grown in the gnotobiotic system in sand infested with spores of both *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* Fo47 at ratios of 1:10; 1:50; and 1:100. Using CLSM, we visualized and analyzed colonization of the tomato root by *F. oxysporum* f. sp. *radicis-lycopersici* after 7 days. Four different stages of root colonization were defined: i) "attachment" to root hairs and main root (Fig. 2A and B); ii) growth along one or two plant cells on the main root (Fig. 2B), defined as "start of colonization"; iii) growth along three or more adjacent cortical cells, defined as "colonization" (Fig. 2C); and iv) dense colonization over the total width of the root surface (Fig. 2D), defined as "heavy colonization". Note the difference in the amount of biomass present on root cells heavily colonized by *F. oxysporum* f. sp. *radicis-lycopersici*, which is much higher compared with cells colonized by *F. oxysporum* f. sp. *radicis-lycopersici* (compare Fig. 2D with C).

Additionally, tomato root colonization was quantified by counting the total number of tomato root cells colonized per colonization stage in the length axes (from crown to root tip). Details on how root colonization was counted are described in Materials and Methods. In short, when *F. oxysporum* f. sp. *radicis-lycopersici* grew between two root cells on the intercellular junctions along 5 cells in the length axes, it was

Table 2. Reduction of tomato foot and root rot disease symptoms by *Fusarium oxysporum* Fo47 in a gnotobiotic system

	Disease index[y]				
Disease severity[z]	**0**	**1**	**2**	**3**	**4**
Ratio 1:2 (n = 19)					
No fungi	19	0	0	0	0
F. oxysporum f. sp. *radicis-lycopersici* alone	0	0	3	16	0
Both	0	2	13	4	0
Fo47 alone	19	0	0	0	0
Ratio 1:10 (n = 16)					
No fungi	16	0	0	0	0
F. oxysporum f. sp. *radicis-lycopersici* alone	0	0	4	11	1
Both	0	7	9	0	0
Fo47 alone	16	0	0	0	0

[y] Disease index of the plants was scored after 7 days of growth on a scale ranging from 0 to 4, where 0 = healthy plants with no visible symptoms of foot and root rot, 1 = plants with pinpoint-size brown spots on the main root or pinpoint-size light-brown spots on the crown, 2 = plants with brown spots on the main root and extensive brown discoloration of the crown, 3 = plants with a wilting appearance and an extensive rot of root and crown, and 4 = dead plants.

[z] Disease severity at two different ratios of *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47 (number of plants grown). Tomato plants were grown in a gnotobiotic sand–nutrient solution system either in the absence of fungi (no fungi), in the presence of *F. oxysporum* f. sp. *radicis-lycopersici* alone (5×10^4 spores/kg of sand), in the presence of both *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 (1×10^5 or 5×10^4 spores/kg of sand for ratios 1:2 and 1:10, respectively), or in the presence of strain Fo47 alone (1×10^5 or 5×10^5 spores/kg of sand for ratios 1:2 and 1:10, respectively).

Table 3. Control of tomato foot and root rot by *Fusarium oxysporum* Fo47 in a gnotobiotic system

	Disease index[x]									
	Exp. 1		**Exp. 2**		**Exp. 3**		**Exp. 4**		**Analysis (χ^2 values)[y]**	
Treatment, fungi present[z]	**0**	**1–4**	**0**	**1–4**	**0**	**1–4**	**0**	**1–4**	**Exp. 1;3**	**Exp. 2;4**
i, No fungi	19	0	19	0	19	0	18	0	…	…
ii, *F. oxysporum* f. sp. *radicis-lycopersici* alone	0	19	0	19	0	19	0	18	…	…
iii, Ratio 1:50	7	12	8	11	…	…	…	…	8.58	10.13
iv, Ratio 1:100	…	…	…	…	7	12	7	11	8.58	8.69
v, Ratio $1:4 \times 10^4$	…	…	…	…	11	8	9	9	10.13	13.33

[x] Plants were scored 7 days after inoculation as healthy (disease index 0) or sick (disease index 1–4).

[y] Statistical analysis of disease control at ratios 1:50, 1:100, and $1:4 \times 10^4$, as compared with treatment ii. Analysis of the biocontrol experiment (Exp.1 to 4) was performed using a χ^2 goodness-of-fit test (Heath 1995) and the calculated χ^2 values are shown. Critical χ^2 value: 3.841. The two compared treatments were significantly different, calculated as $\chi^2 > 3.841$.

[z] Disease severity at *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47 ratios of 1:50, 1:100, and $1:4 \times 10^4$ for 18 or 19 plants grown in a gnotobiotic sand–nutrient solution system in the following treatments: (i) in the absence of fungi, (ii) in the presence of *F. oxysporum* f. sp. *radicis-lycopersici* (5×10^4 spores/kg of sand) or in the presence of both *F. oxysporum* f. sp. *radicis-lycopersici* (5×10^4 spores/kg of sand) and *F. oxysporum* Fo47 at (iii) 2.5×10^6, (iv) 5×10^6, or (v) 2×10^6 spores/kg of sand.

counted as 5 and not as 10. Subsequently, the difference in root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* in the absence and presence of Fo47 was statistically analyzed using a Wilcoxon-Mann-Withney U test. The reduction by Fo47 was analyzed at three different *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47 ratios (Table 4).

Under the disease-reducing condition with an inoculum ratio of 1:10 (Table 2), the nonpathogenic strain Fo47 reduced all colonization stages of the pathogen (Table 4). However, using a Wilcoxon-Mann-Withney U test, it was shown that this reduction of the colonization stages was not significant except for the heavy colonization stage (Table 4). Under disease-controlling conditions with inoculum ratios 1:50 and 1:100 (Table 3), strain Fo47 significantly reduced *F. oxysporum* f. sp. *radicis-lycopersici* in the stage of colonization as well. The heavy colonization stage was not even observed (Table 4). At the ratio 1:100, the pathogen was significantly reduced in the start of colonization (Table 4) as well. Despite the further reduction of the pathogen on the root by Fo47 (Table 4), the higher inoculum concentration (ratio 1:100) did not signify-cantly improve the disease-controlling ability of Fo47 (Table 3).

When a much higher inoculum ratio of *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47 was used ($1{:}4 \times 10^4$), analysis of healthy roots after 7 days showed that root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* was reduced to the initial state of attachment of hyphae to the root hairs ranging from zero to two sites on the root, compared with the root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* in all four colonization stages along more than 300 root cells in the absence of strain Fo47 (Table 4).

Temporal analysis of tomato root surface colonization by *F. oxysporum* f. sp. *radicis-lycopersici* and strain Fo47.

Tomato plants were grown in the gnotobiotic sand system in the presence of spores of *F. oxysporum* f. sp. *radicis-lycopersici* (5×10^4 spores/kg of sand = 5.4×10^1 spores/ml) or Fo47 (2.5×10^6 spores/kg of sand = 2.7×10^3 spores/ml), either alone or together at an inoculum ratio of 1:50. Under the latter condition, Fo47 significantly controlled the disease (Table 3) and significantly reduced root colonization of the pathogen in the colonization and heavy colonization stage (Table 4). Visualization of tomato root colonization in time by Fo47 alone (in two separate experiments with two seedlings per condition) showed that, after 3 days of plant growth, attachment to and start of colonization of the root by Fo47 occurred at two to five sites on the root for each of these stages. Colonization of the tomato root surface was observed after 4 days (Fig. 3), and strongly increased on days six and seven.

Growth of Fo47 hyphae was not targeted strictly to the cellular junctions (Fig. 4A) and occasional penetration of the tomato root by the nonpathogenic strain Fo47 was observed after 3 days (Fig. 4B). The density of the hyphal network reached by strain Fo47 after 7 days (Fig. 4A) was not as high as the heavy colonization network of the pathogen (Fig. 2D).

For the pathogen *F. oxysporum* f. sp. *radicis-lycopersici,* attachment and start of colonization of the root surface after 3 days was observed at maximally one site on the root surface, for each of these stages. After 4 days, colonization of the tomato root surface was observed along 33 tomato cells over the whole main root and strongly increased at days five and six (Fig. 3). Additionally, the pathogenic *Fusarium* sp. heavily colo-

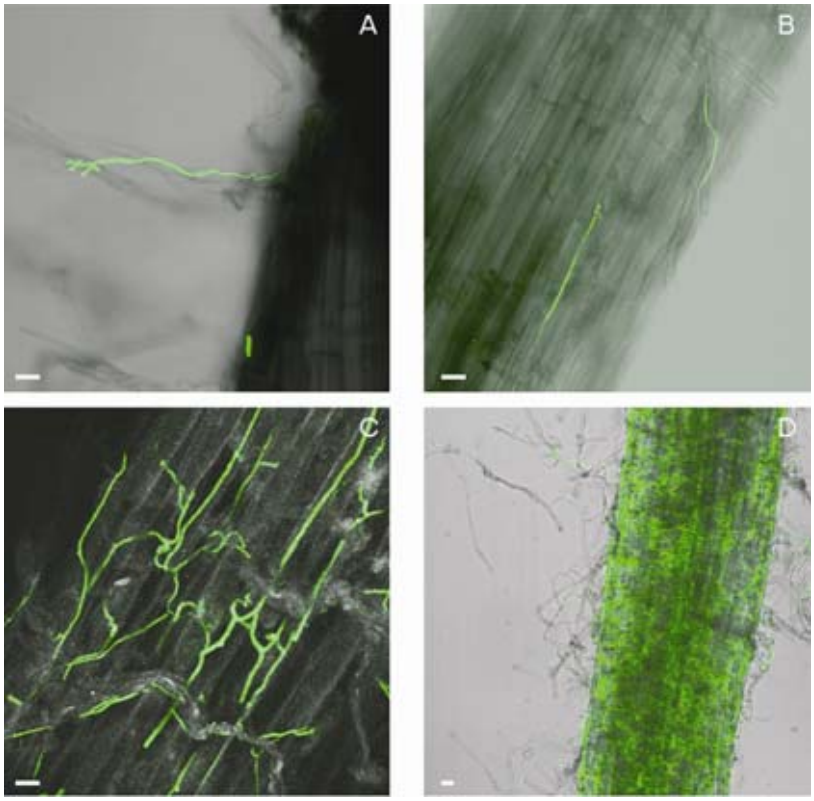

Fig. 2. Confocal laser scanning microscopic analysis of tomato root colonization by *Fusarium* spp. Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing spores of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FCL14), which harbors a constitutively expressed green fluorescent protein (*sgfp*) gene. Walls of tomato root cells appear as gray due to **A, B,** and **D,** contrast light or **C,** reflected light. **A,** Initial colonization of the tomato root by *F. oxysporum* f. sp. *radicis-lycopersici* (similar for Fo47) 'attachment' to root hairs. Subsequent root colonization stages by *F. oxysporum* f. sp. *radicis-lycopersici*: **A,** attachment to root hair; **B,** hyphae growing along the intercellular junctions of two root cells = start colonization stage; **C,** *F. oxysporum* f. sp. *radicis-lycopersici* hyphae growing along the intercellular junctions of more than two root cells = colonization stage; **D,** hyphae growing over the whole root at a very high density and biomass = heavy colonization stage. The size bar represents 10 μm in all panels.

nized the tomato root surface from day five on. The total root surface area heavily colonized by *F. oxysporum* f. sp. *radicis-lycopersici* further increased at days six and seven (Fig. 3). In contrast to the nonpathogenic strain Fo47, growth of *F. oxysporum* f. sp. *radicis-lycopersici* was mainly targeted to the cellular junctions of the root (Fig. 2C).

After inoculation of the sand with a mixture of spores of *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47, Fo47 was observed to be dominantly present on healthy roots (Fig. 4C). With increased disease index of the plants, colonization of the tomato root surface by *F. oxysporum* f. sp. *radicis-lycopersici* appeared to be increased relative to colonization by strain Fo47 (compare Fig. 4C with D). On healthy roots, *F. oxysporum* f. sp. *radicis-lycopersici* was strongly reduced at all colonization stages till day six (Fig. 5). After 7 days, *F. oxysporum* f. sp. *radicis-lycopersici* was strongly reduced at the colonization stage. Heavy colonization was not observed during these 7 days (Fig. 5). Direct cell-to-cell interactions between *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were observed in this period. No stress effects (such as increased branching, swelling of hyphae, or undirected growth of hyphae) (Bolwerk et al. 2003) were observed on any of the fungi upon direct interaction (Fig. 4E and F).

Spore germination on tomato root exudate.

CLSM studies revealed that Fo47 reduced the pathogen at or before the initial stage of attachment and the subsequent colonization stages under disease-controlling conditions (Fig. 3). A high inoculum ratio (1:4 × 10^4 Fo47 spores/kg of sand) arrested *F. oxysporum* f. sp. *radicis-lycopersici* in the attachment stage. To gain more insight into the mechanism causing this strong reduction of *F. oxysporum* f. sp. *radicis-lycopersici*, spore germination of *F. oxysporum* f. sp. *radicis-lycopersici* and strain Fo47 in tomato root exudate was analyzed. The composition of tomato root exudate, with respect to amino acids, sugars, and organic acids, has been described previously (Lugtenberg and Bloemberg 2004). It contains glucose (20 μM) as the major sugar and citric acid (133 μM) as the main organic acid. After incubation overnight in synthetic root exudate, 27% of the *F. oxysporum* f. sp. *radicis-lycopersici* spores germinated, whereas a significantly higher percentage (47%) of Fo47 spores germinated (Fig. 6A). Analysis of spore germination in the major sugar and organic acid showed that a significantly higher percentage of Fo47 spores germinated on both glucose and citric acid (4.4 and 10.7%, respectively) compared with *F. oxysporum* f. sp. *radicis-lycopersici* (0.6 and 6.1%, respectively) (Fig. 6A). Analysis of spore germination in root exudate derived from fresh tomato plant roots confirmed that a significantly higher percentage of Fo47 spores germinate compared with the spores of *F. oxysporum* f. sp. *radicis-lycopersici* (49 and 33%, respectively). Over a period of 7 days, the percentage of spores germinated remained constant and the difference between Fo47 and *F. oxysporum* f. sp. *radicis-lycopersici* was significant (Fig. 6B).

Table 4. Quantification and statistical analysis of the influence of *Fusarium oxysporum* Fo47 on the number of tomato root cells per root colonized by *F. oxysporum* f. sp. *radicis-lycopersici*[y]

		Ratio[z]		
Stage	**Alone**	**1:10**	**1:50**	**1:100**
Attachment	22 a	16 a	15 a	11 a
Start colonization	37 a	31 a	19 a	13 b
Colonization	229 a	118 a	70 b	50 b
Heavy colonization	25 a	7 b	0 b	0 b
Total	313 a	172 a	104 b	74 b

[y] Tomato root colonization stages of *F. oxysporum* f. sp. *radicis-lycopersici* in the absence and presence of Fo47 were classified and quantified after 7 days of growth. The amount of biomass present on root cells heavily colonized by *F. oxysporum* f. sp. *radicis-lycopersici* is much higher compared with cells colonized by *F. oxysporum* f. sp. *radicis-lycopersici*. The total number of plant cells per root colonized by *F. oxysporum* f. sp. *radicis-lycopersici* is an average of four roots. The inoculum concentration of *F. oxysporum* f. sp. *radicis-lycopersici* was 5 × 10^4 spores/kg of sand in all cases. The inoculum concentration of *F. oxysporum* Fo47 was 10, 50, or 100 times higher relative to *F. oxysporum* f. sp. *radicis-lycopersici.*

[z] The difference in the total number of plant cells colonized by *F. oxysporum* f. sp. *radicis-lycopersici* (Alone) or in the presence of strain Fo47 (at three ratios of *F. oxysporum* f. sp. *radicis-lycopersici*:Fo47) is indicated as not significant (a) or significant (b) as determined by the Wilcoxon-Mann-Whitney U test analyzing *F. oxysporum* f. sp. *radicis-lycopersici* colonization data of eight roots.

DISCUSSION

Previous visualization studies of root colonization by pathogenic and biocontrol *Fusarium* strains.

The first reports on visualization focused on the colonization of the root tissue by either a pathogenic (Olivain and Alabouvette 1999; Olivain et al. 2003), or a nonpathogenic *Fusarium* strain (Olivain and Alabouvette 1997, Olivain et al. 2003) of plants growing in nutrient solutions and using electron microscopy. The use of a β-glucuronidase construct allowed quantification of the nonpathogenic *F. oxysporum* SA70 on roots of tomato plants grown in soil or potting material (Bao et al. 2000; Eparvier and Alabouvette 1994). Using histochemical staining, Bao and Lazarovitz (2001) were able to simultaneously visualize the pathogenic *F. oxysporum* f. sp. *lycopersici* and the nonpathogenic *F. oxysporum* SA70 colonizing the outer and the inner root tissue of plants dipped in a spore suspension and subsequently grown in a liquid modified Murashige and Skoog medium. The process of colonization and infection of the tomato root by *F. oxysporum* f. sp. *radicis-lycopersici* was studied at the end of the past century (Brammall and Higgins 1988; Charest et al. 1984); whereas, more recently, further details were revealed using GFP-labeled *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi

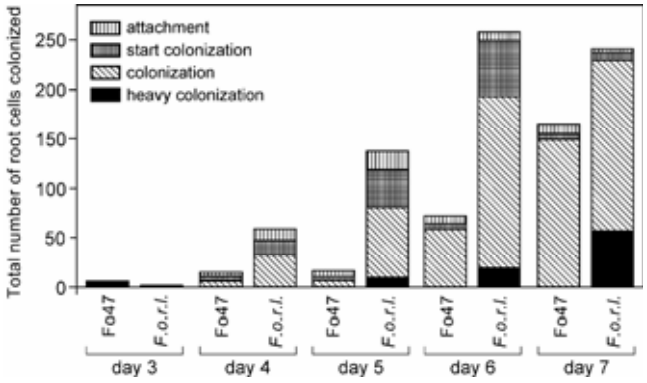

Fig. 3. Quantification of tomato root colonization stages by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and *F. oxysporum* Fo47 in time. Seedlings were grown in sand infested with Fo47 (2.5 × 10^6 spores/kg of sand) or *F. oxysporum* f. sp. *radicis-lycopersici* (5 × 10^4 spores/kg of sand). Plants were scored for tomato root surface colonization after 3, 4, 5, 6, and 7 days of growth. Colonization was classified in four different stages of colonization: attachment, start of colonization, colonization, and heavy colonization. Colonization was quantified by counting the number of plant cells colonized from crown till root tip at the four stages under the following conditions: (i) Fo47 = root colonization of Fo47 in absence of *F. oxysporum* f. sp. *radicis-lycopersici* and (ii) *F. oxysporum* f. sp. *radicis-lycopersici* = root colonization of *F. oxysporum* f. sp. *radicis-lycopersici* in absence of Fo47. Two plants were scored per condition and the average of two experiments is depicted in the figure.

et al. 2002). *F. oxysporum* f. sp. *radicis-lycopersici* initially appears to attach to the root hairs and subsequently starts to colonize the main root, after which it grows along the intercellular junctions (Lagopodi et al. 2002). At the sites of root penetration, hyphae are swollen and heavy colonization of the tomato root is observed at sites where brown lesions are visible on the root (Lagopodi et al. 2002).

Improved visualization of biocontrol of tomato foot and root rot by Fo47 using autofluorescently labeled fungi in a gnotobiotic sand–nutrient solution system.

In the present work, we visualized, for the first time under disease-controlling conditions, tomato root colonization by pathogenic and nonpathogenic *Fusarium* strains simultaneously. Tomato seedlings were grown in a sterile gnotobiotic sand system infested with spores of *F. oxysporum* f. sp. *radicis-lycopersici,* Fo47, or both. This system previously was shown to allow visualization of root colonization by *Pseudomonas* bacteria (Bloemberg et al. 1997, 2000) or *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi et al. 2002) and of the interaction between *F. oxysporum* f. sp. *radicis-lycopersici* and biocontrol *Pseudomonas* bacteria in the tomato rhizosphere (Bolwerk et al. 2003). In order to obtain a better understanding of the biocontrol process, root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* and strain Fo47 was visualized, quantified, and statistically analyzed. It should be noted that competing indigenous bacteria are absent in this gnotobiotic system.

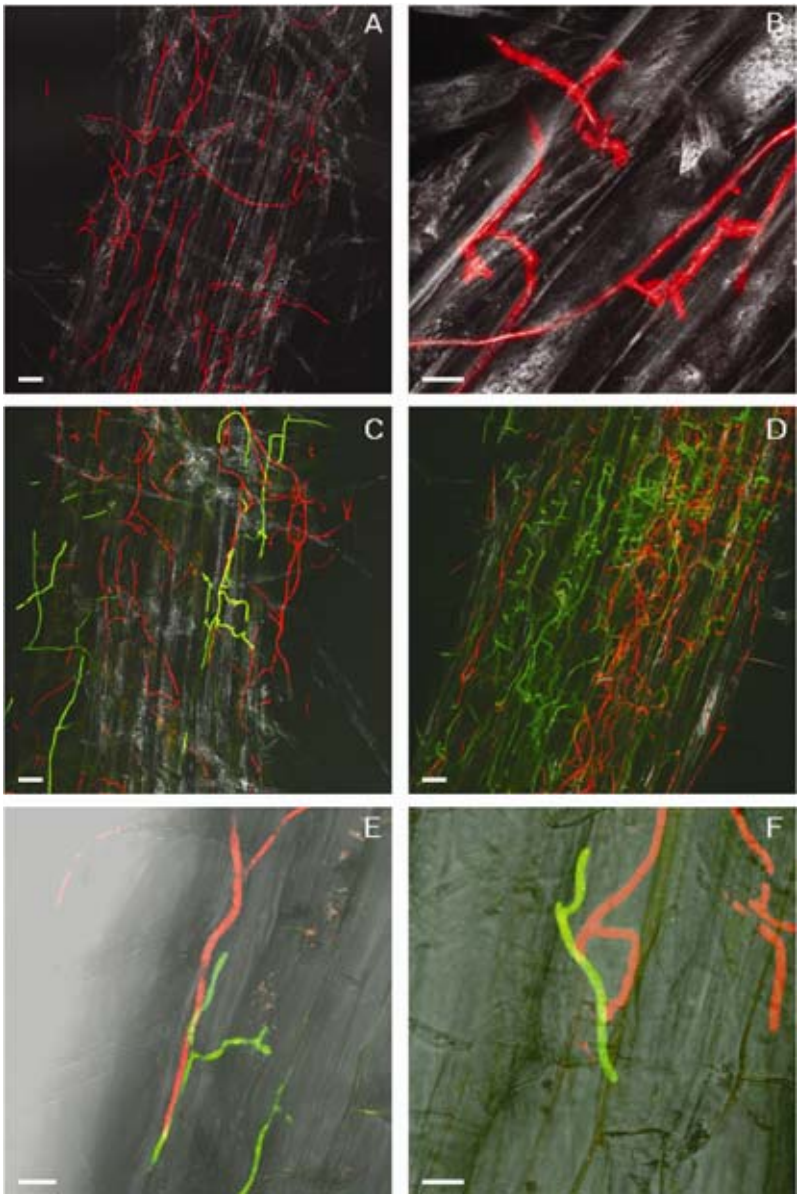

Fig. 4. Confocal laser scanning microscopic analysis of tomato root colonization by the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* and the biocontrol strain Fo47. Two-day-old tomato seedlings were grown in a gnotobiotic sand system containing **A** and **B.** spores of Fo47 (FCL31) or **C–F,** spores of both *F. oxysporum* f. sp. *radicis-lycopersici* (FCL14) and Fo47 (FCL31) at an inoculum ratio of 1:50. *F. oxysporum* f. sp. *radicis-lycopersici* (FCL14) harbors a constitutively expressed green fluorescent protein (*sgfp*) gene and appears as green. Fo47 (FCL31) harbors a constitutively expressed enhanced cyan fluorescent protein (*ecfp*) gene; its emission signal is depicted as red in the shown images. Walls of tomato root cells appear as gray due to **A–D,** reflected light or **E** and **F,** contrast light. Colonization of the tomato root by Fo47: **A,** hyphal growth along cellular junctions and crossing root cells; **B,** penetration of the tomato root by Fo47 (indicated by an arrowhead). **C,** On healthy roots (disease index 0), Fo47 is dominant. **D,** On sick roots with disease index 1, Fo47 and *F. oxysporum* f. sp. *radicis-lycopersici* are equally present. **E** and **F,** Direct cell-to-cell contact between *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 in the rhizosphere. The size bar represents 10 μm in all panels.

Interpretation of the results in relation to mechanisms that could play a role in the control of TFRR by Fo47.

Plate confrontation assays did not show inhibition of the pathogen, and spore germination of *F. oxysporum* f. sp. *radicis-lycopersici* was not affected by the culture supernatant of Fo47; therefore, it is unlikely that Fo47 produces antibiotics or extracellular enzymes seriously affecting the growth of the pathogen. Direct interactions in the rhizosphere between *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were observed but did not cause stress effects in either of the two fungi (Fig. 4E and F) such as undirected growth, increased branching, and hyphal swelling, effects described in *F. oxysporum* f. sp. *radicis-lycopersici* caused by the presence of *Pseudomonas chlororaphis* PCL1391 (Bolwerk et al. 2003). Therefore, we conclude that i) antibiosis and ii) parasitism and predation as mechanisms for biocontrol of TFRR by Fo47 are unlikely. It cannot be ruled out that, under other conditions, Fo47 does produce inhibitory substances.

Paustian and Schnürer (1987) suggested that C-sources are the growth-limiting factor for fungi in soil. Previously, Couteaudier and Alabouvette (1990) showed that glucose, at concentrations 50 times higher than estimated to be present in tomato root exudate, can be consumed more efficiently by Fo47 than by *F. oxysporum* f. sp. *radicis-lycopersici.* In this article, we have analyzed spore germination in tomato root exudate and its major sugar (glucose) and organic acid (citric acid) at concentrations estimated to be present in tomato root exudate (Lugtenberg and Bloemberg 2004). It was observed that a higher percentage of Fo47 spores germinated on these three components (Fig. 6A).

Analysis of spore germination in root exudate collected from roots of fresh tomato plants revealed that, over a period of 7 days, a higher percentage of spores of Fo47 germinated compared with spores of *F. oxysporum* f. sp. *radicis-lycopersici* (Fig. 6B). This would be advantageous for Fo47 in the tomato rhizosphere within the gnotobiotic system, where all nutritional compounds inducing spore germination and supporting hyphal growth are derived from the root exudate. Additionally, the inoculum concentration of Fo47 is 50 times higher than that of *F. oxysporum* f. sp. *radicis-lycopersici.* These two factors combined will reduce the nutrients available for spore germination and growth of *F. oxysporum* f. sp. *radicis-lycopersici*. Consequently, fewer *F. oxysporum* f. sp. *radicis-lycopersici* hyphae will reach the root surface to attach to and colonize the tomato root.

Further reduction of the pathogen, once it has reached the root surface, will be caused by occupation of the root surface by the biocontrol strain Fo47. The root colonization process by the two fungi was shown to contain similar stages and niches. As a consequence, competition for niches on the tomato root involves several sites and stages. The first one is the initial attachment to root hairs (Lagopodi et al. 2002; this study). After 3 days, Fo47 attached to two to five sites on the root, whereas *F. oxysporum* f. sp. *radicis-lycopersici* attached to no to one site. This is likely to be a result of the higher inoculation concentration of Fo47 and of faster germination of its spores and will result in a reduction of C-sources available for spore germination and growth by *F. oxysporum* f. sp. *radicis-lycopersici*. Additionally, this results in a reduction of the number of attachment sites available for *F. oxysporum* f. sp. *radicis-lycopersici*. The second site is the growth of fungi

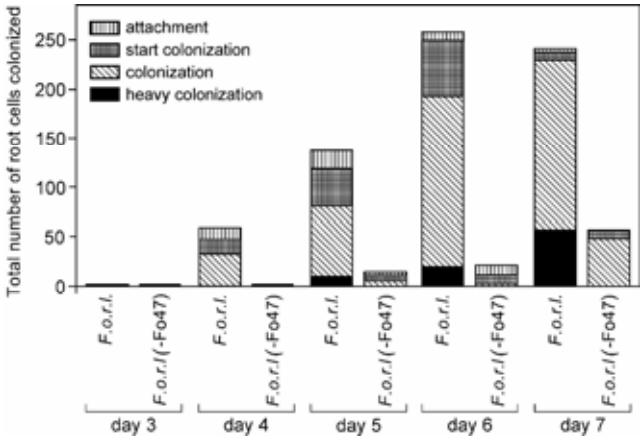

Fig. 5. Quantification of tomato root colonization stages by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) in the absence and presence of *F. oxysporum* Fo47 in time. Seedlings were grown in *F. oxysporum* f. sp. *radicis-lycopersici* (5×10^4 spores/kg of sand) or sand infested with *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 (ratio 1:50). Plants were scored for tomato root surface colonization after 3, 4, 5, 6, and 7 days of growth. Colonization was classified in four different stages of colonization: attachment, start of colonization, colonization, and heavy colonization. Colonization was quantified by counting the number of plant cells colonized from crown till root tip at the four stages under the following conditions: (i) *F. oxysporum* f. sp. *radicis-lycopersici* = root colonization of *F. oxysporum* f. sp. *radicis-lycopersici* in the absence of Fo47 and (ii) *F. oxysporum* f. sp. *radicis-lycopersici* (-Fo47) = root colonization of *F. oxysporum* f. sp. *radicis-lycopersici* in the presence of Fo47. Two plants were scored per condition and the average of two experiments is depicted in the figure.

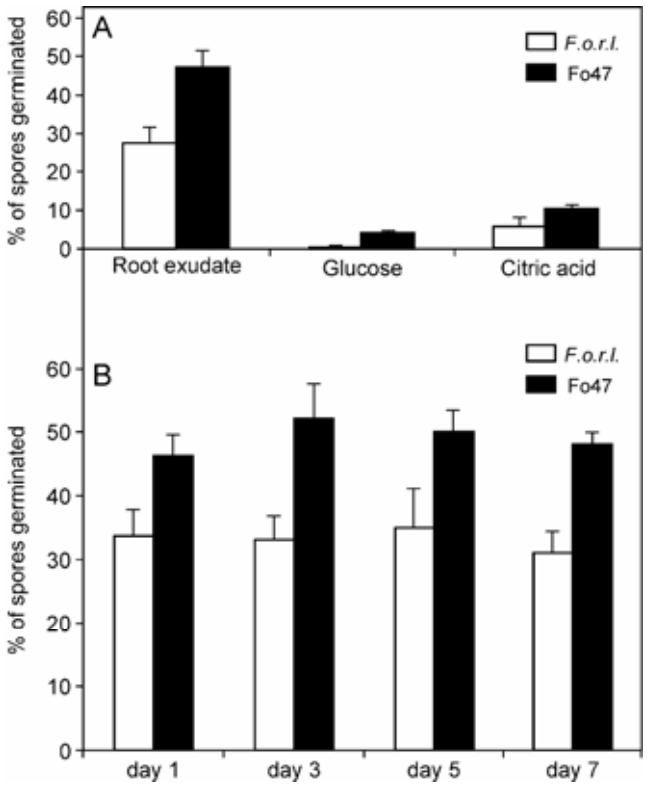

Fig. 6. Germination of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*F.o.r.l.*) and Fo47spores in tomato root exudate and in solutions of its major sugar and organic acid. **A,** Spores of *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were incubated overnight in (i) synthetic root exudate described by Lugtenberg and Bloemberg (2004), (ii) the main exudate sugar glucose (20 µM), or (iii) the main exudate organic acid citric acid (133 µM). The total number of spores and the number of germinated spores were quantified. Subsequently, the percentage of spore germination was calculated. **B,** A time course analysis of the spore germination of *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 in tomato root exudate derived from plants grown in a hydroponic system. Over a period of 7 days, the percentage of spore germination was calculated every 2 days.

along the cellular junctions of the root (Figs. 2C and 4A). The presence of Fo47 at these junctions reduces the sites available for colonization by *F. oxysporum* f. sp. *radicis-lycopersici*. However, root colonization by Fo47 from day four on was slower and to a lower extent compared with that of *F. oxysporum* f. sp. *radicis-lycopersici* despite the 50-fold higher inoculum concentration (Fig. 3), as shown by the following observations. i) Five times more root cells were colonized by *F. oxysporum* f. sp. *radicis-lycopersici* than by Fo47 (colonization was observed along 33 and 6 root cells, respectively) after 4 days of growth. ii) Colonization by *F. oxysporum* f. sp. *radicis-lycopersici* increased most strongly at day four versus at day six by Fo47. iii) The total root area colonized after 7 days of growth was larger for *F. oxysporum* f. sp. *radicis-lycopersici* than for Fo47. iv) The colonization by *F. oxysporum* f. sp. *radicis-lycopersici* was more dense, as indicated by heavy colonization. The third stage, involving penetration of the root, which was observed for both *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi et al. 2002) and Fo47 (Fig 4B), was less frequent for Fo47 than observed for *F. oxysporum* f. sp. *radicis-lycopersici* (Lagopodi et al. 2002) and may have been restricted to specific sites of the root that are more frail. We assume that, due to the occupation of penetration sites by Fo47, fewer sites were available for penetration by *F. oxysporum* f. sp. *radicis-lycopersici.* Consequently, fewer lesions were likely to be formed and no additional nutrients were leaking from the root, thereby preventing the normally extensive growth of *F. oxysporum* f. sp. *radicis-lycopersici* described by Bolwerk and associates (2003). This hypothesis is supported by our observation that, under biocontrol conditions, heavy colonization was not observed.

The results mentioned above suggest that, under biocontrol conditions, Fo47 uses the mechanism "competition for niches and nutrients" as a biocontrol strategy. However, it should be noted that, in order to be effective, Fo47 must be introduced at an at least 50-fold higher inoculum concentration compared with *F. oxysporum* f. sp. *radicis-lycopersici* (Tables 3 and 4). The observation that root colonization by the biocontrol strain from day four on is less aggressive, slower, and to a lesser extent than that of *F. oxysporum* f. sp. *radicis-lycopersici* (Figs. 3 and 4) indicates that Fo47 is not capable of effectively competing with the pathogen for niches and nutrients on the root surface. The higher inoculum concentration presumably is needed to compensate for the poorer root colonization characteristics of Fo47. This is also illustrated by the decrease of root colonization by the pathogen at increasing concentrations of Fo47 (Table 4). In conclusion, our CLSM studies provide strong experimental evidence that the mechanism "competition for niches and nutrients" contributes to the biocontrol by Fo47, as previously suggested by Eparvier and Alabouvette (1994), and that this is the result of excess Fo47 and not of the good colonization properties of Fo47. Because there is a great diversity among strains of *F. oxysporum* f. sp. *radicis-lycopersici*, care should be taken to generalize the interactions described in this work (such as competition for niches and nutrients) for other *Fusarium* strains.

A common strategy for introducing a biocontrol agent is seed coating. Coating seed and seedlings with Fo47 spores resulted in a reduction of disease incidence from 100 to 75%. Under these conditions, Fo47 hyphae could be observed only just below the crown region. Fo47 is not applied to the sand and poorly colonizes the root; therefore, it is likely that other mechanisms in addition to competition contribute to the observed disease reduction. This situation resembles a previous observation by Dekkers and associates (2000) of tomato seed coated with mutants of *P. fluorescens* WCS365; the mutants were impaired in efficient root colonization but were not affected in their ability to protect the plant against TFRR. Biocontrol by strain WCS365 is thought to act via induced systemic resistance. The ability of Fo47 to induce resistance against Fusarium wilt in tomato was shown by Fuchs and associates (1997). Fo47 and the pathogen *F. oxysporum* f. sp. *lycopersici* Fol8 were separated in either space or time, thereby minimizing the role of competition for niches and nutrients in disease control. Inoculation of tomato with Fo47 was correlated with increased levels of PR-1, chitinase, β-1,3-glucanase (classed as PR-2), and β-1,4-glucosidase (Duijff et al. 1998; Fuchs et al. 1997), indicating that Fo47 acts via a systemic acquired resistance (SAR)-like mechanism. Typically, like rhizobacteria, Fo47 did not cause visible symptoms, whereas necrosis was associated with pathogen-induced SAR. Therefore, the observed reduced disease incidence in our experiments could be a result of Fo47 inducing resistance in tomato.

To our knowledge, this is the first time that pathogenic and biocontrol fungi have been visualized simultaneously on the tomato root and that colonization of the tomato root surface by these fungi has been quantified. In this report, new experimental results obtained under disease-controlling conditions are provided that extend our understanding of the mechanism involved in biocontrol of TFRR by Fo47. i) Direct antagonism between the biocontrol fungus and pathogen is unlikely to play a role in biocontrol by Fo47. ii) The preferential germination of Fo47 spores by root exudate components is thought to reduce growth of the pathogen toward the root because more hyphae of Fo47 can compete for nutrients from root exudate, and reduce the number of *F. oxysporum* f. sp. *radicis-lycopersici* hyphae that can compete for attachment sites of the root. iii) The higher inoculum concentration of Fo47 compensates for the less-aggressive growth of Fo47 and, consequently, contributes to effective competition for niches and nutrients on the tomato root. iv) Induced resistance is likely to play a role in controlling TFRR.

MATERIALS AND METHODS

Fungal isolates and inoculum production.

The microorganisms used are listed in Table 1. *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were cultured on potato-dextrose agar (PDA) (Difco Laboratories, Detroit) or shaken at 130 to 160 rpm in Armstrong medium (Singleton et al. 1992) for 2 days at 28ºC. *F. oxysporum* f. sp. *radicis-lycopersici* spores were isolated as described by Lagopodi and associates (2002). The spores were mixed with quartz sand to a concentration of 5×10^4 spores/kg of sand (5.4×10^1 spores/ml) for *F. oxysporum* f. sp. *radicis-lycopersici* and 5×10^4, 1×10^5, 3×10^5, 5×10^5, 2.5×10^6, 5×10^4, and 2×10^9 spores/kg of sand (5.4×10^1, 1.1×10^2, 5.4×10^2, 2.7×10^3, 5.4×10^3, and 2.2×10^6 spores/ml, respectively) for Fo47. For analyzing spore germination on citric acid, *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were grown in modified Armstrong medium: instead of sucrose, citric acid was added as single C-source to a final concentration of 1.3 mM. Supernatant for analyzing spore germination was collected from *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 grown on potato-dextrose broth (Difco Laboratories), Armstrong (Singleton et al. 1992), or synthetic medium (SM) (Lorito et al. 1994) without colloidal chitin and shaken at 130 to 160 rpm for 2 days at 28ºC.

Transformation of *Fusarium* spp.

The construction of the plasmids was carried out using standard cloning techniques (Sambrook et al. 1989). Strains Fo47 and *F. oxysporum* f. sp. *radicis-lycopersici* were transformed by a polyethylene glycol/$CaCl_2$-mediated transformation of protoplasts as described by Kistler and Benny (1988) and modified by Mes and associates (1999) with additional modifi-

cations described by Lagopodi and associates (2002). To select the YFP- or CFP-expressing Hm-B-resistant cotransformants, the colonies were directly observed under a Leica MZFLIII stereo microscope equipped with epifluorescence detection (Leica, Bensheim, Germany). Filter sets tailored to the specific chromophores were used (for EYFP, 500/10-nm with excitation 518/16-nm emission; and, for ECFP, 440/21-nm excitation with 480/36-nm emission).

Control of tomato foot and root rot.

Tomato seed (provided by R. Scheffer, Syntenga, Enkhuizen, The Netherlands) were sterilized (Simons et al. 1996) and incubated at 4ºC for 5 days on plant nutrient solution (PNS) (Hoffland et al. 1989) solidified with 1.8% agar. The seed were incubated for 2 days at 28ºC to allow germination.

The spatiotemporal analyses as well as the disease-controlling 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 spores of *F. oxysporum* f. sp. *radicis-lycopersici* and strain Fo47. Tomato seedlings were placed 5 mm below the surface of the sand. The plants were grown in climate-controlled growth chamber at 21ºC, 40% relative humidity, and 16 h of light per day. In all, 16 to 19 seedlings were grown per treatment. In case of the seed or seedling coating with spores of Fo47, seed or seedlings were incubated in phosphate-buffered saline containing Fo47 spores (1×10^9 spores/ml) for 15 min. After 7 days of growth, the plants were scored for disease development by eye and classified in d.i. 0 to 4. These indexes correspond to the following symptoms: 0 = healthy plants with no visible symptoms of foot and root rot, 1 = plants with pinpoint size brown spots on the main root or pinpoint size light brown spots on the crown, 2 = plants with brown spots on the main root and extensive brown discoloration of the crown, 3 = plants with a wilting appearance and an extensive rot of root and crown, and 4 = dead plants.

CLSM analysis 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 sterile water in order to wash away the sand particles. Whole roots were placed directly on glass slides in drops of water and examined using an inverted fluorescence microscope (DMIRBE; Leica) equipped with filter blocks with spectral properties matching those of ECFP, (440/21-nm excitation with 480/36-nm emission; XF114; Chroma, Brattleboro, VT, U.S.A.) or EGFP (470/20-nm excitation with 515-nm long pass emission; I3; Leica), to which the Leica SP scanhead was attached. Dual color images were acquired by sequential scanning with settings optimal for ECFP (excitation with 457-nm argon laser line, emission detection between 470 and 490 nm), followed by settings optimal for EGFP (excitation with 488-nm argon laser line, detection of emitted light between 500 and 520 nm). Reflected light images were obtained by detection of light at the wavelength used for excitation. The projections of the individual channels were merged in Photoshop 7.0 (Adobe, San Jose, CA, U.S.A.) to facilitate visualization.

To qualify and quantify tomato root surface colonization by *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47, four tomato roots per treatment were analyzed. Four different stages of root colonization were identified: i) attachment to root hairs; ii) growth along one to two plant cells on main root, defined as "start colonization"; iii) growth along three or more adjacent cells in length, defined as "colonization"; and iv) dense colonization over the total width of the root surface, defined as "heavy colonization". By using this classification, colonization by the fungi could be categorized.

Quantification of root colonization.

All epidermis cells of a tomato root were examined from the crown to the root tip (length axis) for one of the four colonization stages (discussed above) of colonization by *Fusarium* hyphae using CLSM. The number of tomato root cells colonized in the length axis (form crown to root tip) was counted. When a hyphae was growing on the intercellular junction between two root cells (in length axes), this was scored as one colonized cell. When five cells in the width axis on the same length-axis position were colonized, it was scored as one colonized cell. If these five cells in the width axis were colonized by *Fusarium* spp. in more than one of the four defined stages (for example, attachment and colonization), the most progressed stage was scored (in this example, colonization). Each experiment was performed at least twice.

Statistical analysis.

Plants were classed as healthy (disease index 0) or sick (disease index 1 to 4). 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 = two conditions tested – 1)(two classes of plants – 1) resulting in the critical χ^2 value of 3.841 ($P < 0.05$). 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. If 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).

Quantification of tomato root colonization was performed by counting the number of root cells colonized by *F. oxysporum* f. sp. *radicis-lycopersici* as described above. To determine whether root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* was significantly reduced by the presence of strain Fo47 after 7 days of incubation, four roots per condition (*F. oxysporum* f. sp. *radicis-lycopersici* alone and *F. oxysporum* f. sp. *radicis-lycopersici* in the presence of Fo47; e.g., two conditions) were analyzed. Within this analysis, eight roots in total were scored from root tip to crown for root colonization by *F. oxysporum* f. sp. *radicis-lycopersici.* A Wilcoxon-Mann-Withney U test (Sokal and Rohlf 1981) was used to determine whether the difference in root colonization by *F. oxysporum* f. sp. *radicis-lycopersici* in the absence and in the presence of Fo47 was significantly different. This statistical analysis was performed on the three different ratios of *F. oxysporum* f. sp. *radicis-lycopersici:*Fo47 (1:10, 1:50, and 1:100). Each ratio was analyzed at least twice.

Plate confrontation assays.

PDA plates were inoculated with agar plugs (four mm) of the fungi, placed 4 cm apart and incubated at 25ºC, and the growth of *F. oxysporum* f. sp. *radicis-lycopersici* was analyzed daily. Additionally, fungal culture supernatant of strain Fo47 and *F. oxysporum* f. sp. *radicis-lycopersici* also was analyzed for its ability to inhibit hyphal growth of *F. oxysporum* f. sp. *radicis-lycopersici*. A 2× concentrated Armstrong overnight culture (100 µl) was plated on one half of a PDA plate and *F. oxysporum* f. sp. *radicis-lycopersici* was inoculated as a stripe of spores on both halves of the plate. The growth of *F. oxysporum* f. sp. *radicis-lycopersici* was analyzed daily.

Spore germination.

Fungal spores of both *F. oxysporum* f. sp. *radicis-lycopersici* and Fo47 were incubated in tomato root exudate (synthetic as

described by Lugtenberg and Bloemberg [2004] and collected from roots of fresh growing tomato plants), 20 μM glucose, or 133 μM citric acid overnight at room temperature. Root exudate was isolated as described previously (Simons et al. 1997). Briefly, 100 ml of sterile seedlings was placed in 100 ml of PNS and allowed to grow in a climate-controlled growth chamber at 20ºC, 40% relative humidity, and 16 h of daylight. After 14 days of growth, root exudate was collected.

Spore germination on root exudate from fresh tomato plants was analyzed for spores isolated from Armstrong cultures containing sucrose (Singelton et al. 1992). Germination in glucose (20 μM) or citric acid (133 μM) was analyzed for spores isolated form Armstrong cultures containing sucrose (Singelton et al. 1992) or citric acid (1.3 μM), respectively. Spore germination in synthetic root exudate was analyzed for spores isolated from Armstrong cultures.

To analyze whether Fo47 could produce inhibitory substances, we allowed spores of *F. oxysporum* f. sp. *radicis-lycopersici* to germinate in culture supernatant of Fo47 grown in nutrient-rich (PDA) or nutrient-poor (SM or Armstrong) medium. The corresponding medium was added to this spore suspension in culture supernatant to a final concentration of 0.1× PDA, 0.02× SM, or 0.02× Armstrong.

The reaction volume was 500 μl and the final concentration of spores was 2.5×10^5/ml. After overnight incubation at room temperature, the number of germinated and total number of spores was counted using a hematocytometer and the percentage of germination was calculated. The germination experiments were carried out in triplicate and were repeated twice. Using a Mann-Whitney U test, differences between spore germination of the pathogen and biocontrol agent were evaluated.

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