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In Vivo Study of *Trichoderma*-Pathogen-Plant Interactions, Using Constitutive and Inducible Green Fluorescent Protein Reporter Systems

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Plant tissue colonization by *Trichoderma atroviride* plays a critical role in the reduction of diseases caused by phytopathogenic fungi, but this process has not been thoroughly studied in situ. We monitored in situ interactions between *gfp*-tagged biocontrol strains of *T. atroviride* and soilborne plant pathogens that were grown in cocultures and on cucumber seeds by confocal scanning laser microscopy and fluorescence stereomicroscopy. Spores of *T. atroviride* adhered to *Pythium ultimum* mycelia in coculture experiments. In mycoparasitic interactions of *T. atroviride* with *P. ultimum* or *Rhizoctonia solani*, the mycoparasitic hyphae grew alongside the pathogen mycelia, and this was followed by coiling and formation of specialized structures similar to hooks, appressoria, and papillae. The morphological changes observed depended on the pathogen tested. Branching of *T. atroviride* mycelium appeared to be an active response to the presence of the pathogenic host. Mycoparasitism of *P. ultimum* by *T. atroviride* occurred on cucumber seed surfaces while the seeds were germinating. The interaction of these fungi on the cucumber seeds was similar to the interaction observed in coculture experiments. Green fluorescent protein expression under the control of host-inducible promoters was also studied. The induction of specific *Trichoderma* genes was monitored visually in cocultures, on plant surfaces, and in soil in the presence of colloidal chitin or *Rhizoctonia* by confocal microscopy and fluorescence stereomicroscopy. These tools allowed initiation of the mycoparasitic gene expression cascade to be monitored in vivo.

Trichoderma spp. are active ingredients in a variety of commercial biofungicides used to control a range of economically important aerial and soilborne fungal plant pathogens (17, 19). The antagonistic activity of biocontrol *Trichoderma* strains is attributable to one or more complex mechanisms, including nutrient competition, antibiosis, the activity of cell wall-lytic enzymes, induction of systemic resistance, and increased plant nutrient availability (16, 18, 19, 24, 30, 31, 42). Many studies, primarily in vitro studies, have shed light on the molecular basis of the three-way relationship among the pathogen, the plant, and the biocontrol agent (14, 35). However, the complexities of these interactions have been poorly studied in situ. For example, many of the factors involved in biocontrol are known (32, 34, 51, 55), but the antifungal mechanisms, including mycoparasitism, and the fate of *Trichoderma* in the soil and on the plant are not well understood. Effective monitoring of biocontrol-related processes in vivo based on the use of vital markers (1, 20, 40) provides a basis for development of new selection methods and improved applications.

The green fluorescent protein (GFP)-encoding gene (*gfp*) (8) is a powerful tool for monitoring the fate and behavior of bacterial and fungal inoculants in situ (1, 5, 29, 43, 48–50). GFP, unlike other biomarkers (22), does not require any substrate or additional cofactors in order to fluoresce. Even a single cell expressing GFP can be easily seen by epifluorescence microscopy or confocal scanning laser microscopy (CSLM) (23, 47, 48). Zeilinger et al. (55) constructed *Trichoderma atroviride* biocontrol strains with fusions of *gfp* to various promoters for constitutive or inducible expression during biocontrol. They found that chitinases were induced by the presence of *Rhizoctonia solani* and other fungal hosts in coculture experiments and that these strains were particularly useful for in vitro studies of the early phases of the interaction with the fungal host (55). Clearly, these mutants could be very useful in in situ studies of the antagonistic process; e.g., they permitted visualization of the mycoparasitic interactions between *Trichoderma* and various pathogenic fungi occurring on plant tissues.

Our objective in this study was to use *gfp*-tagged mutants of *T. atroviride* to study the in situ interaction of *T. atroviride* with the plant pathogens *Rhizoctonia solani* and *Pythium ultimum* in cocultures and directly on cucumber (*Cucumis sativus* L.) seeds, on roots, and in sterile soil. In particular, we were interested in the timing of induction of genes encoding chitinases in the presence of the host fungi or chitin. Our working hypothesis was that chitinase gene promoters would be induced in soil and on plant surfaces in the presence of the host fungus, enabling direct visualization of the mycoparasitic gene expression cascade in vivo. The combination of *gfp* tagging and advanced microscopy for in situ monitoring provides a pleth-

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ora of new possibilities for studying the complex mechanisms of interactions among fungal antagonists, pathogens, and plants.

MATERIALS AND METHODS

Fungal cultures and growth conditions. The following *T. atroviride* mutants (55) of strain P1 were used: the *T. atroviride pki1*::*gfp* strain with the *gfp* gene under control of the constitutive pyruvate kinase promoter; the *T. atroviride ech42*::*gfp* strain with *gfp* under control of the *ech42* promoter, which is induced by cell wall oligosaccharides or digested colloidal chitin; and the *T. atroviride nagl*::*gfp* strain with *gfp* under control of the *nag1* promoter, which is induced by cell wall oligosaccharides or *N*-acetylglucosamine. *T. atroviride* strains were grown on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) supplemented with 100 μg of hygromycin B (Sigma-Aldrich Chemie, Steinheim, Germany) per ml or in SM medium [containing (per liter) 2.8 g of $(NH_4)_2SO_4$, 0.6 g of urea, 4 g of KH_2PO_4, 0.6 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgSO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.0028 g of $ZnSO_4 \cdot H_2O$, 0.0032 g of $CoCl_2 \cdot 6H_2O$, and 1.0 g of sucrose; pH 6.0] supplemented with 100 μg of hygromycin B per ml. *P. ultimum* Trow var. *ultimum* HB2 was provided by John Hockenhull (Department of Plant Biology, KVL, Copenhagen, Denmark). *R. solani* is a pathogenic isolate that was obtained from the Plant Pathology and Biocontrol Unit, The Swedish University of Agricultural Sciences, Uppsala, Sweden. The pathogenic fungal strains were grown on PDA, in potato dextrose broth, on cornmeal agar, or in cornmeal broth (CMB) (Sigma-Aldrich Chemie). All cultures were incubated statically in the dark at 22°C, unless specified otherwise.

Biocontrol assays in vivo. In vivo tests of the biocontrol ability of *T. atroviride* were conducted with *R. solani.* Bean or cucumber seeds were coated with a 10% (wt/vol) aqueous suspension of the adhesive Pelgel (Liphatech, Milwaukee, Wis.) containing 10^9 spores of *T. atroviride* wild-type strain P1 per ml, 10^9 spores of the *pki1*::*gfp* strain per ml, 10^9 spores of the *ech42*::*gfp* strain per ml, or 10^9 spores of the *nagl*::*gfp* strain per ml (1 ml of spore suspension/15 g of seeds) and then air dried in an open petri dish overnight in a laminar flow hood.

An *R. solani* inoculum was prepared in potato dextrose broth liquid cultures that were incubated for 5 days at 25°C with shaking at 150 rpm. The *R. solani* fungal biomass was collected by using a vacuum in a Miracloth-lined Buchner funnel and rinsed twice with 2 volumes of distilled water. Excess liquid was pressed through the filter, and the biomass was weighed. Three grams of the fungal biomass was homogenized in 100 ml of distilled water and mixed with 1 kg of sterile soil.

Treated bean and cucumber seeds were planted 4 and 3 cm deep in the infested soil, respectively. The pots were kept at 25°C in the light and were watered daily with sterile water. The number of plants that emerged or survived and the plant height were evaluated twice weekly for up to 1 month after planting.

For all in vivo biocontrol assays, infected plant material was plated onto acidified PDA to verify the presence of *Trichoderma* and the pathogenic fungi. For all biocontrol assays we used three or more treatment replicates per experiment, the experiments were repeated on three separate occasions, and the results presented below are the average values obtained from all combined experiments. The percentage of survivors was calculated by dividing the number of plants that emerged by the number of seeds planted. The statistical analyses included a one-way analysis of variance of arcsine-transformed percentage values or raw data obtained from measured growth or plant height, and the significance level was <0.05. Due to poor emergence and deformed growth of control plants (seeds treated with Pelgel or water) in the assays with pathogen-infested soil, it was not possible to include the data from these experiments in the analysis of variance. Therefore, unpaired *t* tests were conducted with the wild-type and mutant strains to determine differences.

Fungal interactions in cocultures. Coculture studies of the interaction between the *T. atroviride pki1*::*gfp* strain and *P. ultimum* or *R. solani* and induction of *gfp* in the *T. atroviride ech42*::*gfp* and *nagl*::*gfp* strains by *R. solani* were performed on glass slides on which 150 μl of 25% PDA was spread flat onto an area that was 25 by 15 by 1.5 mm, as previously described (3, 4, 46). After the medium solidified, a plug (diameter, 2 mm) of *P. ultimum* or *R. solani* from the margin of an actively growing culture was inoculated at one end of the slide, and a plug of *T. atroviride* whose size was similar was inoculated at the opposite end. The slide was incubated with the agar side up at 22°C in the dark in a petri dish sealed with Parafilm that contained a layer of sterilized and premoistened Munktell filter paper (Munktell Filter AB, Grycksbo, Sweden). After 24 to 28 h, the fungal hyphae met. The slides were viewed with a confocal scanning laser microscope (model TCS; Leica, Heidelberg, Germany) with excitation wavelengths of 488 nm (Ar) and 633 nm (HeNe). Emission light was collected in the range from 510 to 560 nm for GFP and in the range from 620 to 660 nm for background fluorescence. A ×20 objective with an instrumental zoom factor of ×1 to ×4 was used. Images were obtained by using Leica confocal software (version 2.477). Three-dimensional rendering of the stack of images was obtained by using the three-dimensional software supplied with the confocal microscope system.

Pretreatment of cucumber seeds for in situ study. The surfaces of cucumber seeds were lightly brushed with sterilized water by using a small sterile paintbrush. The seeds were then soaked in 70% (vol/vol) ethanol for 2 min and washed three times with sterilized water. The treated seeds were transferred to sterile filter paper to absorb excess water and then disinfected in 2% (wt/vol) sodium hypochlorite (Sigma-Aldrich Chemie) for 2 min (54). The disinfected seeds were washed five times in sterilized water, and excess water was removed by blotting with sterilized filter paper under aseptic conditions. Thirty disinfected seeds and 30 nonsterile seeds (controls) were incubated on six PDA plates (10 seeds/plate) in the light at 28°C for 5 days to determine the efficacy of disinfection and cucumber seed germination. Ten sterilized seeds were ground dry with a mortar and pestle together with 2 g of sterilized silica (diameter, 1 to 1.5 mm; Sigma-Aldrich Chemie). An 80-μl suspension of the macerated seeds was spread onto a PDA plate and incubated at 28°C for 3 days to identify potential endophytes of cucumber seeds.

Preparation of soil for microcosm studies. The soil utilized was an agricultural soil obtained in Flemingsberg, Sweden (pH 5.5 to 6.5). The soil was autoclaved at 121°C for 1 h on three successive days. A 1-g sample of sterilized soil was added to a sterilized flask containing 20 ml of phosphate-buffered saline (PBS) (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.4 g of Na_2HPO_4per liter, 0.24 g of KH_2PO_4 per liter; pH 7.4) with 20 glass beads (diameter, 5 mm; Sigma-Aldrich Chemie). The flask was shaken at 150 rpm for 30 min. Eighty-microliter portions of the soil suspension were spread on PDA plates. The plates were incubated at 28°C for 3 days to determine contamination. Sterile soil was dried in an oven at 65°C for 3 days before it was used.

Preparation of fungal spores or propagules. A *T. atroviride* spore suspension was prepared by growing *T. atroviride* on PDA plates at 22°C for 7 days and suspending spores in 10 ml of PBS by scraping the plate with an inoculating loop. The suspension was filtered through sterilized glass wool (Merck) in a 10-ml syringe. The spores in the spore suspension were counted by using a Bürker-Türk counting chamber (Karl Hecht Assistent KG, Sondheim/Röhm, Germany) and epifluorescence microscopy (Zeiss, Oberkochen, Germany) with a 485-nm excitation filter and emission at 520 nm.

P. ultimum was grown in a flask containing 20 ml of 2% CMB at 28°C with shaking at 150 rpm for 21 days. Propagules (defined as reproductive units capable of producing colonies) were harvested by centrifugation at $1,650 \times g$ for 15 min, washed three times in PBS by centrifugation under the same conditions, and resuspended in PBS. The resuspended propagules were vortexed to detach the sporangia from the majority of the vegetative hyphae, filtered through sterilized glass wool in a 10-ml syringe, and then counted as described above.

Experimental design for confrontation studies on cucumber seeds. Cucumber seeds were inoculated with a combination of *T. atroviride* spores and *P. ultimum* propagules. Three different treatments were used. For treatment A, a *P. ultimum* propagule suspension was mixed with sterilized soil to obtain 3.1×10^3 propagules/g of soil, and the soil water content was adjusted to 12% (vol/wt) by using sterilized deionized water. Then aliquots (65 g) of the inoculated soil were distributed into petri dishes. Sterilized cucumber seeds were soaked in a *Trichoderma* spore suspension containing 1.1×10^3 spores/ml for 30 min and then blotted dry with sterilized filter paper under aseptic conditions. Twenty-four inoculated cucumber seeds were planted in two petri dishes (12 seeds/dish) that were sealed with Parafilm, and the seeds were grown at 23°C with a cycle consisting of 10 h of light and 14 h of darkness (45). For treatment B, sterilized cucumber seeds were soaked in a *P. ultimum* suspension containing 3.1×10^3 propagules/ml, and a *T. atroviride* spore suspension was mixed with sterilized soil to obtain 10^3 spores/g of soil. The inoculated seeds were planted in petri dishes filled with inoculated soil as described above. For treatment C, *T. atroviride* and *P. ultimum* suspensions were blended together and then mixed with sterilized soil before sterilized cucumber seeds were planted. The concentrations of both spores and propagules and other relative parameters were the same as those described above for the other treatments. For the control, the same volume of PBS that was used for the inoculant suspension was mixed with sterilized soil, and sterilized cucumber seeds were sown in the soil.

Two cucumber seeds were arbitrarily removed from each treatment every 2 days for 20 days in order to observe colonization and coverage by fluorescence stereomicroscopy (model MZ12; Leica AG, Heerbrugg, Switzerland). The colonized cucumber seeds were longitudinally cryosectioned with a cryostat (model CM 3050; Leica, Heidelberg, Germany) to obtain slices that were 20 to 30 μm

thick. One drop of Vectashield mounting medium (Vector Laboratories Inc., Burlingame, Calif.) was added to each slice before a coverslip was added and the preparation was examined by CSLM.

Effect of inoculation method on seed surface colonization by *T. atroviride*. Front and back images of whole seeds were obtained with a Hamamatsu digital charge-coupled device camera (model C4742-95; Hamamatsu Photonics, Hamamatsu City, Japan) installed on an epifluorescence microscope. A grid consisting of 100 squares was superimposed on four sectors (two upper sectors and two lower sectors) of each seed, and the hyphae crossing the intersection points (except the hyphae on two of the side lines) were counted for five different positions of the grid (for a total of 500 crossing points). The fungal aggregates were recorded by using the same method that was used for the hyphae. The percent coverage of cucumber seeds by *T. atroviride* was determined as follows: percent coverage = N (1/5), where N is the number of intersection points occupied by fungal hyphae in five views for 100 square grids (39).

Induction of *T. atroviride* mutants. Ground *R. solani* hyphae were prepared by growing *R. solani* in a 250-ml flask containing 50 ml of 0.2% (wt/vol) CMB at 28°C with shaking at 150 rpm for 14 days. The hyphae were washed five times with sterile double-distilled water in a sterile beaker with stirring and were harvested by centrifugation at 6,000 × *g* for 15 min. Pelleted hyphae were ground in a mortar and pestle, dried overnight at 80°C, and then sieved with a 60-mesh sieve. The sieved hyphal powder was sterilized by autoclaving and stored at 4°C until it was used.

Colloidal chitin was prepared by dissolving 50 g of crab shell chitin (Sigma) in 150 ml of 12 N HCl. Ice-cold sterile distilled water (200 ml) was added, and the pH was adjusted to 7.0 with 5 N NaOH. The suspension was centrifuged at 9,000 × *g* for 10 min, the supernatant was discarded, and 0.2 M phosphate buffer (pH 7.0) was added. The suspension was mixed well and centrifuged again at 9,000 × *g* for 10 min. After the supernatant was discarded, sterile distilled water was added to obtain a chitin concentration of 4% (wt/vol). Finally, the suspension was sterilized by autoclaving and stored at 4°C until it was used.

Spore suspensions of the *T. atroviride ech42*::*gfp* and *nagl*::*gfp* mutants were inoculated to obtain a concentration of 10^3 spores ml^{-1} into 125-ml flasks containing 30 ml of SM medium with 100 μg of hygromycin B ml^{-1}, 0.1% sucrose, and various concentrations of sterile colloidal chitin or the *R. solani* ground hyphae stock (0, 0.125, 0.25, 0.5, 1.5, and 2.5 mg ml^{-1}). The cultures were grown at 28°C with shaking at 150 rpm. Samples (1 ml) of the cultures were taken at zero time and after 24, 48, 72, 96, and 120 h of incubation and were centrifuged at 9,000 × *g* for 10 min. The hyphal pellets were resuspended in 400 μl of sterile double-distilled water and then transferred to separate wells of a 96-well microtiter plate and incubated at 4°C for 30 min. Fluorescence was measured with a microtiter plate spectrofluorometer (BMG LabTechnologies, Offenburg, Germany) by using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. After this, each sample suspension was transferred to a tared plastic weighing dish and dried at 65°C to a constant weight, and the weight of the hyphae was determined with an accuracy of 10^{-4} g.

Induction of *T. atroviride* mutants in soil microcosms. Microcosms in petri dishes contained 65 g of sterile soil (relative moisture content, 65%) blended with 6.5×10^4 spores of the *T. atroviride ech42*::*gfp* or *nagl*::*gfp* strain. *R. solani* was grown on PDA at 22°C until sclerotia that were 2 mm in diameter were formed. *R. solani* sclerotia were inoculated into the soil in the petri dishes with 8 mm between the sclerotia and were covered with 2 mm of soil. The petri dishes were sealed with Parafilm, covered with black cloth, and incubated at 22°C. Soil samples were taken daily for 1 week after inoculation and examined by confocal microscopy.

RESULTS

In vivo biocontrol activity of *gfp*-tagged mutants. Transformation with the *gfp* gene did not affect the biocontrol ability of *T. atroviride* strain P1 (Table 1). Both the emergence (percentage of survival) and the growth of bean plants from antagonist-treated seeds were significantly greater than the emergence and the growth observed for the control treatments (Pelgel or water alone) in soil containing a pathogen ($P < 0.001$). There were no differences among any of the seed treatments, *T. atroviride* strains, or controls if the soil did not contain a pathogen (Table 1).

Mycoparasitism by *T. atroviride* in cocultures. *gfp*-tagged hyphae were easily seen due to their green fluorescence, whereas the pathogenic fungal hyphae were either weakly autofluorescent (red or yellow) or nonfluorescent (brown). With *R. solani*, *T. atroviride* formed a cluster of branches immediately before contact that grew towards the host hyphae (Fig. 1A). Subsequently, the mycoparasite aligned with *R. solani* hyphae (Fig. 1B), which often broke during an attack in a manner not observed with the other fungal hosts tested (Fig. 1C). With *P. ultimum*, *T. atroviride* grew alongside the *P. ultimum* hyphae and branched toward adjacent hyphae (Fig. 1D). *T. atroviride* spores adhered to the host hyphae (Fig. 1E) and germinated after attachment. Then the young *T. atroviride* hyphae extended toward and parasitized the host mycelium (Fig. 1F). The confrontation with *P. ultimum* also was characterized by growth of *T. atroviride* alongside the host hyphae and subsequent coiling around them. The specialized, appressorium-like structures that formed in the presence of the host mycelium and were previously described by Chet et al. (11) with *R. solani* were visualized during parasitism of *P. ultimum* (data not shown). In addition, the hyphal tips of *T. atroviride* swelled and formed papilla-like structures not only during contact with the host hyphae but also before they touched the host mycelium (Fig. 1F).

TABLE 1. Effects of *T. atroviride* wild-type strain P1 and *gfp*-tagged strains on plant survival and plant height of emerged bean seedlings 14 days after planting

Seed treatment	*R. solani*-infested soil		Soil without pathogen	
	% Survival[a]	Height (cm)[b]	% Survival	Height (cm)
Wild type P1 + Pelgel	71	6.6 ± 3.6	100	19 ± 2.8
pki1::*gfp* + Pelgel	76	5.6 ± 5.0	100	17 ± 2.9
ech42::*gfp* + Pelgel	81	4.3 ± 2.8	100	18 ± 2.8
nag1::*gfp* + Pelgel	86	12.0 ± 6.0	100	18 ± 1.4
Pelgel alone	37	NA	100	18 ± 1.1
Water	30	NA	100	18 ± 1.9

[a] The percentage of survival was determined by determining the number of emergent plants during the evaluation period. Analysis of variance for arcsine-transformed values: P1 versus *pki1*::*gfp*, *ech42*::*gfp*, or *nag1*::*gfp* mutant, not significantly different; P1, *pki1*::*gfp* mutant, *ech42*::*gfp* mutant, or *nag1*::*gfp* mutant versus Pelgel or water alone, significantly different ($P < 0.001$). Seed treatments were replicated three times per experiment, and the experiments were repeated on three separate occasions. Data from all experiments were combined for the analysis.

[b] Unpaired *t* test for plant height: for soil treated with pathogen, P1 versus *pki1*::*gfp*, *ech42*::*gfp* or *nag1*::*gfp* mutant, not significantly different: for soil with no pathogen, P1, *pki1*::*gfp* mutant, *ech42*::*gfp* mutant, or *nag1*::*gfp* mutant versus Pelgel or water alone, not significantly different. NA, not applicable (plants in poor condition).

Effect of inoculation method on colonization of the cucumber seed surface by *T. atroviride*. The method of inoculation of *T. atroviride* and *P. ultimum* onto cucumber seeds affected the level of coverage of the seed surface by *T. atroviride*. When *P. ultimum* was mixed with sterilized soil into which seeds that had been soaked in a *T. atroviride* spore suspension were planted (treatment A), *T. atroviride* hyphae appeared 2 days after planting (Table 2). If *T. atroviride* spores were mixed with sterilized soil before seeds that had been soaked in a *P. ultimum* suspension were planted (treatment B) or if sterilized cucumber seeds were sown in soil that was inoculated with the same concentrations of *T. atroviride* spores and *P. ultimum* propagules (treatment C), colonization by the biocontrol agent

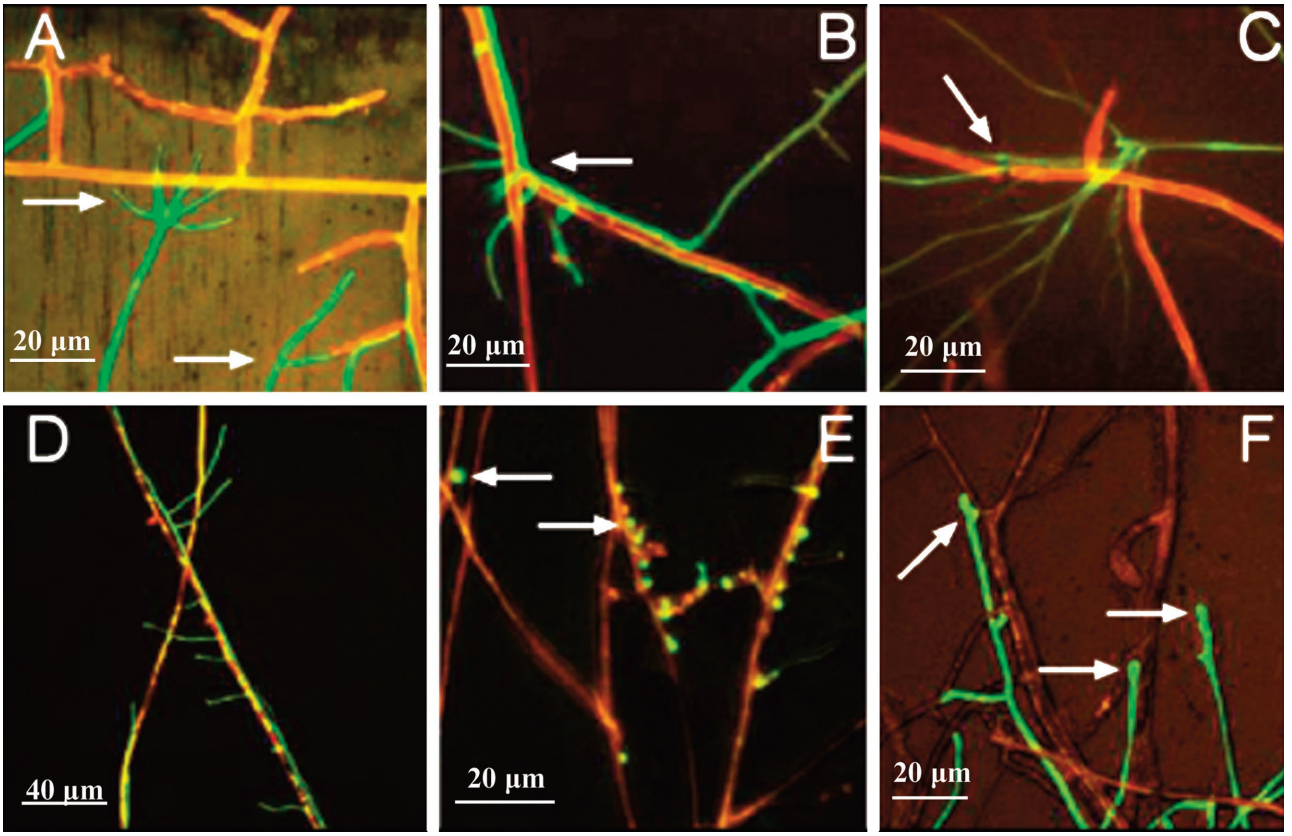

FIG. 1. CSLM images of confrontations between cocultures of the *T. atroviride pkil*::*gfp* mutant and *R. solani* (A, B, and C) and between cocultures of the *T. atroviride pkil*::*gfp* mutant and *P. ultimum* (D, E, and F). The host hyphae are reddish (*R. solani*) or reddish brown (*P. ultimum*), whereas the hyphae of the *T. atroviride pkil*::*gfp* mutant are green. The left arrow in panel A indicates a conidium, and the right arrow in panel A and the arrow in panel B indicate branching in the *T. atroviride* hyphae. In panel C the arrow indicates a breakage point in the *R. solani* hyphae. In panel E the arrows indicate green fluorescent *T. atroviride* spores deposited on *P. ultimum* hyphae. In panel F the arrows indicate papilla-like structures in the *T. atroviride* hyphae. The images were obtained with a ×20 objective.

was not observed until 4 days after planting. Colonization by *T. atroviride* increased for up to 14 days after planting for treatment A and for up to 16 days for treatments B and C. After this, the percent coverage stabilized or slightly decreased for all treatments (Table 2). The *gfp*-tagged *Trichoderma* colonized the outer layer of the roots of the cucumber seedlings (Fig. 2A and B), as well as the soil inoculated with the biocontrol agent and the pathogen together (treatment C) (Fig. 2C).

TABLE 2. Colonization of the cucumber seed surface by *T. atroviride*, expressed as seed coverage[a]

Time (days)	Seed coverage (%)		
	Treatment A	Treatment B	Treatment C
0	0	0	0
2	0.20 ± 0.12	0	0
4	1.7 ± 0.48	0.53 ± 0.67	0.13 ± 0.67
6	5.3 ± 0.73	2.4 ± 0.64	2.6 ± 0.70
8	7.7 ± 3.0	4.1 ± 0.98	3.3 ± 0.75
10	15 ± 1.3	9.8 ± 0.53	9.8 ± 1.1
12	15 ± 1.2	13 ± 0.87	13 ± 0.64
14	17 ± 0.90	13 ± 0.61	13 ± 0.33
16	16 ± 0.71	15 ± 0.98	14 ± 0.46
18	15 ± 1.0	14 ± 1.4	14 ± 1.5
20	16 ± 1.8	14 ± 1.3	13 ± 1.00

[a] The data are means ± standard errors for three seeds. The treatment A cucumber seeds inoculated with *T. atroviride* were planted in *P. ultimum*-infested soil; in treatment B cucumber seeds infested with *P. ultimum* were planted in *T. atroviride*-inoculated soil; and in treatment C both *P. ultimum* and *T. atroviride* were inoculated together into sterile soil before cucumber seeds were planted.

Interaction between *T. atroviride* and *P. ultimum* on cucumber seeds. *P. ultimum* was identifiable as reddish brown hyphae by CSLM. Direct mycoparasitism of *P. ultimum* by *T. atroviride* was observed on the cucumber seed surfaces during seed germination (i.e., 8 days after planting) and for all three inoculation treatments. The morphological changes of the mycoparasite and the relative phases of attack by *T. atroviride* were similar to those observed in cocultures. On the cucumber seed surface, *T. atroviride* hyphae grew alongside the *P. ultimum* mycelium (Fig. 3A). The papilla-like swelling of *T. atroviride* hyphal tips was observed when the hyphae touched the host and also before contact (Fig. 3B). After the first contact with the host was established, *T. atroviride* formed new hyphal branches that further extended toward the *P. ultimum* mycelium (Fig. 3C), and eventually the branches coiled around the host mycelium on the cucumber seed surface (Fig. 3D). Growth of *T. atroviride* hyphae on the cucumber seeds apparently was stimulated by the presence of the host. The hyphae were more concentrated in regions of the seed surface colonized by *P. ultimum* mycelium.

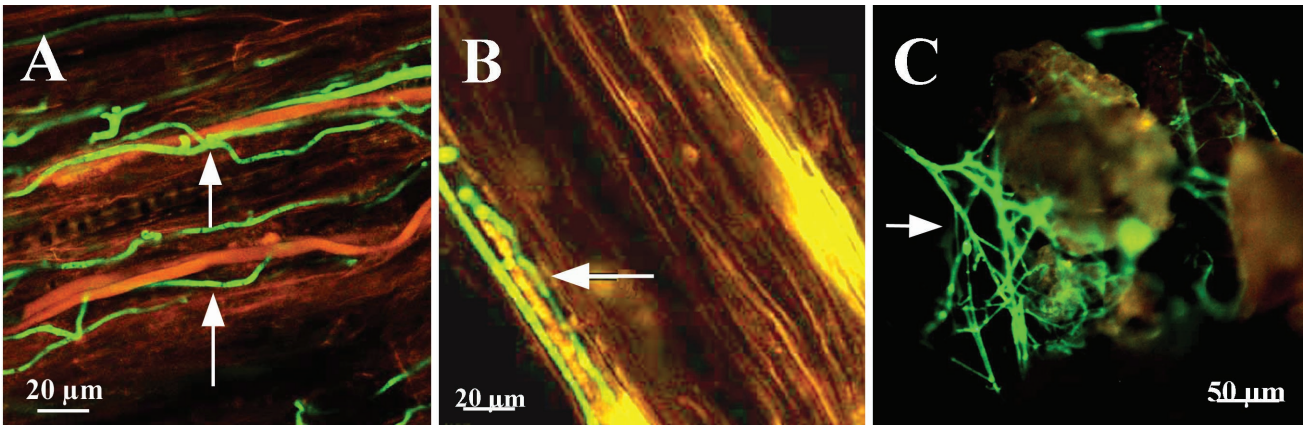

FIG. 2. Images of the *T. atroviride pki1*::*gfp* mutant colonizing a cucumber root (A and B) and soil particles (C) in sterilized soil inoculated with *P. ultimum* after 3 days of incubation. The initial concentrations of *P. ultimum* and *T. atroviride* added to the soil were 3.1×10^3 propagules g^{-1} and 3×10^3 spores g^{-1}, respectively. Cucumber seeds were also planted at the time of inoculation. The images in panels A and B were taken with a CSLM with a ×20 objective. The arrows indicate green fluorescent *T. atroviride* hyphae alongside *P. ultimum* hyphae (yellow or reddish brown) on the outer surface of a cucumber root (reddish brown). The image in panel C was taken with a fluorescence steromicroscope by using a ×6.9 objective and shows green fluorescent *T. atroviride* hyphae (arrow) adhering to soil particles (brownish or yellow).

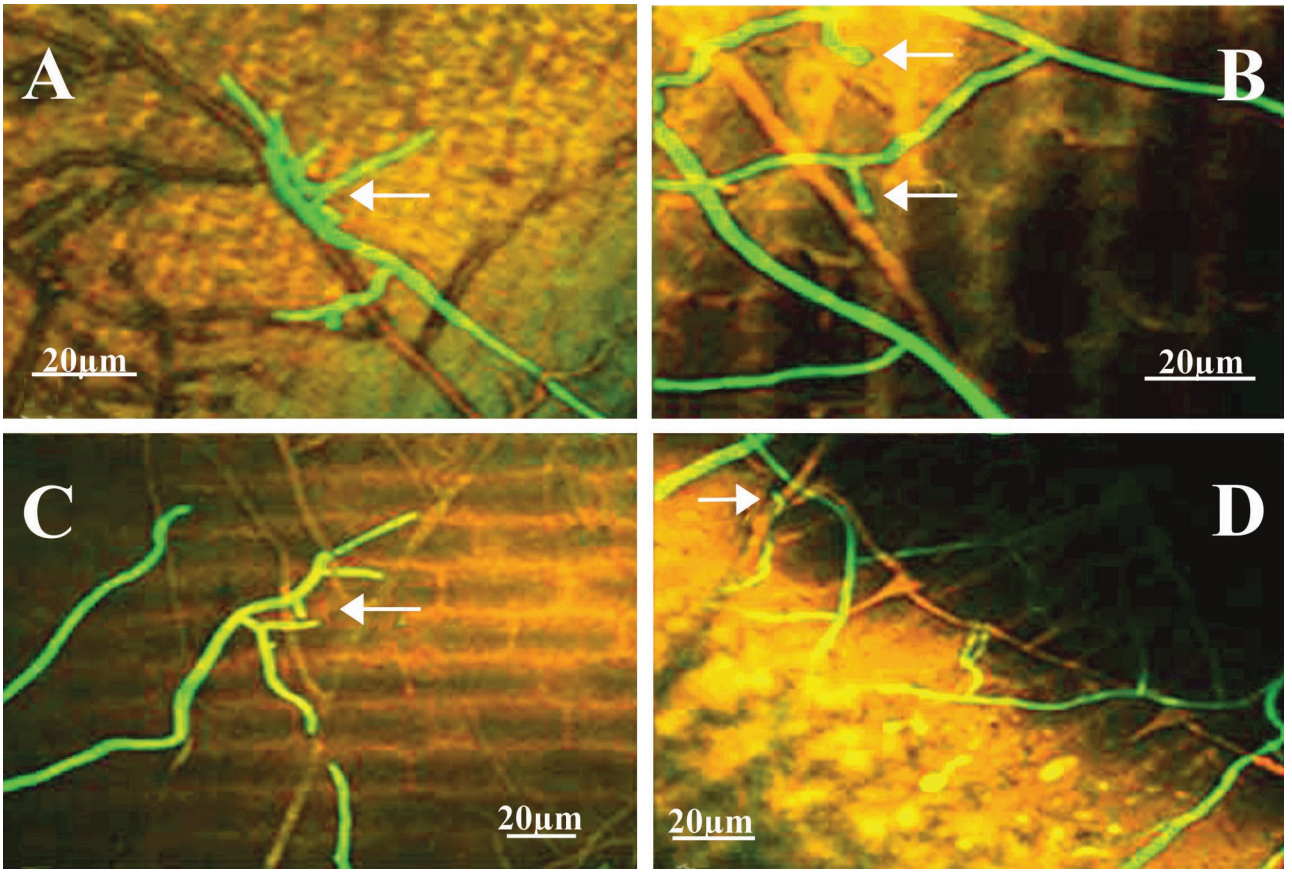

FIG. 3. CSLM images of cucumber seed cryosections prepared 10 days (A), 12 days (B), 14 days (C), and 18 days (D) after planting in sterile soil inoculated with *P. ultimum* at a concentration of 3.1×10^3 propagules g^{-1}. The arrow in panel A indicates an aggregation of *T. atroviride* hyphae on *P. ultimum* hyphae. The arrows in panel B indicate papilla-like swelling of *T. atroviride* hyphal tips. The arrow in panel C indicates branching *T. atroviride* hyphae extending towards *P. ultimum* hyphae. The arrow in panel D indicates coiling of *T. atroviride* hyphae around *P. ultimum* hyphae. All images were taken with a ×20 objective.

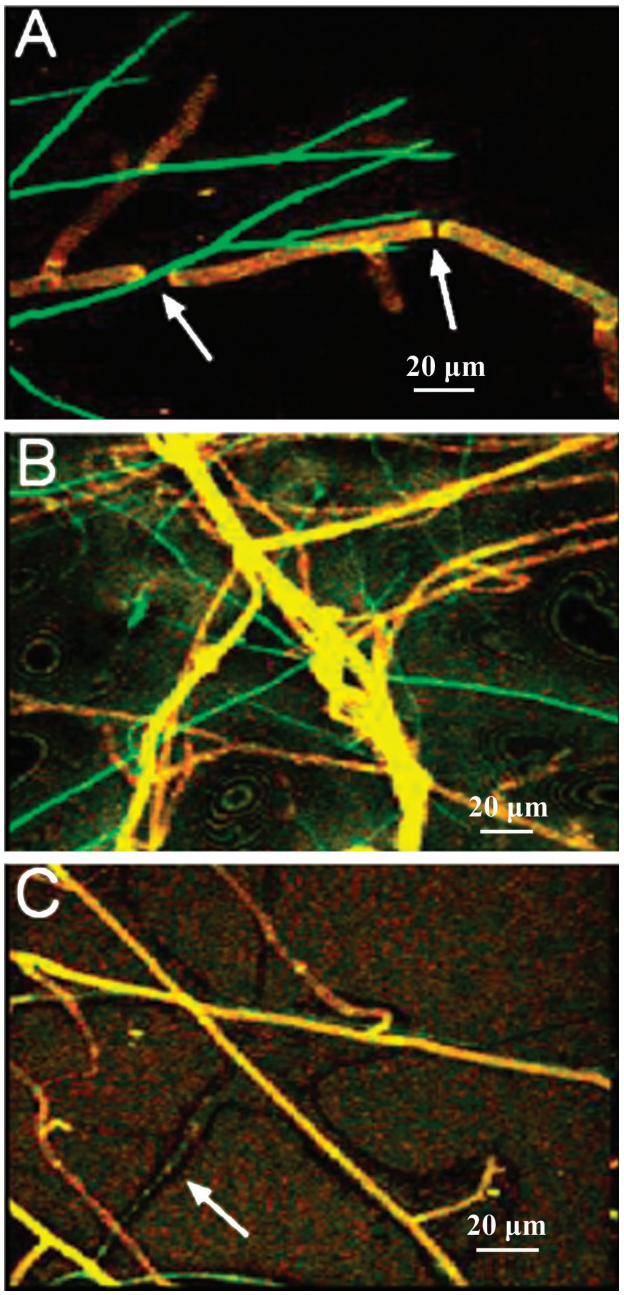

FIG. 4. CSLM images of induction of chitinase genes in the *T. atroviride nag1*::*gfp* mutant (A) and the *T. atroviride ech42*::*gfp* mutant (B) by *R. solani* hyphae. In panels A and B the *T. atroviride* hyphae are green fluorescent due to contact with the *R. solani* hyphae, and the *R. solani* hyphae are reddish yellow. The arrows in panel A indicate breakage points in the *R. solani* hyphae. (C) Coculture of *R. solani* and wild-type *T. atroviride* (no *gfp*) (arrow) for comparison. All images were taken with a ×20 objective.

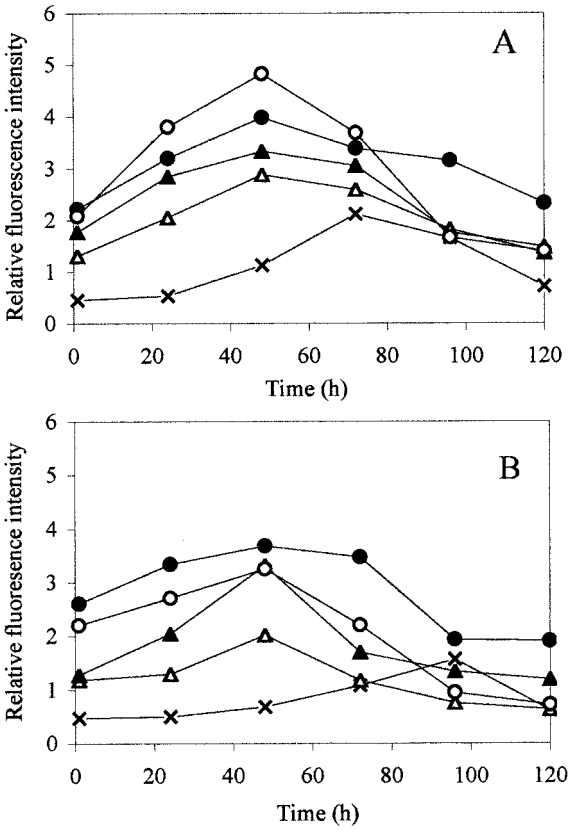

FIG. 5. Relative fluorescence intensity, expressed per 0.1 mg (dry weight) of *Trichoderma* hyphae, upon chitinase gene induction of the *T. atroviride nag1*::*gfp* mutant (A) or the *T. atroviride ech42*::*gfp* mutant (B) in the presence of ground chitin at concentrations of 0.5 mg ml^{-1} (△) and 2.5 mg ml^{-1} (○) or in the presence of *R. solani* hyphae at concentrations of 0.5 mg ml^{-1} (▲) and 2.5 mg ml^{-1} (●). Controls (×) consisted of *T. atroviride* mutant strains with no chitin or *R. solani* hyphae added. Each data point represents the mean of triplicate samples, which did not vary more than 10% from the mean.

Induction of expression of *T. atroviride* mutants by *R. solani* in cocultures. Induction of expression of chitinase genes resulted in a GFP fluorescent phenotype in both *Trichoderma* mutants (the *ech*::*gfp* and *nag1*::*gfp* mutants) 2 days after inoculation when the *Trichoderma* hyphae contacted *R. solani* hyphae (Fig. 4). The *nagl*::*gfp* mutant (Fig. 4A) was more intensely green fluorescent than the *ech42*::*gfp* mutant (Fig. 4B) upon contact with *R. solani* hyphae in PDA. The fluorescence intensity of both mutants became fainter as hyphal growth and sporulation continued, and 2 to 3 days after contact, the older hyphae lost green fluorescence. Prior to contact of the hyphae only weak autofluorescence was observed, which was comparable to the autofluorescence of the wild-type *T. atroviride* strain (Fig. 4C).

Induction of expression of *T. atroviride* mutants by colloidal chitin or ground *R. solani* hyphae. The *T. atroviride* mutants were incubated in the presence of colloidal chitin or ground *R. solani* hyphae, and fluorescence was monitored over time. The greatest fluorescence intensity appeared 48 h after inoculation of either the *nagl*::*gfp* or *ech42*::*gfp* mutant into SM medium supplemented with colloidal chitin or ground *R. solani* (Fig. 5). Up to the time of maximum intensity (48 h), the increase in fluorescence intensity was proportional to the concentration of colloidal chitin or ground *R. solani* for both mutants (the results for the highest concentration and an intermediate concentration of each are shown in Fig. 5). The fluorescence intensity of the *nag1*::*gfp* mutant in SM medium was greater than that of the *ech42*::*gfp* mutant after induction with colloidal

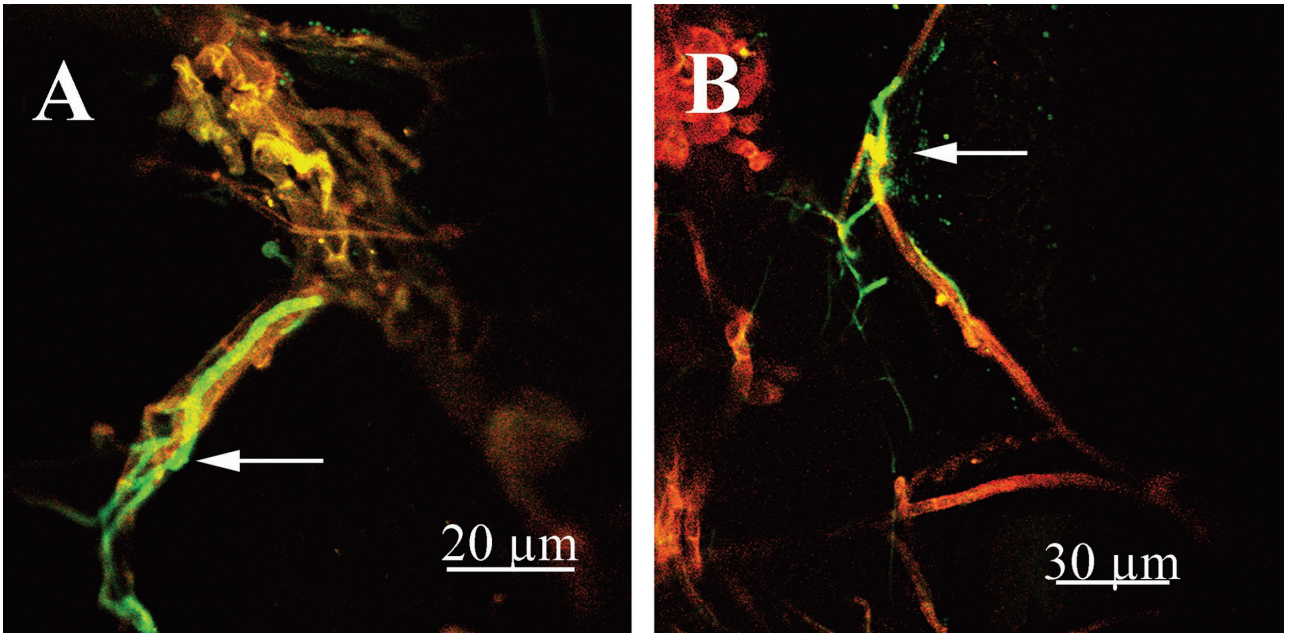

FIG. 6. CSLM images of induction of chitinase genes after 3 days of incubation of the *T. atroviride nag1*::*gfp* mutant (A) and after 4 days of incubation of the *T. atroviride ech42*::*gfp* mutant (B) upon contact with *R. solani* hyphae (yellow or reddish brown) in sterile soil. The arrows indicate *T. atroviride* hyphae. Both images were taken with a ×20 objective.

chitin (Fig. 5), which is consistent with the confrontation studies performed with the *T. atroviride* mutants and *R. solani* (Fig. 4). However, the difference between the mutants was less obvious when they were induced by *R. solani* hyphae (Fig. 5). The relative fluorescence intensity increased over time for the controls without added hyphae or chitin as well, but to a much lesser extent, and the greatest fluorescence intensity was observed after 72 or 96 h of incubation (Fig. 5). The fluorescence observed in the controls was autofluorescence, not induction of *gfp* gene expression, and it was similar to that seen in wild-type *T. atroviride* strains that lacked the *gfp* gene (data not shown).

Microcosm studies of inducible expression of *T. atroviride* mutants. Hyphae of both *T. atroviride* mutants were observed growing in soil 2 days after inoculation. Three days after inoculation, GFP-fluorescing hyphae of the *nagl*::*gfp* mutant were observed growing alongside *R. solani* hyphae (Fig. 6A). After 4 days, the *gfp* gene of the *ech*::*gfp* mutant was expressed when the mutant was in contact with *R. solani* hyphae (Fig. 6B). More *T. atroviride* hyphae interacted with *R. solani* hyphae during the rest of the 7-day sampling period. On the seventh day the *Trichoderma* strain began to sporulate, and the spores were green fluorescent (data not shown). After sporulation the green fluorescence of the *Trichoderma* hyphae began to fade.

DISCUSSION

The mycoparasitic mode of action of *Trichoderma* spp. against fungal plant pathogens has been studied extensively in two-culture assays and under other in vitro conditions (9, 10, 12, 21, 31). This study, performed in situ, allowed direct observation of the development of a mycoparasitic attack that occurred in sterile soil and on plant roots and seeds, conditions in which the physical effects of the complex soil matrix and plant tissues were taken into account. The wild-type strain and the *gfp*-tagged mutants of *T. atroviride* strain P1 had similarly high levels of biocontrol activity, indicating that transformation and expression of the *gfp* gene did not reduce the biocontrol capability of the fungus.

Our study revealed both the in vivo occurrence of and the sequence order for the various phases and gene expression of a necrotrophic parasitic interaction between *T. atroviride* and *P. ultimum*. The chemotactic growth of *T. atroviride* toward the host and the coiling around the host hyphae were the most common observations. We found, for instance, that development of the helix-shaped hyphae by the mycoparasite (44) occurred not only in the presence but also in the absence of direct contact with the host (data not shown).

Previous in vitro studies have shown that *Trichoderma harzianum* hyphae grow and branch directly towards their host (9). Here we found that in situ the branching of *T. atroviride* hyphae is an active, probably chemotactic, response to the presence of the host. We also observed papilla-like structures at the *T. atroviride* hyphal tips, which occurred both in the presence and in the absence of direct contact with *P. ultimum*. Bartnicki-Garcia et al. (2) speculated that papilla formation may be caused by exudates released from the host mycelium capable of displacing the Spitzenkörper (a phase-dark body found at the tip of elongating hyphae of higher fungi), which results in the apex becoming rounded and increasing in diameter. Since the fungal tip is an active growing area and very sensitive to many types of disturbances and stimuli, an alternative explanation may be that these morphological alterations are due to the effect of osmotic pressure changes (13). Our results support the hypothesis that papilla formation can occur due to environmental factors other than contact with host fungi. Alterna-

tively, exudates released from the host mycelium could diffuse and induce distant papilla formation in *Trichoderma*.

In coculture experiments, *T. atroviride* spores adhered to the hyphae of *P. ultimum*, where they germinated and parasitized the host. Adhesion of fungal spores to the host surface is generally thought to be a necessary step for germination of the spores of a fungal mycoparasite and establishment of a successful parasitic interaction (25, 27, 28). There may be specific compounds released from the host hyphae that induce germination of *T. atroviride* spores and induce the later steps of mycoparasitism (12, 52, 53). This process may be quite complex. For instance, studies of *Cochliobolus heterostrophus* (6) showed that adhesion of fungal spores to leaves and artificial surfaces is accomplished through a variety of passive and active mechanisms.

The plant seed surface usually is a microbe-rich habitat in which multiple interactions among the germinating seeds, soil pathogens, and antagonists occur. Our in situ study showed that mycoparasitism of *P. ultimum* by *T. atroviride* takes place on the seed surface. We used sterile conditions so that the nontagged fungal hosts could be specifically identified in soil and on plant surfaces. On the seed surface, the mycoparasite usually formed hyphal branches that grew towards the host and resulted in intense mycelial growth around the host mycelium. This active growth may have been supported by the production of extracellular enzymes capable of releasing cell wall components that provided nutrients and/or further stimulated host colonization (4, 12, 33, 35, 52, 53).

The inoculation method affected colonization of the cucumber seed surface by *T. atroviride*. Rapid and extensive coverage of the surface was observed with seeds pretreated with *T. atroviride* spores (treatment A). Early colonization by a biocontrol agent often is required to fill the critical niches and to effectively compete against pathogenic fungi (38). Thus, seed coating with bacterial and fungal biocontrol agents often is utilized or required to control aggressive, rapidly growing soilborne pathogens, such as *P. ultimum* and *R. solani* (15, 38, 41).

Colonization by *T. atroviride* was observed during all growth stages of the young cucumber plant. Before cucumber seeds germinated, the hyphae colonized the seed surfaces. Subsequently, *T. atroviride* colonization extended to the cucumber radicle (Fig. 2), and this organism colonized the rhizosphere of the young cucumber root. This active colonization process may be related to the ability of *T. atroviride* to suppress *P. ultimum*- or *R. solani*-caused diseases (Table 1). Although we found no evidence of the presence of the mycoparasite inside the seeds, we cannot exclude the possibility that there is a direct relationship between the biocontrol agent and the plant.

We observed induction of the biocontrol-related *ech42* and *nag-1* genes during mycoparasitism by fusing the promoters to *gfp* (7, 31, 55). The transformants were activated by the presence of the host, chitin, and chitoligomers (37, 55) and fluoresced during the early phase of the interaction. This interaction occurred during coculture in vitro with *R. solani* in medium containing colloidal chitin or *R. solani* hyphal fragments (55) and in situ in the soil and in the presence of the host (Fig. 6). This is the first observation of in vivo expression of a fungal biocontrol-related gene, a phenomenon that has been predicted by various molecular studies (51; see reference 26 for a review) but has never been observed microscopically previously. GFP fluorescence was detectable within 24 h after *T. atroviride* started to colonize the soil, indicating that induction of both *nag1* and *ech42* is a rather early event during the interaction with *R. solani* and that both endo- and exochitinases may be used by *T. atroviride* to mycoparasitize the living host rather than to simply degrade dead hyphae.

The data presented here and in other studies clearly indicate that biocontrol- or mycoparasitism-related promoters associated with vital markers, such as GFP or DsRed (40), can be effectively used to study microbial interactions. For instance, it is possible to discern patterns of gene induction and to observe fungal interactions in vivo that occur in the soil and around the plant (36). This methodology may provide a way to monitor biocontrol activity (20) and the plant-*Trichoderma* interaction, thereby improving the selection of useful strains and the effectiveness of biopesticide and biofertilizer treatments.

In this work, *gfp* tagging was effective for monitoring in situ interactions between *T. atroviride* and other microbes grown in cocultures or on a plant (1, 23). In our experiments with cucumber, we observed a direct, mycoparasitic interaction between *T. atroviride* P1 and *P. ultimum* on the seed surface, which of course does not rule out the possible involvement of other antagonistic mechanisms (e.g., antibiosis, competition for nutrients or space, induction of resistance in the plant, etc.). On the basis of our CSLM-based study, we concluded that direct mycoparasitism and colonization of plant roots have roles in the biocontrol of *P. ultimum* by *T. atroviride*. In addition, we observed the presence and colonization of the tagged *Trichoderma* in the soil without killing the microorganisms. Such in situ monitoring studies of fungal antagonists should improve both our understanding of the ecology and the agricultural applications of these useful microbes.

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