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Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum*

Mariola R. Chacón,[1,¶] Olga Rodríguez-Galán,[2] Tahía Benítez,[3] Sonia Sousa,[1] Manuel Rey,[4] Antonio Llobell,[1] Jesús Delgado-Jarana[1]*

[1]Institute of Plant Biochemistry and Photosynthesis, Research Center Isla de la Cartuja, CSIC, University of Sevilla, Spain. [2]National Center for Biotechnology, Madrid, Spain. [3]Department of Genetics, University of Sevilla, Spain. [4]Newbiotechnic. Bollullos de la Mitación, Sevilla, Spain

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Summary. The capacity of the fungus *Trichoderma harzianum* CECT 2413 to colonize roots and stimulate plant growth was analyzed. Tobacco seedlings (*Nicotiana benthamiana*) transferred to Petri dishes inoculated with *T. harzianum* conidia showed increased plant fresh weight (140%) and foliar area (300%), as well as the proliferation of secondary roots (300%) and true leaves (140%). The interaction between strain CECT 2413 and the tomato-root system was also studied during the early stages of root colonization by the fungus. When *T. harzianum* conidia were inoculated into the liquid medium of hydroponically grown tomato plants (*Lycopersicum esculentum*), profuse adhesion of hyphae to the plant roots as well as colonization of the root epidermis and cortex were observed. Confocal microscopy of a *T. harzianum* transformant that expressed the green fluorescent protein (GFP) revealed intercellular hyphal growth and the formation of plant-induced papilla-like hyphal tips. Analysis of the *T. harzianum*-tomato interaction in soil indicated that the contact between *T. harzianum* and the roots persisted over a long period of time. This interaction was characterized by the presence of yeast-like cells, a novel and previously undescribed developmental change. To study the molecular mechanism underlying fungal ability to colonize the tomato-root system, the *T. harzianum* transcriptome was analyzed during the early stages of the plant-fungus interaction. The expression of fungal genes related to redox reactions, lipid metabolism, detoxification, and sugar or amino-acid transport increased when *T. harzianum* colonized tomato roots. These observations are similar to those regarding the interactions of mycorrhiza and pathogenic fungi with plants. [**Int Microbiol** 2007; 10(1):19-27]

Key words: *Trichoderma harzianum* · plant–fungus interaction · tomato · tobacco · mycorrhiza · gene expression

Introduction

The fungal genus *Trichoderma* is cosmopolitan in soils, and the ecological adaptability of *Trichoderma* species is evidenced by their widespread distribution, including under different environmental conditions and on various substrates. This physiological flexibility together with the antagonistic action of *Trichoderma* spp. against phytopathogenic fungi and the ability of these fungi to promote plant growth have made them attractive biological control agents [14]. The ability of *Trichoderma* to recognize and parasitize phytopathogenic fungi in the rhizosphere [4,11,26,40] has been ascribed to several complex mechanisms, such as nutrient competition, antibiosis, mycoparasitism, induction of systemic resistance, and increased plant-nutrient availability [21,28,37].

Species of the *Trichoderma* genus are characteristically saprophytes [14]; however, root colonization by some *Trichoderma* strains is a common phenomenon in the field [11]. The relationship between *Trichoderma* and plants has

***Corresponding author:** J. Delgado-Jarana
Centro de Investigaciones Científicas Isla de la Cartuja CSIC, Universidad de Sevilla, Spain.
Tel. +34-954557109. Fax +34-954557104
E-mail: jesusdelgado1@supercable.es

[¶]Present address: Neurosciences Institute of Alicante, UMH-CSIC, San Juan Campus, Alicante, Spain

long been studied [18] and the promotion of plant growth, under axenic conditions and in natural soils [11,37], is among the many reported effects of the plant-fungus interaction [14,21]. *Trichoderma* spp. are thought to promote plant growth by at least two different mechanisms: (i) by controlling the population of pathogenic microorganisms in the rhizosphere [3,4], and (ii) by influencing plant physiology through mineral solubilization [1,11] or hormone secretion [5]. Furthermore, plant-cell-wall-degrading enzymes produced by *Trichoderma*, such as xylanases and cellulases, are able to directly induce ethylene biosynthesis in plants, a well-known response to the presence of pathogens [14]. There is extensive evidence for the induction of plant defense systems by *Trichoderma* species in a number of plants [10,12,14]. Taken together, these data indicate that *Trichoderma* is able to colonize plant roots, inducing changes in plant defense systems and plant physiology without destroying plant tissues [38]. However, most such studies have focused on plant physiology without studying the fungal component of the association.

The aim of this study was to analyze the morphological and genetic changes in *Trichoderma* following plant-root colonization.

Materials and methods

Organisms, media and growth conditions. *Trichoderma harzianum* CECT 2413 (Colección Española de Cultivos Tipo, Burjassot, Valencia, Spain) was maintained at 4ºC in the dark on potato dextrose agar (PDA) (Difco, USA). Tobacco (*Nicotiana benthamiana*) and tomato (*Lycopersicum esculentum*, Manitu) seeds (Semillas Ramiro Arnedo, Calahorra, Spain) were maintained in the dark at 4ºC. Seeds were sterilized with 10% (v/v) sodium hypochlorite solution (Scharlau, Barcelona, Spain) for 15 min, washed three times with sterile distilled water and vigorous shaking, and submerged in autoclaved distilled water for 24 h at room temperature to synchronize germination. Tomato seeds were germinated and grown on perlite for 3 weeks in a growth chamber at 70% hygrometry, with a photoperiod of 16 h light at 90 μE m^{-2} s^{-1} and 25ºC, and 8 h dark at 23ºC. Tobacco seedlings were obtained by seed germination in MS medium (Murashige & Skoog Medium, Duchefa Biochemie, Netherlands) with 3% (w/v) sucrose and 0.8% (w/v) agar at pH 5.6, 25ºC in the dark. After the cotyledons had completely emerged (5 days), the seedlings were transplanted.

Plant-growth promotion by *T. harzianum* root colonization. Square Petri dishes (150-mm side) were half-filled with 0.5× MS medium containing 0.8% agar (Murashige & Skoog Medium, Duchefa) and then allowed to solidify with a slope of 30º. On the lower part of the slanting surface, ten drops, each containing ten conidia of *T. harzianum*, were dotted linearly after which the dishes were incubated for 24 h. Tobacco (or tomato) seedlings (obtained as described above) were then transplanted to each dot showing germinated conidia by gently inserting the plant roots in the agar medium (see Fig. 1A). Dishes were then sealed with laboratory film, half-covered with aluminum foil to keep the roots in the dark and the cotyledons in the light, and incubated in a vertical position in a growth chamber at 70% hygrometry, with a photoperiod of 16 h light at 90 μE m^{-2} s^{-1} and 25ºC, and 8 h dark at 23ºC. The seedlings were checked after 20 days.

To determine the effect of *T. harzianum* on tobacco plants, the true leaves were counted and the secondary root number was quantified by stereomicroscopy. In addition, fresh weight was determined by gently removing the plants and then weighing them. Foliar areas were measured by removing the first leaf of several plants and then photographing the collected leaves using an Olympus SZ4045TR zoom stereo microscope with an adapted Leica DFC 300 FX camera. Surface areas (mm^2) were measured by Image J software [http://rsb.info.nih.gov/ij/index.html]. Samples from independent experiments were processed and imaged under identical conditions. Experiments aimed at assessing the promotion of plant growth were done at least twice, with eleven replicates for each condition and ten plants per replicate. Overall significant differences between conditions were determined by analysis of variance (ANOVA, 5% significance level).

Plant-fungus interactions in hydroponic culture. *T. harzianum* germlings were obtained by inoculating 10^6 conidia/ml in 200 ml of potato dextrose broth (PDB) (Sigma, Madrid, Spain) and then further incubated for 15 h at 28ºC and 200 rpm. The germinated conidia were centrifuged at 4,400 rpm for 10 min, washed three times with sterile distilled water, suspended in 25 ml of sterile water, and used immediately to study fungal-plant interactions in hydroponic cultures. Bunches of 15 tomato plants, tied together with strings, were placed inside 250-ml flasks containing 150 ml of salt minimal medium MM [24] supplemented with 0.2% (w/v) glycerol as the sole carbon source and 20 mg ammonium sulfate/l. The roots were submerged in the medium up to the hypocotyls. The flasks were then inoculated to a final concentration of 10^5 germlings/ml, prepared as described above, and incubated for 10–24 h at 25ºC with shaking at 115 rpm for early colonization studies. To study later stages, the plants were transferred immediately to sand pots and grown in a growth chamber, as described above, until harvest. The same experiment but without inoculation of the fungus was done as a control. Microscopy was carried out in five independent experiments.

Bright-field microscopy. Roots colonized by *T. harzianum* were taken from the hydroponic cultures after 18 h, thoroughly washed with distilled water, and fixed by immersion in 4% (v/v) paraformaldehyde–0.25% (v/v) glutaraldehyde in 0.05 M potassium-phosphate buffer (pH 7.2) for 3 h at 4ºC. These samples were further incubated under vacuum for 1 h at 4ºC, washed with 2% (v/v) paraformaldehyde in 0.05 M potassium-phosphate buffer (pH 7.2) for 15 min at 4ºC, kept at 4ºC, and washed twice with 0.05 M potassium phosphate buffer and gentle shaking for 15 min before use. They were then gradually dehydrated in an ethanol series (50–100%) without shaking, 15 min per step, and finally in 100% ethanol for 30 min, infiltrated in 100% ethanol/SPURR (Fluka, Sigma-Aldrich, Madrid, Spain) medium grade (1:1) for 4 h, and maintained in SPURR (Fluka, Sigma-Aldrich, Madrid, Spain) medium grade overnight at 4ºC. This was followed by embedding in SPURR medium grade overnight at 60ºC. Ultra-thin sections (1μm) were cut with an ultra microtome (Reichert-Jung ultracut E, Leica) and stained with aqueous 1% (w/v) toluidine blue (Sharlau, Barcelona, Spain). Microscopy of the samples was carried out using an Olympus BX60 microscope. Control plants underwent the same procedures.

Confocal laser scanning microscopy (CLSM). A transformant from *T. harzianum* CECT 2413 that expressed the *gfp* gene, encoding green fluorescent protein (GFP), from *Aquarea victoria* was analyzed by CLSM. This strain was obtained by protoplast cotransformation of *T. harzianum* [24]. The pZEGA1 vector carried the *gfp* gene under the control of the constitutive promoter *pki1* (pyruvate kinase) of *T. reesei* [39]; the p3SR2 vector carried the *amdS* gene, which allows growth with acetamide as the sole nitrogen source [13]. Transformant strains that expressed *gfp* and showed the same physiological and morphological characteristics as the wild-type in several culture media were isolated, and one of the transformants, GFP22, was chosen for CLSM analysis.

CLSM with a Leica TCS SP2 was also used to study the plant-fungus interaction in vivo. Tomato roots colonized by *T. harzianum* GFP22 were taken from the hydroponic cultures after 10 and 24 h incubation or harvested from the sand after 24, 48, 72, 96 144, and 158 h of growth and thorough-

ly washed with distilled water. Samples were then excised and stained with 10 μM propidium iodide (Sigma, Madrid, Spain) for 10 min to label the plant cell wall. The excitation wavelength was 488 nm (argon/krypton laser) and the emission wavelengths were 500–550 nm for GFP and 600–750 nm for propidium iodide. A dichroic filter RSP 500 was also used. A panoramic view was obtained with a ×20 objective and detailed views with ×40 and ×63 objectives and a zoom factor. Images were acquired by Leica Confocal Software 2.5.

Macroarray preparation. DNA to be spotted was obtained by PCR using universal primers from the library vector and cDNA clones isolated from *T. harzianum* CECT 2413 grown in different cultures covering a wide range of conditions (glucose or chitin as carbon sources, nitrogen and carbon starvation, fungal cell walls, plant cell walls, and acidic pH). PCR reactions (100 μl) were carried out in 96-well microtiter plates, with standard amplification parameters. The PCR products (1 μl) were separated on an agarose gel to assess the quality and quantity of the product. The DNA was denatured in 50% DMSO and then spotted using a BIOMEK 2000 robot (Beckman, USA) onto 12 × 8 cm Hybond N+ membranes (duplicate spots) (Amersham, Madrid, Spain). The deposited DNA was then cross-linked to the membranes by UV radiation (125 mJ) in a Vilber-Lourmat cross-linker (Marne La Vallée, France). Membranes were stored at room temperature until use. A set of filters was prepared containing all unique sequences available at the time of analysis [33] [http://www.trichoderma.org]. Each filter contained 672 unique sequences organized in an overall grid of 12 columns × 8 rows and subgrids of 4 × 4 spots, with every spot repeated twice as indicated above. Twenty-six microtiter plates, each containing 96 clones (2496 total spots), were spotted in duplicate on four membranes replicated three times. Additionally, the membranes included positive controls (expressed sequence tags (ESTs) corresponding to known *T. harzianum* genes, i.e., genes encoding cell-wall-degrading enzymes, cell-cycle proteins, etc. [33], negative controls (library vector, PCR buffer), and loading controls composed of RNA obtained by in vitro transcription of several plant-specific genes. RNA was isolated as described below. Filters containing 288 spots with homology to known DNA sequences [33] organized in an overall grid of 12 columns × 8 rows and interleaved subgrids of 2 × 4 spots were used for positive controls. Controls of known amounts of DNA were spotted in some of the wells in order to normalize and quantify the intensity of the dots.

Macroarray hybridization. Probes were prepared from fungal samples harvested from the hydroponic cocultures after 10 and 24 h. Mycelium grown on the plant roots was separated from plant material by vigorous shaking in ice-cold water and filtered immediately. Samples were also harvested without eliminating the plant roots, to obtain mRNA from fungus growing inside the plant tissues. A culture of *T. harzianum* growing in the absence of plant roots was used as the control. For RNA extraction, mycelia were harvested, frozen in liquid nitrogen, lyophilized, and ground to a fine powder. RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). Probes were prepared by reverse transcription using 12.5 μg of total sample RNA, 0.15 ng of each plant-specific control RNA, 7.5 μCi [^{32}P]-dCTP, and 1 μl of PowerScript Reverse Transcriptase enzyme (Clontech, Palo Alto, CA, USA) per membrane (9 × 12 cm) following the manufacturer's indications. After the reaction, free [^{32}P]-dCTP was removed by filtration through a Microspin S400-HR column (Amersham, Madrid, Spain), and the reaction efficiency was quantified by means of a scintillation counter. Triplicate cDNA probes were prepared from three independent experiments. Membranes were prehybridized at 65ºC in Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 10 mM EDTA, 7% [w/v] SDS) for 1 h and then hybridized at 65ºC using 10–15 ml of fresh buffer per 9 × 12 cm membrane. Radioactive probe was added to obtain 5 × 10^5 cpm per ml hybridization buffer. The membranes were hybridized overnight, washed three times with washing buffer (0.04 M sodium phosphate buffer, pH 7.2, 0.1% [w/v] SDS) at 65ºC, wrapped using a single layer of transparent film, and exposed for 3 h on a Packard Multipurpose MP intensifying screen. The image was obtained using a Cyclone Storage Phosphor System (Perkin Elmer, Boston, MA, USA). Three independent hybridizations were done using different probes and membranes.

Data analysis. The image of the hybridized membrane was analyzed using Phoretix Array v3.1 (Nonlinear Dynamics, USA) as follows: First, a background control subtraction of the median spot-edge average was carried out, followed by normalization of the intensities of the dots on the basis of the intensities of the internal controls. Dots with intensity values below the mean intensity of the negative controls were discarded. Each EST was dotted in duplicate, and each membrane was hybridized three times. Dot and replica averages and standard deviations were calculated for the entire membrane in order to determine the accuracy of the experiment. Finally, the ratio of the intensity values of the test (plant-fungus interaction) and control (fungus growing alone) conditions was calculated. An arbitrary threshold of a two-fold difference between intensity levels was used to define genes as induced during plant-fungus interaction.

Results

Promotion of plant growth by *T. harzianum* root colonization. Plant-growth promotion by *T. harzianum* CECT 2413 was assayed under sterile conditions after 20 days of incubation. Effects due to the solubilization of soil nutrients were excluded by the use of complete specific plant-culture medium. Plants inoculated with *T. harzianum* developed better than control plants (Fig. 1A), as evidenced by a three-fold increase in foliar area (5.32 ± 1.88 for control vs. 17.72 ± 9.82 for *T. harzianum*; Fig. 1E), a 1.4-fold increase in the number of true leaves (3.34 ± 0.45 for control vs. 4.74 ± 0.40 for *T. harzianum*; Fig. 1B), a three-fold increase in the number of secondary roots (1.3 ± 0.37 for control vs. 3.89±1.20 for *T. harzianum*; Fig. 1D), and a 1.4-fold increase in fresh weight (16.5 ± 5.6 for control vs. 23.2 ± 6.6 for *T. harzianum*; Fig. 1C). A comparison between plants treated with *T. harzianum* and untreated plants yielded *P* values of 0.0183 for fresh weight, 0.0001 for secondary root number, 0.0001 for foliar area, and 0.0001 for the number of true leaves. Although in some cases the standard deviations reached these values, ANOVA test results and the low SEM values (bars in Fig.1) indicated significant differences between *T. harzianum* and control treatments. Hence, *T. harzianum* CECT 2413 promoted tobacco plant growth by increasing the foliar area, the number of leaves and secondary roots, and the plant fresh weight. These effects were clearly not due to nutrient solubilization or to the control of plant pathogenic microorganisms. Experiments with tomato plants showed that inoculation with *T. harzianum* yielded effects on growth promotion similar to those detected in tobacco plants inoculated with the fungus (data not shown). Furthermore, compared to tobacco plants, tomato seeds germinated more synchronously, plant sizes were more homogeneous, and the root system grew more profusely. For these reasons, further experiments were carried out with tomato plants.

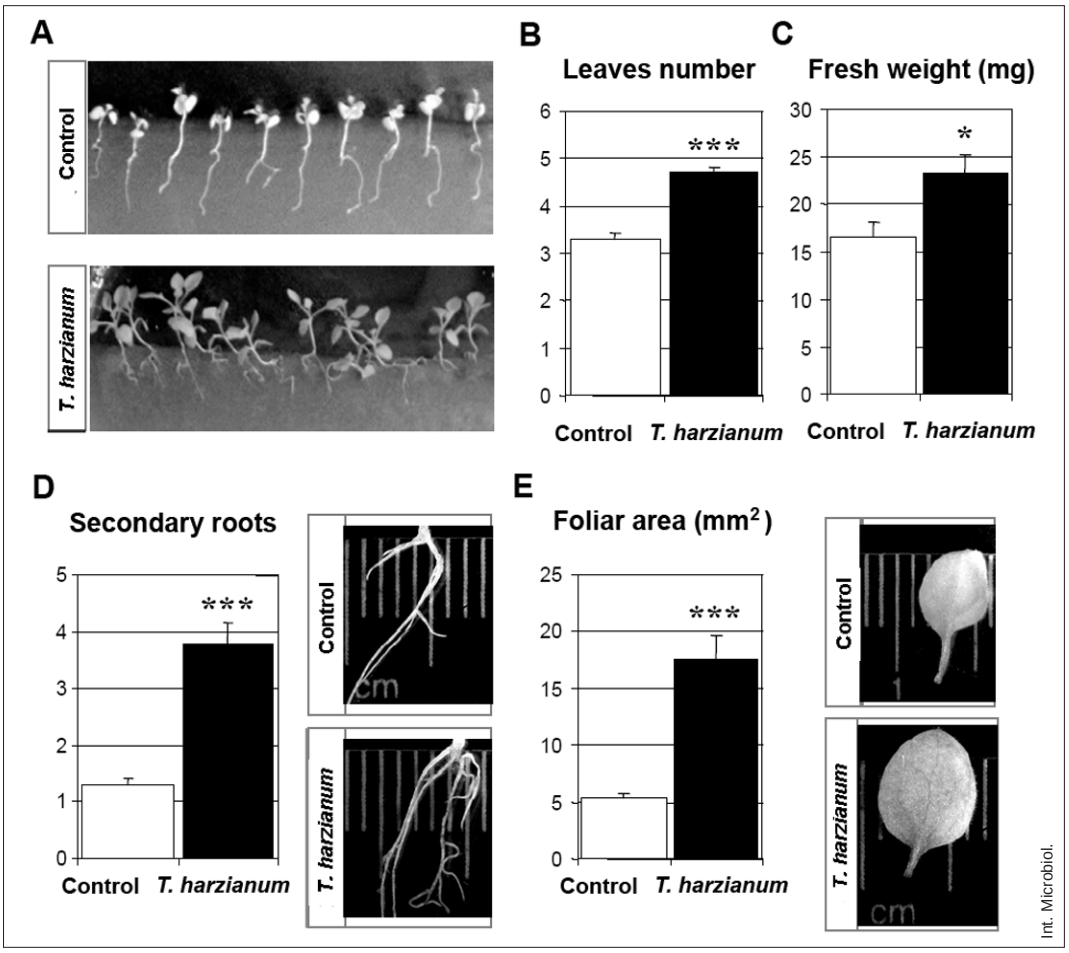

Fig. 1. Plant growth promotion assay. (**A**) Tobacco germlings planted in Petri dishes inoculated with *T. harzianum* (10 spores per root) or non-inoculated. The promotion of plant growth was quantified after 20 days (**B**), fresh weight (**C**), length of secondary roots (**D**), and foliar area (**E**). The results are based on at least two independent experiments. Data are expressed as mean values ± standard error of the mean (SEM). Asterisks indicate results of ANOVA test (B–E): $*P < 0.05$ and $***P < 0.001$. D and E are sample representative images.

Bright-field microscopy of root cortex colonized by *T. harzianum* hyphae. Tomato roots colonized by *T. harzianum* were obtained using a hydroponic culture, as described in Materials and methods. Cross-sections of *T. harzianum* hyphae (toluidine-blue-stained) and plant cells were prepared 18 h after inoculation (Fig. 2A) and showed that most fungal cells grew parallel to the longitudinal root axis. *T. harzianum* colonized the root epidermis and cortex but not the vessels (Fig. 2A). Hyphae grew within the tissues through intercellular spaces (Fig. 2B). While the non-inoculated roots showed intact cellular structures, roots inoculated with *T. harzianum* showed a subtle disorganization of the epidermis (Fig. 2A). Moreover, the cell walls of some root cells were highly stained, indicating that plants responded to fungal penetration by depositing dense matter at these sites.

In vivo examination of tomato-root colonization by *T. harzianum* using CLSM. A GFP-expressing transformant of *T. harzianum*, GFP22, was used to visualize the fungus by its fluorescence. Samples of 3-week old plants co-cultured hydroponically with *T. harzianum* germlings were collected after 10 and 24 h and analyzed by CLSM. The epidermis, cortex, and vessels of the root cells were intact or only minimally altered. After 10 h of co-culture, hyphae had entered the roots and grown in the intercellular space of the epidermis (Fig. 3A). The green fluorescent hyphae had grown between the epidermal cells and were surrounded by red fluorescent plant cell walls (Fig. 3A, B). In some cases, the elongated zone of the hypha showed a swollen tip, which was identified as a non-septated papilla-like structure (Fig. 3A, C). Extensive colonization of the root surface was observed 24 h after co-inoculation, so that a

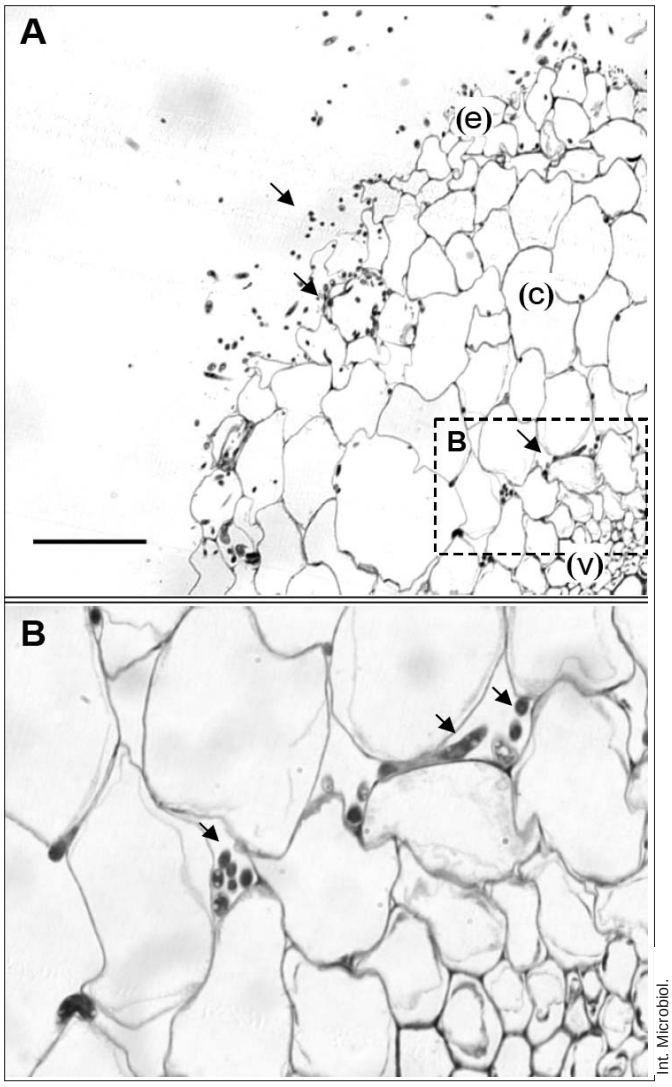

Fig. 2. Light micrographs of ultra-thin cross-sections of tomato root 18 h after inoculation with *T. harzianum*; magnified ×1000 (**A**) or ×2000 (**B**), inset. Hyphae (arrows) are shown by toluidine-blue staining. More intense staining of the cell walls is indicated by arrowheads; e, epidermis, c, cortex; v, vessels. Cross-sections of the tomato root control showed the same organization. Scale bar: 10 mm (A).

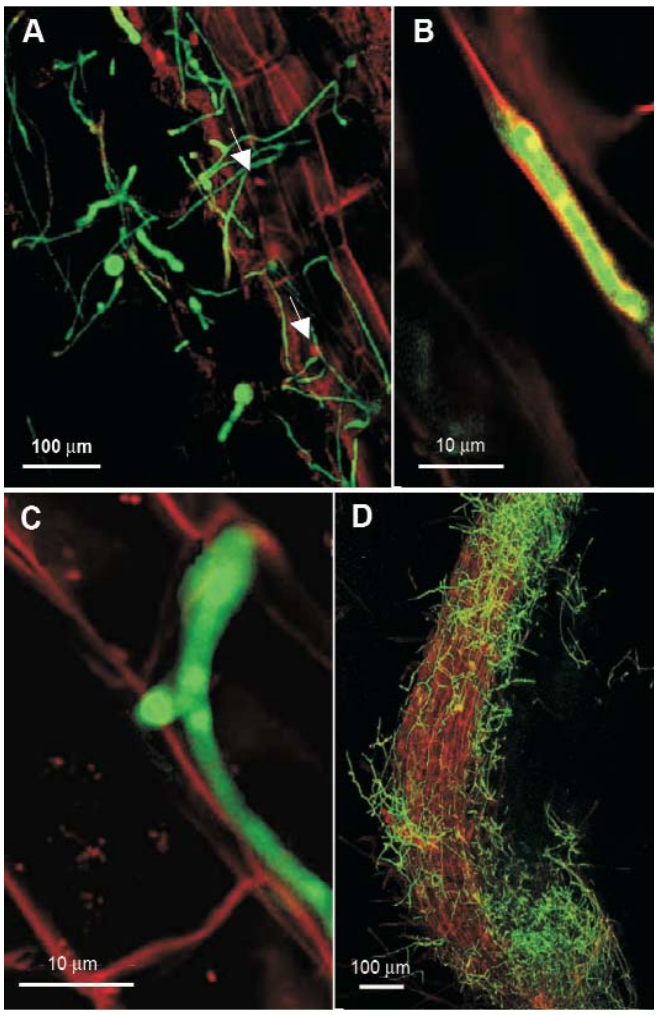

Fig. 3. CLSM analyses of tomato root colonization with *T. harzianum* GFP22. Green fluorescence caused by GFP expression indicates the fungus. Propidium-iodide-stained plant cells are shown as red fluorescence. (**A**) Germlings growing in contact with root cells after 10 h. Arrows indicate propidium-iodide-stained root cell nuclei in the area of contact between plant and fungus. (**B**) Hypha growing between red fluorescent plant cell walls. (**C**) Hyphal tip showing a papilla-like structure. (**D**) Extensive colonization of the tomato root surface after 24 h co-culture.

dense network of hyphae covered the entire surface of the main root (Fig. 3D).

Hyphal tips from *T. harzianum* incubated for 10 h with or without tomato plants were counted and compared. Swollen, papilla-like structures were seen in 69% (± 2.83 SD) of the hyphal tips from plant-fungus samples compared to 25% (± 5.65 SD) in control samples. This result indicated that the fungus had undergone specific morphological changes in response to the plant roots.

Long-term colonization of tomato roots by *T. harzianum* in soil. To analyze root colonization over a longer period of time, plants inoculated with *T. harzianum* germlings were immediately planted in soil sand. After one day, the fungus showed the same pattern of colonization previously described for hydroponic cultures; that is, hyphae were attached to the root surface and had settled in the grooves between epidermal cells (Fig. 4A). After 48 h, the fungus had grown inside the roots, within the intercellular spaces, and some root cells were colonized intracellularly by *T. harzianum* (Fig. 4B). After 72 h, *T. harzianum* underwent further morphological changes, switching to a yeast-like cell type (Fig. 4C). Outside the roots, these yeast-like cells remained attached to hair roots or emergence sites (Fig. 4D). At 120 h (Fig. 4E), some hyphae were still observed, togeth-

er with yeast-like cells. Many yeast-like cells were attached to the roots even after 144 and 168 h (Fig. 4F, G). These were of different sizes and some were septated (Fig. 5A). When *T. harzianum* was inoculated into soil in the absence of tomato plants, the mycelial mass lysed and the fungus sporulated rapidly, showing fluorescent conidia (Fig. 5B) different in size and shape from those of the yeast-like cells (Fig. 5A).

***T. harzianum* gene expression profiling of early colonization of tomato roots.** In order to unravel the changes in *Trichoderma* gene expression that may be related to root colonization, transcriptome analysis was carried out. RNA samples of *T. harzianum* incubated under control conditions were compared with those of *T. harzianum* grown with plant roots (hydroponic cultures). The arrays contained 2496 unique ESTs from *T. harzianum* grown under conditions covering a wide range of transcriptional profiles. ESTs that showed at least a two-fold increase under plant-*Trichoderma* conditions were considered plant-induced ESTs. According to this criterion, 5–6% of the ESTs were identified as up-regulated during plant-*Trichoderma* interactions (see Table 1, ONLINE). Some of these ESTs showed high similarity to genes involved in different cellular processes, such as redox metabolism, morphological changes, or membrane and vesicle synthesis. ESTs corresponding to genes involved in lipid metabolism, vesicle trafficking, membrane fusion, and cell-wall synthesis were induced during the early stages of root colonization: an integral membrane protein (L02T34P058R05413, entry n.11); a cold-active sterase (L06T34P031R02957, entry n.39); an endosome cargo

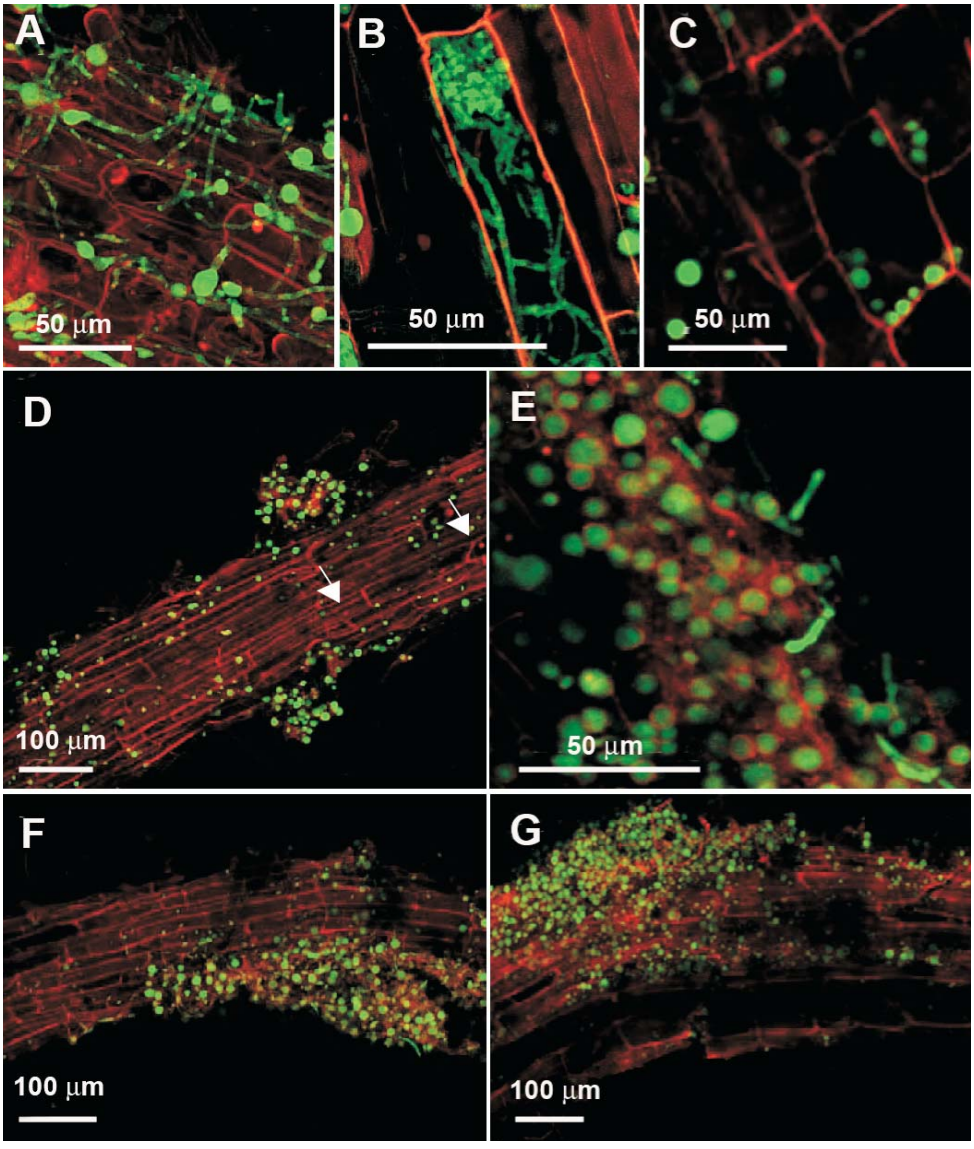

Fig. 4. CLSM analyses of long-term tomato root colonization by *T. harzianum*. (**A**) Root colonization after 24 h of inoculation. (**B**) Hyphae growing inside a plant cell after 48 h. (**C**) Yeast-like cells inside plant cells after 72 h of inoculation. (**D**, **F**, **G**) Yeast-like cells adhered to hair roots or emergence sites. D: 96 h, F: 144 h, G: 168 h. Arrows point to some root cell nuclei stained with propidium iodine in the area of contact between plant and fungus. (**E**) Yeast-like cells and hyphae after 120 h.

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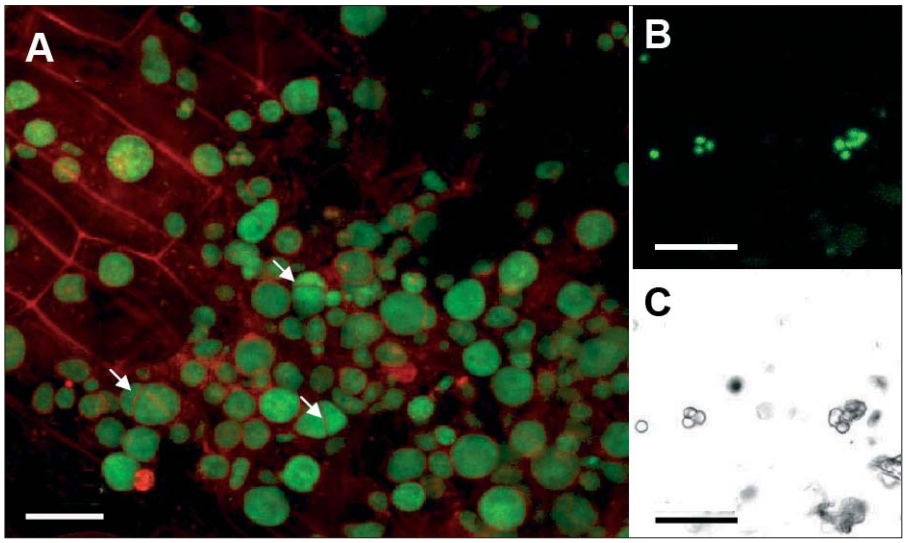

Fig. 5. CLSM analysis of fungal specific structures during colonization in sand soil. (**A**) After two days yeast-like cells were observed. Some of them were septated (arrows). (**B**) After four days, fungus inoculated without tomato plants lysed and sporulated. (**C**) Light microscopy picture of conidia shown in B. Scale bars represents 20 µm, which demonstrates the different sizes of yeast-like cells and conidia.

receptor protein (L02T34P020R01878, entry n.57); a cell-wall glucanase (L05T34P052R04860, entry n.68); a clathrin-coated vesicle protein (L02T34P058R05376, entry n.92); a multispanning membrane protein (L02T34P054R05069, entry n.110); an EST related to fatty acid hydroxylase (L02T34P050R04655, entry n.134); and a sterol desaturase (EST L05T34P052R04838, entry n.16). Other ESTs related to *Aspergillus* sugar and amino-acid transport proteins were also induced: an amino-acid transporter (L02T34P058R0 5377, entry n.85) and a monosaccharide transporter (L91 T34P045R04147, entry n.109). Several ESTs up-regulated during plant colonization referred to energy-related processes, such as genes related to ATPases (L05T34P052R04801, entry n.90; L06T34P027R02501, entry n.93; L06T34P032R0 3008, entry n.127; L02T34P018R01662, entry n.136). Finally, two ESTs involved in redox metabolism were also induced: a NAD-dependent malate dehydrogenase (L02T3 4P054R05017, entry n.12) and a NAD-dependent formate dehydrogenase (L06T34P029R02721, entry n.42).

Discussion

T. harzianum CECT 2413 promoted the growth of tomato and tobacco plants, increasing foliar area and secondary roots, among other parameters. Moreover, changes in the root-system architecture were detected (Fig.1C, D). The experiments were carried out in sterile MS medium, so that protection of the plants by the fungus against other microorganisms of the rhizosphere and/or metal solubilization could be ruled out as reasons for the increased growth. The significant promotion of plant growth observed in this study was likely due either to the secretion of phytohormones by *T. harzianum* [5] or to the development of a plant-fungus relationship similar to that described for mycorrhizal fungi, in which plant growth is promoted by nutrient exchange [2,22].

Plant-root colonization by *T. harzianum* was monitored by light microscopy and CLSM, and resembled that of plant associations involving mycorrhizal or phytopathogenic fungi. Hyphae adhered to roots and emergence sites, as described for other fungi [15,22,23,34]. A slight disorganization of the epidermal cell layer was observed at those sites where fungal germlings had attached to the roots (Fig. 2A). Nevertheless, the cortex was colonized mostly through the intercellular spaces and without disturbing cell integrity (Fig.2B). In some cases, hyphae penetrated living plant cells since plant nuclei were not accessible to the dye and remained unstained (Fig. 4). Fungal growth was observed mainly in intercellular spaces, and fungal colonization was not associated with disruption of the host cell wall, in contrast to reports for various host-pathogen interactions [27,31]. A similar behavior was reported for cucumber roots after inoculation with *T. harzianum* strain T-203, i.e., extensive colonization of the root surface, appressoria-like structures, and hyphal filaments penetrating the root epidermis between adjacent cells, generating a lytic zone around the penetrated area [38]. The increased staining of the cell wall (Fig. 2) may have been a consequence of lignin and/or glucan deposition, characteristic of the systemic resistance response induced in plants [10,16]. Similar wall appositions have also been described for interactions between plant and nonpathogenic microorganisms [16,37].

Swollen hyphal tips described as papilla [19,23] were seen during the interaction of *T. harzianum* with tomato roots

(Fig. 3A, C), but have not been reported before in this species [14]. The frequency of these papilla-like formations increased three-fold in the presence of plant roots. Specific morphological structures have also been described during early interactions with plants in mycorrhizal fungi, where they are thought to play a role in nutrient exchange [34]; in phytopathogenic fungi, where they are involved in the recognition and/or penetration of plant cells [31]; and in *T. harzianum* strain T-203, in which appressoria-like structures related to biological control were frequently detected as part of the interaction of T-203 with plants [38]. Here, when experiments were done in soil, some hyphae penetrated the plant cells but no changes indicative of cell death were detected. After 72 h, a sudden morphological switch from filamentous growth to a yeast-like shape—a cell structure that has not been described for this fungus before [14]—was observed (Figs. 4, 5). The yeast-like cells maintained fluorescent activity for at least 168 h (Fig 4G), which suggested that they were metabolically active and related to the survival of the fungus in soil. Survival structures (i.e., chlamydospores) have been reported in fungi pathogenic for plants [7] and mammals [17], although chlamydospores are usually formed within hyphae or at hyphal tips [17]. Sporangia and conidia of some fungi, such as the phytopatogen *Uromyces fabae*, have been described as septated yeast-like cells and egg-shaped, respectively. In addition, two components of a fungal structure called the hyphopodium are thought to be remnants of appressoria and haustoria [32]. An alternative hypothesis for the role of yeast-like cells is that they provide an additional mechanism of enhancing surface exchange between the fungus and the plant.

In order to follow those changes in *Trichoderma* gene expression that may control events observed during the early colonization of plant roots, a transcriptome analysis was carried out. Array analysis of early plant-root colonization (19 and 24 h cocultures) showed that 5–6% of the 2496 unique ESTs were induced. Some of the up-regulated genes showed similarities to those described in mycorrhizal and pathogenic fungi during plant-fungus interactions [9,20,22,36]. An EST with homology to sterol desaturase (entry n.16) was highly induced in *T. harzianum*, as was reported for mycorrhizal formation between *Eucalyptus globulus* and *Pisolithus tinctorius* [35]. ESTs corresponding to genes involved in lipid metabolism, vesicle trafficking, as well as membrane and cell-wall synthesis were also induced during early root colonization (entries n.11; n.39; n.57; n.68; n.92; n.110; n.134). These results suggested a reorganization of both the membrane and the fungal cell wall. Proteins with transmembrane domains were shown to be differentially expressed in ectomycorrhiza [35]. *T. harzianum*–plant interactions may require an increase in membrane formation that favors the exchange of nutrients between the fungus and the plant, as reported for mycorrhizal symbiosis [29] and other biotrophic interactions [36]. This hypothesis was supported by the high-level expression of fungal genes related to sugar and amino-acid transport (entries n.85 and 109).

Other ESTs up-regulated during plant colonization were related to ATPases. Similarly, appressorium formation induced an increase in the expression of the H^+-ATPase gene HA5 in the mycorrhizal fungus *Glomus mossae* [25], in a process related to Ca^{2+}-dependent signaling [6,23]. A high demand for metabolic energy was also detected in the plant-fungal pathogenic interactions of *Alternaria* and *Arabidopsis* [8]. The relevance of redox metabolism in plant-*Trichoderma* interactions was highlighted by the up-regulation of genes involved in such processes, e.g., EST entries n.12 and 42, which encode NAD-dependent malate dehydrogenase and NAD-dependent formate dehydrogenase. These genes were also induced in *Alternaria* in its pathogenic interaction with *Arabidopsis* [8]. EST n.73, which is homologous to the Gβ subunit of *Fusarium oxysporum*, related to calcium signaling and hyphal development [9], was also induced during early root colonization, as was an ABC multidrug transporter (entry n.61). The ABC transporter Mg, Atr4 in the phytopathogenic fungus *Mycosphaerella graminicola*, has been described as a virulence factor involved in wheat colonization [30]; its function is probably related to neutralizing toxic metabolites produced by plant cells during colonization.

The results showed that *T. harzianum* CECT 2413 is able to promote plant growth and colonize roots. Furthermore, the fungus undergoes morphological changes that are specific for plant-fungus interactions, such as the formation of papilla-like hyphal tips and yeast-like cells. Transcriptome analysis showed that the gene-expression patterns accompanying these interactions are similar to those of phytopathogenic and mycorrhizal fungal-plant associations. Our results provide further evidence that, in their interactions with plants, fungi invoke a range of interactive responses based on several common mechanisms. *Trichoderma* is a biotrophic symbiont that is more closely related to mycorrhyzal than to plant pathogenic fungi, since it does not damage host plants, but instead promotes plant growth. This observation, together with studies carried out over the last decade, may contribute to the development of *T. harzianum* as a biocontrol agent.

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