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Colonization of *Arabidopsis* roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonic acid/ethylene and salicylic acid pathways

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Abstract *Trichoderma* spp. are common soil fungi used as biocontrol agents due to their capacity to produce antibiotics, induce systemic resistance in plants and parasitize phytopathogenic fungi of major agricultural importance. The present study investigated whether colonization of *Arabidopsis thaliana* seedlings by *Trichoderma atroviride* affected plant growth and development. Here it is shown that *T. atroviride* promotes growth in *Arabidopsis*. Moreover, *T. atroviride* produced indole compounds in liquid cultures. These results suggest that indole-acetic acid-related indoles (IAA-related indoles) produced by *T. atroviride* may have a stimulatory effect on plant growth. In addition, whether colonization of *Arabidopsis* roots by *T. atroviride* can induce systemic protection against foliar pathogens was tested. *Arabidopsis* roots inoculation with *T. atroviride* provided systemic protection to the leaves inoculated with bacterial and fungal pathogens. To investigate the possible pathway involved in the systemic resistance induced by *T. atroviride*, the expression profile of salicylic acid, jasmonic acid/ethylene, oxidative burst and camalexin related genes was assessed in *Arabidopsis*. *T. atroviride* induced an overlapped expression of defence-related genes of SA and JA/ET pathways, and of the gene involved in the synthesis of the antimicrobial phytoalexin, camalexin, both locally and systemically. This is the first report where colonization of *Arabidopsis* roots by *T. atroviride* induces the expression of SA and JA/ET pathways simultaneously to confer resistance against hemibiotrophic and necrotrophic phytopathogens. The beneficial effects induced by the inoculation of *Arabidopsis* roots with *T. atroviride* and the induction of the plant defence system suggest a molecular dialogue between these organisms.

Keywords Plant–fungus interaction · Systemic resistance · Camalexin · PR proteins

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Abbreviations

ATPCA	*Arabidopsis thaliana* Peroxidase A
IAA	Indole Acetic Acid
ISR	Induced Systemic Resistance
LOX-1	Lipoxygenase 1
NPR1	Non-Expressor of PR genes 1
PAD3	Phytoalexin Deficient 3
PDF1.2	Plant Defensin 1.2
PR	Pathogenesis Related Protein
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
JA	Jasmonic Acid

Introduction

Plants have developed sophisticated defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. When under attack, a plant is capable of enhancing its resistance, a condition often referred to as induced, or acquired resistance. Acquired disease resistance is thought to involve an enhancement of basal resistance (Ton et al. 2002; Van Loon 2007). In this response, salicylic acid (SA) plays a crucial role in plant defence and is generally involved in the activation of defence responses against biotrophic and hemibiotrophic pathogens, as well as the establishment of Systemic Acquired Resistance (SAR) (Van Loon 2007). Mutants that are affected by the accumulation of SA or are insensitive to SA show enhanced susceptibility to biotrophic and hemibiotrophic pathogens. Recently, it has been shown that SA, which is induced upon pathogen infection, acts as a mobile inducer of SAR in tobacco (Park et al. 2007). SA levels increase in pathogen-challenged tissues of plants and exogenous applications result in the induction of pathogenesis-related (*PR*) genes and enhanced resistance to a broad range of pathogens (Bari and Jones 2009).

In contrast to SAR, jasmonic acid (JA) and ethylene (ET) mediate the induced systemic resistance (ISR), this response is usually associated with defence against necrotrophic pathogens and herbivorous insects (Bari and Jones 2009). In this response, several JA/ET-dependent genes that encode PR proteins, including plant defensin1.2 (*PDF1.2*), thionin2.1 (*THI2.1*), hevein-like protein (*HEL*), and chitinase B (*CHIB*) are expressed. *PDF1.2* is commonly used to monitor JA/ET-dependent defence responses (Van Loon et al. 2006).

Although, SA and JA/ET defence pathways are mutually antagonistic, evidence of synergistic interactions have also been reported mediated by the transcription factor NPR1 (Non-expressor of *PR* genes 1). The NPR1 protein is an important transcriptional co-activator of SA-responsive *PR* genes; NPR1 is also a key regulator in SA-mediated suppression of JA/ET signalling. Furthermore, NPR1 has been implicated in several other JA/ET-dependent defence responses, including beneficial rhizobacteria-mediated ISR (Van Loon 2007) and JA/ET-dependent resistance against the soil-borne fungus *Verticillium longisporum* (Pieterse et al. 2009); however, the pathway involved in the response to beneficial microorganisms has not been elucidated.

Trichoderma spp. are common soil fungi widely used as biocontrol agents against plant pathogens of major agricultural importance (Chet and Inbar 1994; Harman et al. 2004). The biocontrol mechanism exerted by *Trichoderma* spp. is comprised by different mechanisms, including the production of antibiotics, competition for space and nutrients with other rhizosphere microorganisms, as well as the direct attack of phytopathogenic fungi by means of mycoparasitism (Chet and Inbar 1994; Harman et al. 2004).

In addition, some *Trichoderma* rhizosphere-competent strains can colonize either the root surfaces or the entire plant, a process that has been shown to bestow significant beneficial effects to plants, such as root growth, plant growth enhancement, and increases in productivity (Yedidia et al. 1999; Bailey et al. 2006). Moreover, root growth induced by *Trichoderma* spp. increases nutrient uptake, drought and soil packing tolerance, and fosters germination and vigour of the seeds (Harman et al. 2004; Shoresh et al. 2010). Furthermore, *Trichoderma virens* promotes plant growth by the production of phytohormones and IAA-related indoles (Contreras-Cornejo et al. 2009).

Activation of plant defence responses by *T. harzianum* has been reported. During root colonization, *T. harzianum* induces the defence system in cucumber, by increasing chitinase and peroxidase activity in leaves and roots (Yedidia et al. 1999). This response involves recognition of the fungus through the ISR pathway. This response is the closest analogue of induced resistance activated by rhizobac-

teria (Bakker et al. 2003; Van Loon 2007). In addition, *Trichoderma longibrachiatum* induces the expression of *PR* genes whose response is mediated by SA, which is also triggered by necrotizing pathogens (Martinez et al. 2001). In *Trichoderma spp.* a number of elicitors of the plant defence system have been characterized as well as proteins with enzymatic activity, Avr homologues, oligosaccharides and low molecular weight compounds (Shoresh et al. 2010).

In this work the *Arabidopsis thaliana* root colonization by *T. atroviride* and its implication on growth, induction of systemic protection against hemibiotrophic and necrotrophic pathogens, as well as on the induction of defence related genes mediated by SA, JA/ET, and the synthesis of camalexin was studied. Moreover, the production of IAA-related indoles by this fungus in liquid cultures was investigated.

Materials and methods

Organisms and growth conditions

Arabidopsis thaliana ecotype Col-0 was used for this study. *Arabidopsis* seeds were sterilized with a 10% (*v/v*) sodium hypochlorite solution for 10 min and washed three times with sterile distilled water, then seeds were germinated and grown on agar plates containing MS medium (Murashige and Skoog 1962).

Fungal strains *Trichoderma atroviride* IMI 206040, the *T. atroviride* transformant *pki1*::*gfp* TaGFP22 and *Botrytis cinerea* were grown at 28°C on potato dextrose agar (PDA) (DIFCO) for 7 days and conidia were collected with sterile distilled water and adjusted to a concentration of 1×10^6 conidia ml^{-1}. The bacterium *Pseudomonas syringae pv tomato* DC 3000 (*Pst* DC3000) was grown at 28°C on Kings B medium (King et al. 1954).

The TaGFP22 transformant was obtained by using the pHYG-GFP vector carrying the *gfp* gene from *Aequorea victoria* under the control of *T. reesei* constitutive promoter *pki1* (pyruvate kinase) and the hygromycin phosphotransferase gene (*hph*) from *E. coli* under the control of *Aspergillus nidulans trpC* promoter (Zeilinger et al. 1999), which was used for the transformation of *T. atroviride* protoplast, as described by Baek and Kenerley (1998). Several transformants that expressed *gfp* gene and showed similar morphological characteristics when compared with the wild type strain were selected. The TaGFP22 transformant was chosen for the colonization assay. *B. cinerea* strain was isolated from a tomato field at San Luis Potosi, Mexico, and identified by PCR amplification of 18S rDNA using the oligonucleotides ITS1 and ITS4 (White et al. 1990).

Plant-growth promotion assay

Arabidopsis seeds were grown on 0.3× MS medium and, 4 days after germination, seedlings were transplanted to flowerpots containing peat moss as substrate (LAMBERT™), and inoculated with 20 µl of 1×10^6 spores ml^{-1} of *T. atroviride.* Twenty-four h postinoculation, flowerpots were irrigated with MS (0.3×) to allow the fungus to colonize the rhizosphere. Six days postinoculation, plants were supplied with nutrient solution HUMIFERT (Cosmocel, Monterrey, México) (0.3%). Twenty days postinoculation, *T. atroviride* treated plants were carefully removed from containers and roots were washed in sterile distilled water. Plant length was measured with a ruler and fresh weight was measured on analytical scale. Then, plants were air-dried at 70°C for 72 h to further measure the dry weight on analytical scale. Each treatment consisted of 15 plants, and the experiment was repeated three times.

Production of IAA-related indoles by *T. atroviride*

IAA-related indoles production by *T. atroviride* was determined in the presence and absence of L-tryptophan. *Trichoderma virens* was included as IAA-related indoles producer (Contreras-Cornejo et al. 2009). The fungi were grown in 200 ml^{-1} of MS medium supplemented with filter sterilized solution of L-tryptophan at 200 µg ml^{-1}. Flasks were inoculated with 1×10^8conidia and incubated at 28° C for 8 days. Culture supernatants were recovered by centrifugation at 3,000 rpm by 10 min and filtered through a 1,000 ml Vacuum Filter/Storage Bottle, 0.22 µm (Corning Cat. Number 430186, Corning, New York, USA) and stored at 4°C until used for the assays. One ml of supernatant was mixed with 2 drops of orthophosphoric acid and 2 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 1 ml

0.5 M $FeCl_3$) (Glickmann and Dessaux 1995). After room temperature incubation in the dark for 20 min, absorbance was read at 530 nm using a Schimadzu UV-1700 PharmaSpect spectrophotometer. IAA-related indoles concentrations were estimated using triplicate standard curve for comercial IAA (Sigma Aldrich) prepared in MS medium.

Root colonization by *T. atroviride* assay

Seven-day-old *Arabidopsis* plants were inoculated with *T. atroviride* and plugs of actively growing mycelium were taken from the co-culture of *A. thaliana* with *T. atroviride* at 48 and 72 h postinoculation and washed with sodium hypochlorite for 5 min and placed on Petri dishes containing fresh MS medium. Roots and shoots of *Arabidopsis* seedlings were also washed with sodium hypochlorite for 5 min and placed on a Petri dish with MS medium. Plates were visually inspected to evaluate the *T. atroviride* actively growing mycelium emerging from the *Arabidopsis* roots or shoots. Seven-day-old *Arabidopsis* plants were inoculated with TaGFP22 and roots were visualized at 48 h and 72 h postinoculation using an inverted Laser Scanning Confocal Microscope (LSCM) (Zeiss LSM-510 NLO META). GFP expression was imaged with Argon-2 laser, Abs/Em 488/515–530 nm. Confocal images were captured using LSM-510 software (version 3.2; Carl Zeiss) and evaluated with an LSM-510 Image Examiner (version 3.2).

Protection assay against fungal and bacterial phytopathogens induced by *T. atroviride*

Arabidopsis plants used for protection assays were treated as described for plant growth promotion experiments. Fifteen days *T. atroviride* postinoculation, 3 leaves from each plant were inoculated with 10 μl of a suspension of *Pst* DC 3000 grown at an OD=0.2, or with 10 μl of a suspension of 1×10^6 conidia ml^{-1} of *B. cinerea*. The lesion area was evaluated 7 days post-pathogen inoculation. Percentage of leaves damage was calculated obtaining the total leaf area and the total damaged leaf area, the ratio between these values gave the percentage of damaged area. Each treatment consisted of 10 plants, and the experiment was repeated three times with similar results.

Expression analysis of *Arabidopsis* defence related genes

Twenty-day-old *Arabidopsis* plants were grown on Petri dishes and inoculated in between the roots (3 cm) with 15 μl of a suspension of 1×10^6 conidia ml^{-1} of *T. atroviride*, allowing the interaction for 72 h and 96 h. Mock plants were included as control. *Arabidopsis* roots and leaves were harvested, separated and frozen in liquid nitrogen at the indicated times. Total RNA was extracted using the Concert RNA extraction solution (Invitrogen) as described by the manufacturer. Seven *Arabidopsis thaliana* genes related to different plant defence pathways were selected. Genes selected were: *PR-1a* and *PR-2* (Systemic Acquired Resistance), *LOX-1*, *PDF1.2*, *ATPCA* (related to Induced Systemic Resistance and oxidative burst), *PAD3* (terpenoid phytoalexin pathway) and actin 8 as the housekeeping gene (Table 1). Expression of plant defence-related genes was assessed by quantitative real-time RT-PCR (qRT-PCR). The *Arabidospsis* gene specific primer pairs were designed with primer express 3.0 program (Applied Biosystems) based on sequences available in GenBank database (Table 1). Total RNA was DNase-treated using rDNase I (Ambion), and 2 μg of total RNA was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen). The qRT-PCR reaction was performed using the kit Fast Syber Green Master Mix (Applied Biosystems) with 10 ng of cDNA. Experiments were performed using an Abiprism 7500 fast Real-Time PCR system (Applied Biosystems) following the conditions suggested by the manufacturer. The absence of primer-dimmers was confirmed in reactions without cDNA. The experiments were independently repeated twice and each reaction was performed in triplicate using a relative quantification analysis. The expression of each specific gene was normalized versus the reference control with the formula $2^{-\Delta\Delta CT}$.

Results

T. atroviride promotes growth of *Arabidopsis* seedlings

With the aim of analyzing the interaction of *Trichoderma atroviride* with *Arabidopsis thaliana*, *Arabidopsis* seedlings were root inoculated with *T. atroviride* and allowed to interact for 20 days.

Table 1 Oligonucleotides used in this study for qRT-PCR analysis of defence-related genes in *Arabidopsis thaliana*

Primer name	Sequence (5′-3′)	Gene amplified	Accession number
PR-1F PR-1R	ATCTAAGGGTTCACAACCAGGCAC TGCCTCTTAGTTGTTCTGCGTAGC	*PR-1a*	M90508
PR-2F PR-2R	AGGAGCTTAGCCTCACCACC GAGGATGAGCTCGATGTCAGAG	*PR-2*	NM_115586.2
LOX-1F LOX-1R	AGACGTTCCAGGCCATGGCAG CTTGGGTAAGGATACTCCTGTG	*LOX-1*	NM_104376.2
ATPCA-F ATPCA-R	AGACGTTCCAGGCCATGGCAG GGAGAGCGCAACAAGATCAG	*ATPCA*	NM_114770.2
PAD3-F PAD3-R	CGATGGAGATGCTCTCAAGTTC GTCTCCTTGACCACGAGC	*PAD3*	NM_113595.3
PDF1.1-F PDF1.1-R	CACCCTTATCTTCGCTGCTC GGAAGACATAGTTGCATGATCC	*PDF1.2*	NM_123809.3
ACT8-F ACT8-R	GACTCAGATCATGTTTGAGACC CATGTAACCTCTCTCGGTAAGG	*ACTIN 8*	NM_103814.3

Arabidospsis treated seedlings were bigger than the untreated control plants (Fig. 1a and b). Clearly, there was an increase in foliar area and plant growth (Fig. 1c). An increase in plant biomass, which was measured as fresh (Fig. 1d) and dry weight (Fig. 1e) was also determined. Fresh weight almost doubled control untreated seedlings, whereas dry weight results of treated plants were one third higher than that of the untreated control. These results indicate a beneficial effect on *Arabidopsis* growth and development by the inoculation of roots with *T. atroviride*.

T. atroviride produces IAA-related indoles

To identify the molecule secreted by the fungus involved in plant growth promotion, the ability of *T. atroviride* to produces IAA-related indoles was analyzed. Briefly, *T. atroviride* and *T. virens* (included

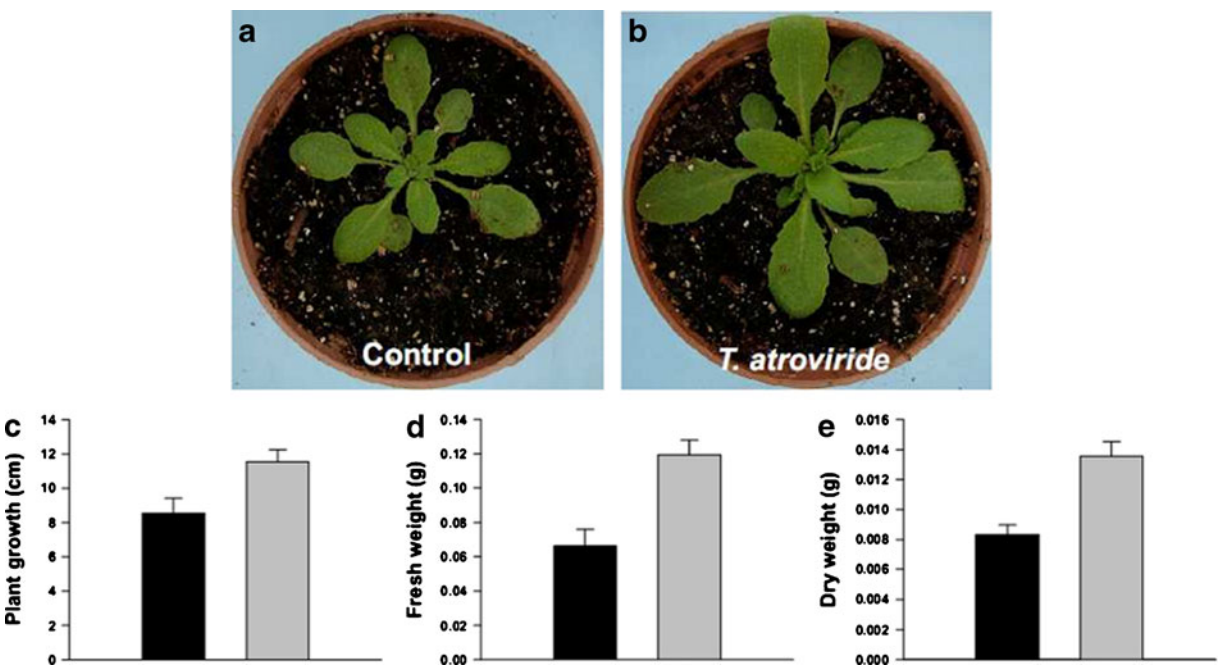

Fig. 1 Effect of *T. atroviride* colonization on plant growth of *Arabidopsis*. a and b, *Arabidopsis* plant growth of not inoculated and inoculated with *T. atroviride,* respectively. c, length of plants, d, Fresh weight, e, Dry weight. *Black bars* show *T. atroviride*-untreated seedlings, whereas *grey bars* represent *T. atroviride*-treated seedlings

as IAA-related indoles producer) were grown in MS liquid medium amended or not with L-tryptophan. IAA-related indoles were detected at concentrations of 11.9 and 19.0 μg ml^{-1} in culture filtrates of *T. atroviride and T. virens* grown in MS alone respectivelly (Fig. 2). Supplementation of MS medium with tryptophan considerably enhanced IAA-related indoles production by these fungi. *T. atroviride* produced 23 μg ml^{-1} whereas *T. virens* produced 27 μg ml^{-1} (Fig. 2).

T. atroviride colonize *Arabidopsis* roots

To know if the *T. atroviride* effect on *Arabidopsis* growth was associated with colonization of roots, a colonization assay was performed by inoculating in vitro the *Arabidopsis* roots and allowing them to interact for 48 and 72 h. No growth of the fungus was observed on plates where the plugs were washed with sodium hypochlorite and placed, whereas an actively growing mycelium was observed emerging from the *Arabidopsis* roots, no actively growing mycelium was observed emerging from leaves (data not shown). To further study the *Arabidopsis* root colonization by *T. atroviride*, several GFP-expressing transformants of *T. atroviride* were generated. Seven-day-old *Arabidopsis*

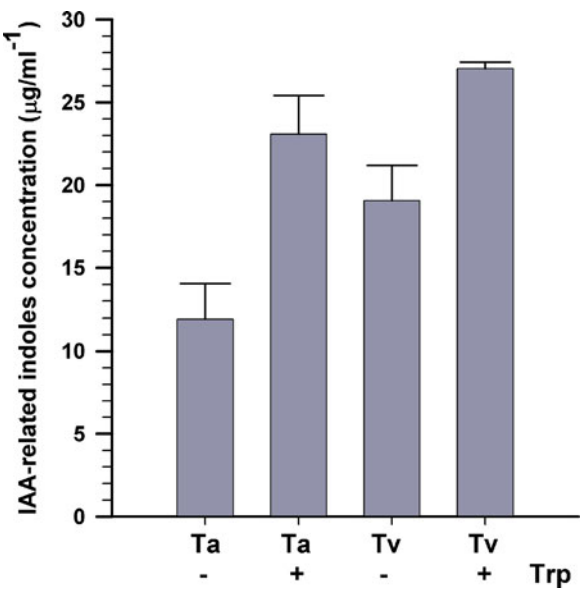

Fig. 2 Effect of L-tryptophan on the production of IAA-related indoles by *T. atroviride*. Production of IAA-related indoles by *T. atroviride* (Ta) in liquid cultures amended (+) or not (−) with 200 μgml^{-1} of L-tryptophan. *Trichoderma virens* (Tv) was included as control of IAA-related indoles production. Each value represents a mean of 4 replicates

seedlings were inoculated with conidia of TaGFP22-expressing transformant. Roots of seedlings were collected and analyzed by LSCM at 48 and 72 h of fungus-plant interaction. The epidermis, cortex, and vessels of the root cells were intact or only minimally altered. After 48 h of co-culture, hyphae had entered the roots and grown in the intercellular space of the epidermis (Fig. 3a–c). The green fluorescent hyphae entered into the epidermal cells. In some cases, the elongated zone of the hyphae showed structures similar to an appressorium (Fig. 3a–c). Extensive colonization of the root surface was observed even at the root tip (Fig. 3d–f). Together, these results showed that the fungus is able to colonize *Arabidopsis* roots but not the aerial parts and that the fungus forms appressorium-like structures in the plant epidermis.

Arabidopsis roots colonization by *T. atroviride* induces resistance against foliar plant pathogens

To test whether *Arabidopsis* root colonization by *T. atroviride* provides protection against fungal and bacterial pathogens, protection assays using the hemi-biotrophic bacteria *Pst* DC3000 and the necrotrophic fungus *B. cinerea* were conducted. *Arabidopsis* seedlings were root inoculated with *T. atroviride* and leaves were treated with *Pst* DC3000 or *B. cinerea* 2 weeks postinoculation. After 8 days of plant-pathogen interaction, the control plants, not treated with *T. atroviride* but inoculated with *Pst* DC3000, showed the typical bacterial speck disease provoked by this pathogen (Fig. 4a). In contrast, plants inoculated with *T. atroviride* showed reduced lesion area compared with mock plants. In the case of the *T. atroviride-Arabidopsis thaliana* protection assays against *B. cinerea*, a marked reduction in lesion area was observed on leaves of treated seedlings compared with mock control plants (Fig. 4b). Based on these results, it can be concluded that colonization by *T. atroviride* induces systemic resistance in *Arabidopsis* against foliar pathogens with different lifestyle.

T. atroviride induces the expression of *Arabidopsis* SA, JA/ET, oxidative burst and camalexin defence-related genes both locally and systemically

Protection assays showed that *T. atroviride* enables *Arabidopsis* to counteract pathogens with different life styles, which trigger different resistance response

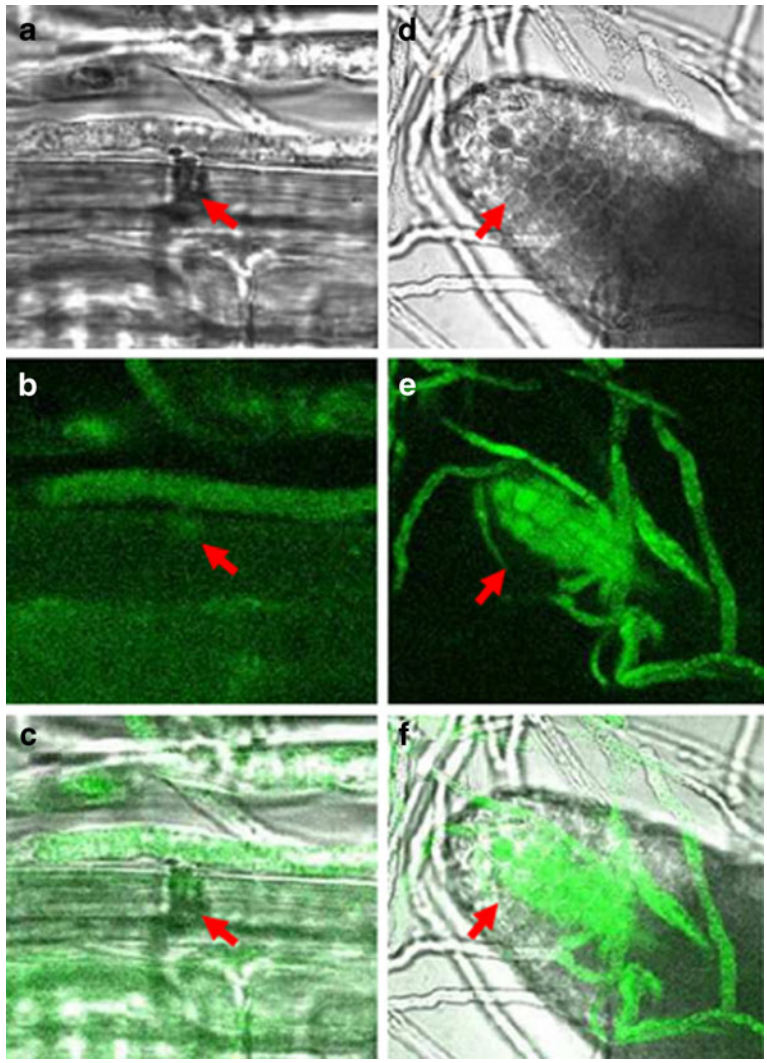

Fig. 3 Confocal images of *T. atroviride* TaGFP22 transformant and *Arabidopsis* co-cultures. **a**, **b**, and **c** show the growth of the green fluorescent hyphae entering into the epidermal cells (*arrows* indicate the appressorium-like structure). In some cases, the elongated zone of the hyphae showed structures similar to an appressorium indicated by the arrow. **d**, **e** and **f**, show the extensive colonization in the root tip surface (arrows indicate the hyphae penetrating the root tip)

pathways. It is well known that hemibiotrophic pathogens, such as *Pst* DC3000, trigger the SA pathway, whereas *B. cinerea*, a necrotrophic microorganism, triggers the JA/ET pathway (Ton et al. 2002;

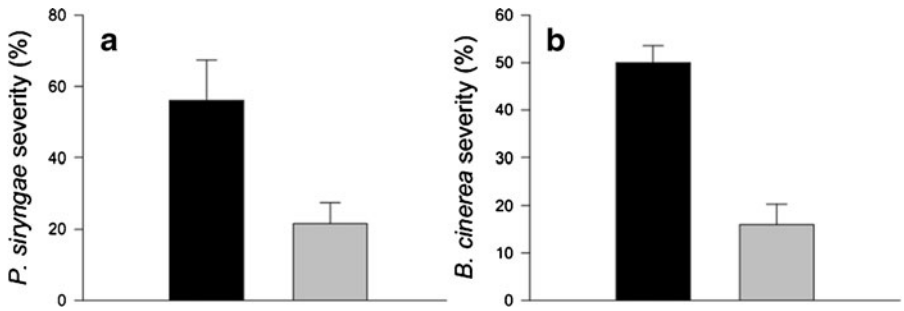

Fig. 4 Effect of *T. atroviride* on induced systemic resistance in *Arabidopsis* seedlings against the phytopathogens, *B. cinerea* and *Pst* DC3000. The graphs illustrate the levels of systemic disease protection observed against *Pst* DC3000 (**a**) or *B. cinerea* (**b**). *Black bars* show *T. atroviride*-untreated seedlings, whereas *grey bars* represent *T. atroviride*-treated seedlings

Pieterse et al. 2009). With the aim of exploring the possible pathway involved in the protection against such pathogens in *Arabidopsis*, the expression profiles of SA, JA/ET, oxidative burst defence-related genes, as well as of the gene involved in the synthesis of the antimicrobial compound camalexin at different times post *T. atroviride* inoculation (72 h and 96 h) were assessed. Mock plants were included as control. Expression of defence-related genes was first examined locally at the site of colonization (roots) at 72 and 96 h after *T. atroviride* inoculation (Fig. 5). Figure 5 shows that *PR-1a* (unknown function) was not induced at 72 h, whereas *PR-2* (β-1, 3-glucanase), *PDF1.2* (Plant Defensin), *LOX-1* (Lipoxygenase 1), and *ATPCA* (Peroxidase a) were slightly induced as compared with mock plants. The gene *PAD3* (Cytochrome P450 monooxygenase) that codes for the last enzyme involved in the synthesis of camalexin was induced 50-times in roots at 72 h. Expression of *PR-1a*, *PR-2*, *PDF1.2*, and *PAD3* were up-regulated in roots at 96 h postinoculation, whereas *LOX-1* and *ATPCA* underwent no significant changes. To analyze the induction of systemic defence response in *Arabidopsis*, mRNA levels of defence-related genes were analyzed in leaves. Expression of *PR-1a* and *PDF1.2* was not induced in leaves at 72 h. Figure 5 shows that *PR-2*, *LOX-1*, and *ATPCA* were slightly up-regulated at 72 h postinoculation with *T. atroviride*, whereas *PAD3* was induced almost 175-fold at the same time (Fig. 5). At 96 h post-treatment, all genes reached their maximum level of expression, excluding the *ATPCA* gene whose levels decreased compared with 72 h. Together, these data indicate that *T. atroviride* activated systemic and local expression of SA and JA/ET defence-related genes, as well as the gene encoding the last enzyme involved in the synthesis of camalexin in *Arabidopsis*. Contrasting with other reports (Yedidia et al. 1999; Shoresh et al. 2005; Segarra et al. 2009), this work clearly demonstrated that *T. atroviride* induces the simultaneous expression of SA- and JA/ET- related genes in *Arabidopsis*.

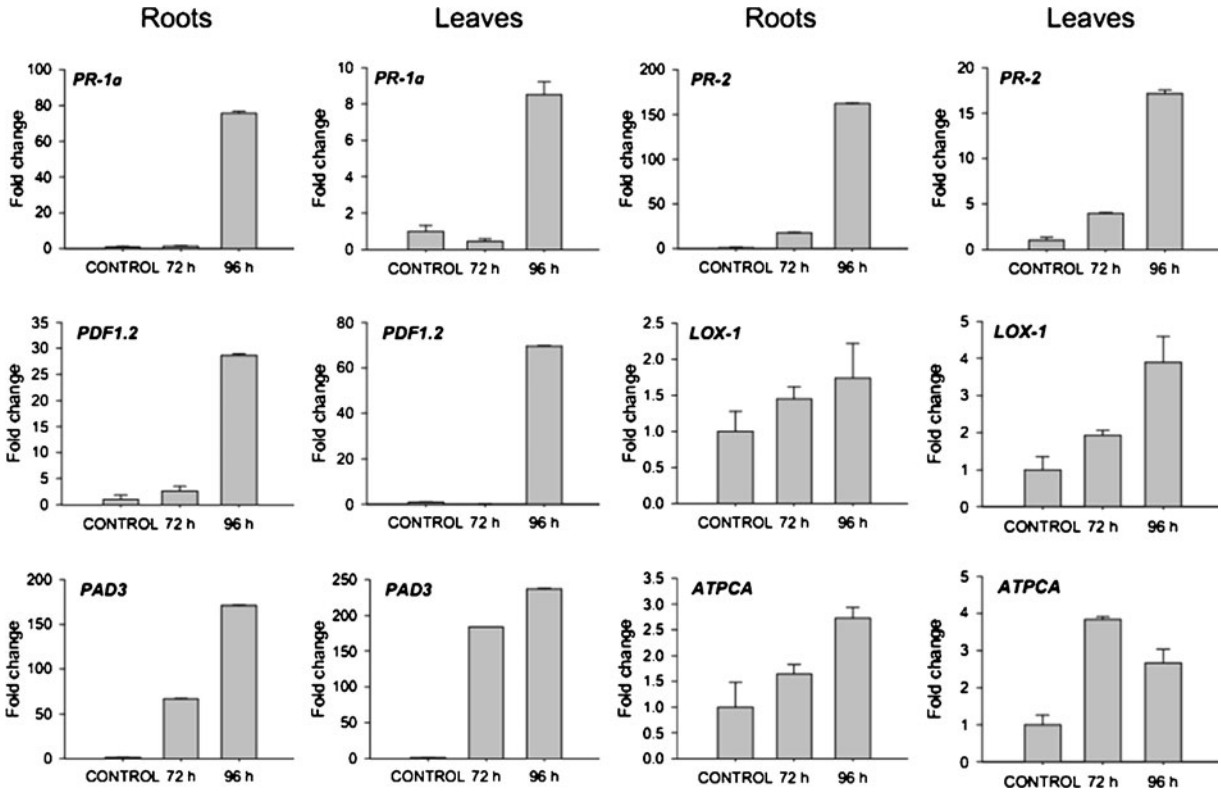

Fig. 5 Relative expression analysis of defence-related genes in *Arabidopsis* seedlings inoculated with *T. atroviride*. Total RNA from roots and leaves of *Arabidopsis* plants inoculated with *T. atroviride*, was subjected to qRT-PCR to quantify six genes related to different plant defence pathways: *PR-1a* and *PR-2* (SAR), *PAD3* (synthesis of camalexin), *ATPCA* (oxidative burst), and *PDF1.2* and *LOX-1* (ISR)

Discussion

It has been demonstrated that plant growth promotion by *Trichoderma* spp. is dependent on either root colonization or colonization of the entire plant (Kleifeld and Chet 1992; Chacón et al. 2007; Shoresh et al. 2010); however, for *Trichoderma* spp.-*Arabidopsis thaliana* interaction there are only few reports (Korolev et al. 2008; Contreras-Cornejo et al. 2009; Segarra et al. 2009). Here it is shown that, *T. atroviride* promotes growth in *Arabidopsis* when applied to roots, revealing that growth enhancement might depend on root colonization. In this sense, it has been suggested that the mechanism involved in growth promotion could be due to root colonization and the ability of *Trichoderma* spp. to provide nutrients and phytohormones (Harman et al. 2004; Contreras-Cornejo et al. 2009; Shoresh et al. 2010) or by changing the internal phytohormone homeostasis in the plant. It is well known that several plant beneficial microbes can synthesize phytohormones, which can be used by plants and it may be one of the mechanisms of plant growth promotion by microorganisms (Van Loon 2007; Shoresh et al. 2010). However, most of the reports have been focused on rhizobacteria and there are few reports on IAA or IAA-related indoles production by beneficial fungi (Contreras-Cornejo et al. 2009; Shoresh et al. 2010). IAA or IAA-related indoles production by fungi from the *Trichoderma* genera has not been studied at all. In this work, it was demostrated that *T. atroviride* can produce IAA-related indoles that may be utilized for plant growth promotion. Plant growth promotion by *T. atroviride* effects on *Arabidopsis* were demonstrated and might be caused mainly by IAA-related indoles, which is a direct plant growth promotion mechanism. Production of other phytohormones, such as gibberellins and cytokinins by *T. atroviride* should be investigated.

To determinate if *T. atroviride* is able to colonize *Arabidopsis* roots, root colonization experiments were carried out. To this end, a *T. atroviride* GFP-expressing transformant to visualize the fungus-plant interaction was generated. Indeed, it was observed through LSCM that the fungus is able to colonize *Arabidopsis* roots, the intercellular space of the epidermis forming appressoriun-like structures. This is in agreement with previous studies, where *T. harzianum* hyphae penetrated and colonized the epidermal layers and the intercellular spaces in cucumber and tomato roots. It was also demonstrated that, during those interactions, *T. harzianum* induced systemic resistance on those plants (Yedidia et al. 1999; Chacón et al. 2007). *Arabidopsis* root colonization by *T. atroviride* resulted in an increase in biomass of the entire plant, demonstrating that the effect on seedling growth is systemic; this observation was confirmed because the fungus was not recovered from the aerial parts of the plant. Moreover, it was demonstrated that *T. atroviride* is able to synthesize IAA-related indoles in vitro, which supports the hypothesis that indole compounds produced by this beneficial fungus could be involved in the growth promotion in greenhouse or field conditions. Taken together the results, indicated that *T. atroviride* is able to colonize *Arabidopsis* roots and promote growth systemically by providing IAA-related indoles to plants, as described for other *Trichoderma* species (Yedidia et al. 2001; Harman et al. 2004; Contreras-Cornejo et al. 2009; Shoresh et al. 2010).

The effect of plant growth promoting rhizobacteria on the induction of plant systemic resistance is well known, however the effect of plant growth promoting fungi has been just recently launched. The *Trichoderma* research community has focused its efforts mainly on the study of the mechanisms of mycoparasitism and antibiosis, devoting less attention to the induction of systemic resistance induced by *Trichoderma* spp. (Harman et al. 2004). *Trichoderma* spp. are able to induce systemic changes in plants which are frequently related with increased levels of PR proteins and/or the accumulation of phytoalexins (Shoresh et al. 2010). Yedidia et al. (2003), demonstrated that the application of *Trichoderma asperellum* T-203 to cucumber roots reduced considerably the disease provoked by *Pseudomonas syringae* pv. *lachrymans*, furthermore there was production of antifungal compounds in leaves. De Meyer et al. (1998) demonstrated that inoculation of strain *T. harzianum* T-39 on bean roots reduced considerably the lesion area provoked by *B. cinerea*. Here it is shown that *T. atroviride* induced protection in *Arabidopsis* against both the hemibiotrophic bacteria *Pst* DC3000 and the necrotrophic fungus *B. cinerea*. In this work, the pathogens were applied on leaves, which ensure the spatial separation of the pathogen from *T. atroviride* applied to the root, which secretes antibiotics and has mycoparasitic

activity. Development of *Pst* DC3000 and *B. cinerea* on *Arabidopsis* leaves was significantly reduced in plants treated with *T. atroviride* compared with the untreated plants, which resembles the typical SAR and ISR responses respectively. These results together suggested that SA and JA/ET pathways could be the main reason of such pathogen growth suppression in *Arabidopsis*. It can be concluded that suppression of disease development was systemic and that mycoparasitic activity or production of antimicrobial molecules by the fungus were not involved in such diseases suppression.

A number of investigations have reported the induction of systemic resistance for several plants including *Arabidopsis* (Beckers and Spoel 2006; Pieterse et al. 2009). Pharmacological analysis using specific inhibitors of JA/ET pathways on *T. asperellum*-cucumber interaction showed that these signal transduction pathways are involved in the protective effect conferred by *T. asperellum* against *P. syringae* pv *lachrymans*. Accumulation analysis of SA in roots and leaves of cucumber treated with *T. asperellum* did not show differences when compared with non-inoculated plants. Expression analysis of JA/ET-regulated genes also showed that *T. asperellum* modulates the local and systemic expression of these genes in cucumber (Shoresh et al. 2005). Korolev et al. (2008) showed that *T. harzianum*-induced resistance against *B. cinerea* is dependent on JA/ET pathways, by using mutants impaired in such transduction pathways. Later, it was demonstrated that the defence pathways induced by *T. asperellum* and the beneficial bacteria *Pseudomonas fluorescens* WCS417r are very similar and both of them are independent from SA but require NPR1 and MYB72 (Segarra et al. 2009).

With the aim of closely studying the possible signal transduction pathway involved in the induction of systemic resistance against pathogens with different lifestyles in *Arabidopsis*, the expression profile of a set of SAR, ISR, oxidative burst and camalexin defence-related genes at 72 and 96 h postinoculation with *T. atroviride* was assessed. In the present study, almost all genes were induced by *T. atroviride* both locally and systemically, achieving their maximum expression in both, roots and leaves, at 96 h postinoculation. This work showed increased expression of *PR* genes at 96 h post treatment with *T. atroviride* both locally and systemically. The β-1-3-glucanase (*PR2*)-encoding gene is highly induced in leaves in response to inoculation with *T. atroviride*. Several studies have indicated that root colonization by *Trichoderma* strains results in increased levels of defence-related enzymes in plants, including peroxidases, chitinases, β-1-3-glucanase (Howell et al. 2000; Yedidia et al. 1999, 2003; Harman et al. 2004). These results suggest that PR proteins could be involved in systemic response to suppress diseases in *Arabidopsis* when inoculated with *T. atroviride*. The expression levels of *ATPCA,* a class III peroxidase-encoding gene involved in generating hydrogen peroxide and in conferring resistance against pathogens were not significantly affected in roots at 72 h, but increased at 96 h. When measured in leaves, *ATPCA* increased almost 3-fold and decreased 2-fold at 96 h as compared with 72 h postinoculation. Peroxidases accumulate as a response to reactive oxygen species (ROS) generation provoked by pathogen attack; the increase in enzyme activities in leaves suggests a systemic defence response to the presence of *T. atroviride*. Expression levels of JA/ET and of pathogen-induced genes, *PDF1.2* and *LOX-1*, were different at both times. *PDF1.2* was up regulated at 96 h postinoculation with *T. atroviride* in both roots and leaves, whereas *LOX-1* underwent no significant changes in roots, but reached four- times the level of expression in leaves. The gene expression data of JA/ET defence related genes suggest that *T. atroviride* induces systemic resistance in *Arabidopsis* through JA/ET pathway. Expression analysis of the *PAD3* gene that encodes the last enzyme involved in the synthesis of the antimicrobial compound, camalexin, showed an up-regulation in both roots and leaves after treatment of *Arabidopsis* with *T. atroviride*. Camalexin is a phytoalexin, whose members are low-molecular-weight compounds that have antimicrobial activity and are produced by plants in response to attack by pathogens. In addition, *Arabidopsis PAD* mutants loose their ability to restrict the growth of bacterial pathogens (Glazebrook and Ausubel 1994). *PAD3* transcript is induced by infection with virulent *Pst* DC3000 or avirulent *P. syringae* pv. *maculicola* ES4326 and SA treatment in *Arabidopsis*. Moreover, *PAD3* mutation has no effect on resistance to *Pst* DC3000 but compromises resistance to the fungal pathogen *Alternaria brassicicola* (Zhou et al. 1999). Yedidia et al. (1999) showed that *T. asperellum* might activate metabolic pathways in cucumber, leading to

the systemic accumulation of phytoalexins. Altogether these results suggest that camalexin is involved in suppression of grey mould symptoms provoked by *B. cinerea* in *Arabidopsis* but not on those caused by *Pst*. In contrast with the works of Segarra et al. (2009) and Korolev et al. (2008), who suggest that JA/ET pathways are responsible for the systemic resistance in *Arabidospis* induced by *T. asperellum*, in this work an overlapping of SAR and ISR gene expression was observed. Thus, the systemic resistance induced by *T. atroviride* root colonization seems to have similarities and differences with that of *T. asperellum* and *P. fluorescens* WCS417r, but appear to be distinct from either of them. Each microorganism mediates systemic resistance mostly by ISR, although others mechanisms may also be involved (Shoresh et al. 2010). Our findings suggest that *T. atroviride* induces an overlapping in the expression of SA- and JA/ET-dependent genes, oxidative burst and the synthesis of camalexin-related genes both locally and systemically to suppress pathogen growth in *Arabidopsis*. Although many reports describe an antagonistic interaction between SA- and JA/ET-dependent signalling, synergistic interactions have been described as well. Recently, the effect of co-treatment with various concentrations of SA and JA were assessed in tobacco and *Arabidopsis,* finding a transient synergistic enhancement in the expression of genes associated with either JA/ET (*PDF1.2* and *Thi1.2*) or SA (*PR-1a*) signalling when both signals were applied at low (typically 10–100 mM) concentrations (Mur et al. 2006). Antagonism was observed at more prolonged treatment times or at higher concentrations (Mur et al. 2006). Simultaneous activation of ISR and SAR enhanced induced disease resistance against *Pst* DC3000 was demonstrated (Van Wees et al. 2000). Induction of ISR did not affect the expression of the SAR marker gene *PR-1a* in plant expressing SAR. These results demonstrated that SAR and ISR pathways are compatible (Van Wees et al. 2000). Shoresh et al. (2010) suggest that mediation of systemic resistance in plants by microorganisms depends on which elicitors are involved. There are *Trichoderma* strains that induce defence but not growth and vice versa, suggesting that the signalling pathways leading to these plants responses are different (Shoresh et al. 2010).

In conclusion, this study has demonstrated that inoculation of *Arabidopsis* roots with *T. atroviride* promotes growth and development of *Arabidopsis* seedlings, and systemically inhibits proliferation of *Pst* DC3000 and *B. cinerea*. In addition, it was demonstrated that *T. atroviride* produces IAA-related indoles, which may have a stimulatory effect on *Arabidopsis* growth and development. The reduction in *Arabidopsis* foliar damage appeared to be associated with simultaneous transcript accumulation of SA, JA/ET defence-related genes, as well as with the expression of genes involved in the oxidative burst and with the synthesis and accumulation of the antimicrobial compound camalexin in *Arabidopsis*. The beneficial effects induced by the inoculation of *T. atroviride* on *Arabidopsis* roots and the induction of plant defence system suggests a molecular dialogue between the plant and the fungus.

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