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REGULAR ARTICLE

Trichoderma harzianum T6776 modulates a complex metabolic network to stimulate tomato *cv.* Micro-Tom growth

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Abstract
Background and aims *Trichoderma harzianum* 6776 is a novel and beneficial tomato fungal isolate. To investigate the mechanisms underlying the *T. harzianum* 6776-tomato interaction, several physiological and biochemical responses were explored on dwarf tomato plants, *cv.* Micro-Tom.
Methods Growth of treated and untreated plants was evaluated by measuring the height and biomass production of plants. The leaf pigment content and sugar partitioning in plant organs were evaluated by biochemical analysis. The photosynthetic parameters were measured by a miniaturized PAM fluorometer and a portable gas-exchange system. The hormonal analysis in root and xylem sap was performed by gas cromatography-mass spectrometry (GC-MS).
Results *T. harzianum* 6776 positively affected plant growth, increasing the leaf pigment content and improving the photosynthetic activity at both stomatal and non-stomatal levels. Differences in pigment composition and photosynthetic performance were reflected in the carbohydrate content and their partitioning. In the absence of a pathogen, root and xylem vessel stress and growth-related hormone balance were affected by the interaction with *T. harzianum* 6776, with an increase in jasmonic and indoleacetic acids and a decrease in salicylic acid content.
Conclusions This study shows the complex connection between increased hormone accumulation and transport, altered sugar partitioning and enhanced photosynthetic efficiency induced by *T. harzianum* 6776, and how growth promotion is the result of the combination of these drastic changes in Micro-Tom plants.

Keywords Beneficial fungi · Endophytism · Photochemical efficiency · Pigment content · Sugar partitioning, Hormonal profiling

Abbreviations

T6776	*Trichoderma harzianum* strain 6776
MT	Micro-Tom plants
JA	Jasmonic acid
SA	Salicylic acid
IAA	Indol-acetic acid
DPI	Days post inoculum
DPS	Days post sowing
PSII	Photosystem II

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Introduction

Towards the middle of this century, the global human population is estimated to reach around nine billion people. The challenge is to increase food production while reducing the environmental impact. This entails sustainable crop production, that limits the use of chemical inputs, such as pesticides and fertilizers. *Trichoderma* (teleomorph *Hypocrea*) is a well-known ascomycete fungal genus prevalent in many ecosystems, which also include species commonly used as active ingredients in the formulation of commercial biofertilizers and biopesticides. More than 60 % of all registered biopesticides are *Trichoderma*-based, due to the abilities of these fungi to improve plant defences against biotic and abiotic stresses and also to act as growth promotion agents on different plant species (Mastouri et al. 2010; Shoresh et al. 2010; Contreras-Cornejo et al. 2014).

Some *Trichoderma* rhizosphere-competent strains can penetrate and colonize the roots of different plants, acting as endophytic plant symbionts and leading to substantial changes in plant physiology and in its defence system. Generally, the recognition between these two partners activates the mitogen-activated protein kinase (MAPK) plant cascade, thus triggering the plant hormone signalling network of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). In addition, abscisic acid (ABA) biosynthesis has been reported to have been triggered (Hermosa et al. 2012). JA and ET are associated with induced systemic resistance (ISR), whereas SA is involved in systemic acquired resistance (SAR) (Pieterse et al. 2009). The response pattern mediated by JA/ET or SA in plants by *Trichoderma* spp. is isolate/inoculum dependent (Hermosa et al. 2013). ABA affects both abiotic and biotic plant responses (Asselbergh et al. 2008) and its role in *Trichoderma* induced responses is still controversial. It is commonly associated with callose priming and with the regulation of the defence gene expression through the activation of JA biosynthesis (Hermosa et al. 2012).

Some *Trichoderma* spp. strains are able to produce auxins (IAA) themselves and/or to induce an IAA accumulation in the plants they interact with (Contreras-Cornejo et al. 2009). Indeed, the increase in IAA plant content is one of the mechanisms by which biocontrol agents promote growth in tomato plants (Gravel et al. 2007; Chowdappa et al. 2013). Photosynthetic efficiency and carbohydrate metabolism, as well as respiratory rates, are improved, leading to an enhanced growth response (Harman et al. 2004a; Chacón et al. 2007; Brotman et al. 2013), especially when combined with a greater uptake of macro and micro-nutrients (Altomare et al. 1999). Plant-derived sucrose is key both to root colonization and to increasing the rate of leaf photosynthesis in maize plants (Vargas et al. 2009). Plant responses and transduction signalling pathways activated by *Trichoderma* spp. depend on both plant cultivars and fungal isolate genotypes, with strains stimulating defence responses but not growth and vice-versa (Bailey et al. 2006).

Tomato (*Solanum lycopersicum L.*) is the second-most important vegetable crop in the world (after potato), with a global production of about 160 million tons (FAOSTAT Database, 2012). Tomato is an alternative model plant to *Arabidopsis thaliana* due to its diverse developmental traits and its agronomical important plant-insect and plant-pathogen interactions not found in *Arabidopsis* (Campos et al. 2010). In addition, its genome has been recently released (Tomato Genome Consortium 2012). Originally bred for home gardening purposes (Scott 1989), the laboratory dwarf tomato cultivar Micro-Tom (MT) has also been proposed as a model system (Meissner et al. 1997; Arie et al. 2007; Campos et al. 2010). Many works have demonstrated the biostimulating and biocontrol activity of *Trichoderma* spp. in several tomato cultivars (Gravel et al. 2007; Nzanza et al. 2012; Chowdappa et al. 2013), however no data are available for the dwarf tomato *cv.* Micro-Tom.

Trichoderma harzianum strain 6776 (T6776) is a novel and beneficial isolate that acts as a biocontrol agent against soil-borne pathogens and as a growth promoter on different tomato cultivars (Sarrocco et al. 2013). In order to investigate the genetic potential of this promising isolate, its genome has been sequenced and recently annotated (Baroncelli et al. 2015).

Since the beneficial effects depend on the genotype of both partners involved in the interaction (Harman et al. 2004b; Tucci et al. 2011; Bharti et al. 2012) and no data are available on the dwarf tomato *cv.* Micro-Tom interacting with *Trichoderma* spp., in the present work we explored the T6776-MT interaction for the first time. We evaluated the effects of T6776 on growth and developmental rate, photosynthetic activity and pigment composition, the content of several hormones and soluble sugars in MT seedlings. As the xylem vessel ensures the communication and the translocation of a lot of

nutrients and signals from the roots to all aboveground organs and tissues, we also analysed the xylem vessel hormone composition to gain information on the physiological status of the plant, as well as information on long distance hormone transport.

The ability of hormones to act as mobile signals is still controversial, because little is known about the hormonal content in the vascular system of the plant that interact with microorganisms (Matsuura et al. 2012; Furch et al. 2014). To the best of our knowledge, this is the first study that provides information on how different metabolic pathways are modified in the same plants after *Trichoderma*/tomato interaction. It is also the first time that hormone transport through the xylem vessel of tomato plants co-cultivated with *Trichoderma* spp. has been evaluated. Since the cross-talk between hormones requires the simultaneous study of several hormones, the JA, SA, ABA and IAA root composition and transport in xylem sap of MT interacting with *T*6776 were analysed. Finally, we demonstrate that the *cv.* Micro-Tom is a good plant model to study the interaction between tomato and beneficial fungi.

Materials and methods

Fungal strain and inoculum preparation

The T6776 strain was isolated from soil in Colignola, Pisa (Italy) and deposited in the fungal collection of the Plant Pathological Mycology Lab of the Department of Agriculture, Food and Environment, University of Pisa. One mL of spore suspension (10^6 spore mL^{-1}) collected from 7 day old T6776 cultures on Potato Dextrose Agar (PDA, Difco Laboratories) was inoculated in a 50-mL flask containing 20 mL of sterilized (121 °C, 20 min) tomato hydroponic solution (THS, see below) with the addition of yeast extract (0.5 %). Conidia were allowed to grow for 44 h (150 rpm; 25 °C), after which the biomass was collected by centrifugation (7000 g; 15 min), washed twice in distilled water and re-suspended in fresh THS. One mL of inoculum suspension was added to the root system of 7 day old seedlings of treated plants (T6776). One mL of un-inoculated THS was added to the root system of 7 day old seedlings of untreated plants (CNT). The experiment, therefore, consisted of two treatments (inoculated and not inoculated), and for each treatment 45 plants were sown. The experiment was repeated three times (three replicates) in different moments. The plants were grown, sampled and analysed as described in the next paragraphs.

Plant material and growth condition

Tomato (*Solanum lycopersicum* L.) *cv.* Micro-Tom (MT) was used in this work. Seeds (Domina srl, panamseeds.com) were sown in a Rock-wool plot placed over a 2 L tank supplemented with THS ($Ca(NO_3)_2$ 846 g; KNO_3 149 g; Fe-EDDHA 15.2 g; Mg $(NO_3)_2$ 378 g; KH_2PO_4 136 g; KNO_3 89 g; K_2SO_4 220 g; KCl 183 g; H_3BO_3 1.2 g; $CuSO_4$ 0.2 g; $ZnSO_4$ 1.5 g; $MnSO_4$ 0.2 g, per liter). The THS was changed completely once a week. Plants were grown in a growth chamber for five weeks under a long day regime (16 h light), with 200 μmol m^{-2} s^{-1} PAR, 25 °C/19 °C temperature.

Endophytic root ability of T6776

Root colonization ability of T6776 was evaluated on MT following Alonso-Ramírez et al. (2014), with some modifications. Briefly, whole root, cut from 5 week old plants (28 days post inoculation, DPI) with T6776 inoculation and without, as indicated above, were washed under tap water, surface sterilized by immersing in a sodium hypochlorite – 50 % Ethanol solution (5 min; 1 % active chloride), and then washed three times with sterile water (5 min). Samples were dried on sterile filter paper in a sterile laminar flow chamber. Five roots per treatment per replicate were analysed (*n=15*). Four or five mm root segments from each root were placed on *Trichoderma* selective medium P190 (TSM-P190) (Sarrocco et al. 2009) and plates were incubated for seven days in order to monitor the presence of T6776 emerging from the root tissues. Macro and microscopic observations were performed to confirm the identification of the isolate growing out from the tissues.

Effect of T6776 on MT growth and development

Shoot length and leaf area were measured every two days from day 10 (3 DPI), to day 35 (28 DPI) after sowing (DPS). Leaf area was calculated by measuring one fully expanded leaf per plant by Image J 1.2. Both leaf area and shoot length values were used to create a growth curve in the presence/absence of T6776 using Sigma Plot 11 (Sigma Plot Software, Inc.; Chicago, IL, USA). Data were subjected to variance regression

analysis in order to compare the slope of the curves between treatments for each parameter measured. A comparison between regression lines was performed by Graph Pad 5 (Graph Pad software Inc., La Jolla, CA, USA). Twelve plants per treatment per replicate were randomly measured (*n=36*).

Both fresh and dry weights were measured on 5 week old plants (28 DPI) in one pool of five randomly chosen plants per treatment per replicate (*n*=3). After washing with water, roots, stems and leaves were separated, and fresh and dry weight (60 °C; 1 week) were measured.

Photosynthesis measurements and chlorophyll fluorescence

Chlorophyll a fluorescence was measured on fully expanded and exposed leaves using a miniaturized pulse-amplitude-modulated fluorometer (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) at different times: at 21 and 28 DPS (14 and 21 DPI, respectively) on 12 plants per treatment per replicate (*n=36*) and at 35 DPS (28 DPI) on 3-4 plants per treatment per replicate (*n=10*). Leaves were pre-darkened for 30 min before starting the experiments. The potential efficiency of PSII photochemistry was calculated on dark-adapted leaves as Fv/Fm=(Fm-Fo)/Fm, where Fv represents the variable fluorescence in the dark, Fo is the minimum fluorescence yield in the dark and Fm is the maximum fluorescence yield in the dark after application of a saturation flash of light that completely closes all the PSII reaction centres. The actual photon yield of PSII (Φ_{PSII}) in the light was determined as Φ_{PSII}=(Fm'-F)/Fm' after approximately 30 min of illumination at an actinic photosynthetic photon flux density (PPFD) of 200 or 300 μmol m^{-2} s^{-1} when steady state was achieved. This is where Fm' is the maximum fluorescence yield with all the PSII reaction centres in the reduced state obtained by superimposing a saturating light flash during exposure to actinic light and F is the fluorescence yield at the actual reduction state of PSII reaction centres during actinic illumination. Non photochemical quenching was determined according to the Stern-Volmer equation as NPQ=Fm/Fm'-1 (Bilger and Björkman 1990). Fluorescence nomenclature was according to van Kooten and Snel (1990).

Gas-exchange measurements were taken on two or three 5 week old plants (28 PDI) per treatment per replicate (*n=8*), using a photosynthetic portable system Li6400 (LiCor, Lincoln, NE, USA). Chamber conditions were set at 25 °C and 400 μmol mol^{-1} of CO_2 concentration. The PPFD inside the chamber was set at 200 or 300 μmol m^{-2} s^{-1} with a red/blue LED light source. Light response curves of the photosynthetic CO_2 assimilation rate were performed on one 5 week old plant per treatment per replicate (*n=3*) over a range of incident PPFD between 25 and 800 μmol m^{-2} s^{-1}. Experimental data were fit using a non-rectangular empirical function to estimate the maximum apparent quantum efficiency and the light saturated rate of photosynthesis at growth CO_2 concentration (A_{max}).

Pigment analysis

Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (chl tot) and total carotenoid (xanthophylls+β-carotene; x+c) leaf contents were determined on 5 week old MT (28 DPI). Samples were prepared following a slightly modified method previously described (Quach et al. 2004). Nine plants per treatment per replicate were used to prepared three extracts, each one obtained from three plants (*n=9*).

Leaf tissue samples were mixed with anhydrous magnesium sulphate (1:1 *w/w*) and silica sand (1:2 *w/w*). The mixture was ground in a mortar, stirred after adding 2.0 mL of 100 % acetone and extracted (10 min.). Extracts were centrifuged (4000·*g*; 5 min.) and diluted (1:60 *v/v*) with 100 % acetone. The quantitative determination of photosynthetic pigments was carried out using a Hitachi U-3200 spectrophotometer (Hitachi, Tokyo, Japan), following the method described by Lichtenthaler (1987). Chla and chl b were determined at 661.6 nm and 644.8 nm, respectively whereas x + c were determined at 470 nm. Pigment concentrations (C) were determined by the following equations:

$$C_a = (11.24 \cdot \text{Abs } 661.6) - (2.04 \cdot \text{Abs } 644.8);$$
$$C_b = (20.13 \cdot \text{Abs } 644.8) - (4.19 \cdot \text{Abs } 661.6);$$
$$C_{a+b} = (18.09 \cdot \text{Abs } 644.8) + (7.05 \cdot \text{Abs } 661.6);$$
$$C_{x+c} = (1000 \cdot \text{Abs } 470 - 1.90 \cdot C_a - 63.14 \cdot C_b)/214.$$

The numbers in the equations are based on the determined specific absorption coefficients in 100 % acetone (pure solvent), as reported by Lichtenthaler (1987). Values were expressed as mg g^{-1} fresh weight (FW).

Hormonal profiling of xylem sap and roots

Xylem exudates and root extracts were purified by high performance liquid chromatography (HPLC), and the contents of indole-3-acetic acid (IAA), abscissic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) were analysed by gas chromatography–mass spectrometry (GC-MS). Plants were sampled five weeks after sowing (28 DPI). Two plants per treatment per replicate were analyzed ($n=6$).

Xylem sap samples were collected as previously described (Furch et al. 2014) with some modifications. Plants without the aerial part were placed on a Petri dish with distilled water, and each excised stem was tightly inserted into a PVC tube. Exudates were sampled every 30 min for a total time of 5.5 h (330 min). Xylem sap volumes collected from each plant were measured and stored at –20 °C until analysis. After being carefully washed, roots were dried, cut at the base of the stem, fresh weighted and stored at –20 °C until analysis.

Extraction and purification

Roots were ground in a mortar with 80 % MeOH (1:5 *w/v*) and $^{13}C_6$ IAA (Cambridge Isotopes Laboratories Inc., Andover, MA, USA), [$^2H_6$]-ABA (OlChemlm Ltd, Olomouc, Czech Republic), [$^2H_4$]-SA (CDN Isotopes Inc., Quebec, Canada) and [$^2H_5$]-JA (CDN Isotopes Inc., Quebec, Canada) were added as internal standards. Methanolic extracts were centrifuged (4000·*g*; 5 min), surnatants were collected and pellets were eluted with 80 % MeOH. The extraction was repeated three times. Extracts were reduced to water by a rotary evaporator, acidified (pH=2.8–3), and partitioned three times with ethyl-acetate (1:1 *v/v*). Organic phases were collected and stored at 4 ° C in darkness until HPLC analysis. Xylem sap samples (500 μL) were added with the aforementioned internal standards and processed as with the root samples.

HPLC analysis

Hormones (IAA, ABA, SA, JA) were separated by reversed phase HPLC, as previously described (Mariotti et al. 2011). Analysis were performed with a Kontron instrument (Kontron Instruments, Munich, Germany) equipped with a variable wavelength UV detector SpectroMonitor 3100 (Milton Roy, Florida, USA) operating at 214 nm at a flow rate=1 ml min^{-1}. Samples were applied to a 150 mm×4.6 mm i.d. column, packed with Hypersil C18 particle size 5 μm (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each fraction was dried in a rotary evaporator and resolved in MeOH until GC-MS analysis.

GC-MS analysis

Samples were dried, trimethylsilylated with 10 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, USA) at 70 °C for 1 h, and analysed by GC-MS. Quantitative determination of IAA, ABA, SA and JA was performed as previously described by Mariotti et al. (2011), using a Saturn 2200 quadrupole ion trap mass spectrometer coupled to a CP-3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a Mega 1MS capillary column (30 m×0.25 mm i.d., 0.25 m film thickness) (Mega, Milano, Italy). Plant hormones were identified by comparing full mass spectra with authentic compounds. Quantification was carried out by reference to standard plots of concentration ratios versus ion ratios, obtained by analysing known mixtures of standard compounds. Hormonal root content was expressed in ng g^{-1}FW, while the hormonal xylem content was expressed in μM.

Sugar partitioning of different plant organs

Analyses were performed on 5 week old plants. One sample per organ type per treatment per replicate was analysed ($n=3$). Each sample was obtained by pooling five plants per treatment per replicate.

Analysis of soluble carbohydrates

Samples were rapidly frozen in liquid nitrogen and ground to a powder, then extracted as described by Tobias et al. (1992) and assayed for glucose, fructose and sucrose contents through coupled enzymatic assay methods as described by Pompeiano et al. (2013).

Analysis of starch

Root samples were rapidly frozen in liquid nitrogen and ground to a powder, then extracted in 10 mL of boiling 10 mM KOH for 1 min. After cooling, extracts were neutralized with 100 μL 1 M HCl. After centrifugation

(10 min, 10000 g) an aliquot was incubated for 5 min with 1 ml K_2 0.13 % - KI 0.3 % and Abs 595 was read. Values were compared with a known amount of soluble starch which had been treated in the same way as the samples.

Statistical analysis

Gas-exchange measurements and plant heights values were used to create regression curves using Sigma Plot 11. All the other data were analysed using one-way ANOVA. Pairwaise comparisons were performed using the Tukey test by SYSTAT 1.2 assuming $P \leq 0.05$ as the significance level. Percentage data were subjected to angular transformation before analysis to normalize the distribution and stabilize the residual variance.

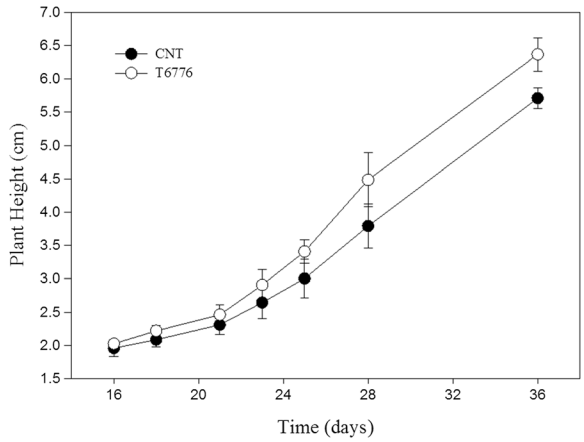

Fig. 1 Increase in height of inoculated (T6776) and non inoculated (CNT) plants. Each point represents mean ± SD of 3 replicates, 12 plants each (n=36), from the 15th day post sowing (DPS) to 35th DPS (from 5 to 28 days post inoculum-DPI)

Results

Colonization ability of T6776 on MT roots

Fungal mycelium emerged from 80 % (SE=±4.13) and 10.7 % (SE=±3.90) of root segments of T6776 and CNT plants, respectively (P=0.000). Further morphological and microscopic observations confirmed the identification of the fungal isolate as T6776. The T6776 outgrowth mycelium was also found in CNT plants, but in very small amounts 10.7 % ±3.9.

Effects of co-cultivation of MT with T6776 on plant growth rate and biomass production

In order to evaluate growth promotion of T6776 on MT, analyses were performed on T6776 and CNT plants. Figure 1 shows the growth curves of plants, expressing the plant height grown with or without T6776. The analysis of regression variance showed that curves derived from MT height values with or without T6776 inoculation differed significantly in terms of their slope (P_{slope}<0.0001; CNT_{slope}=0.1920±0.0062, R^2=0.80; $T6776_{slope}$=0.2265±0.0053, R^2=0.88), confirming the growth stimulating effect of T6776. Conversely, there was no significant difference in the curves in terms of leaf area, suggesting that this parameter is not influenced by T6776 in these growth conditions or on this *cv.* (data not shown).

ANOVA analysis of the biomass at the end of the experimental period (28 DPI) supports the results of the growth curves. FW and DW of the whole plants treated with T6776 were significantly higher compared to CNT plants (+46.66 % and 31.86 %, respectively). When the different plant portions were evaluated separately, FW of stems (+60 %), FW of roots (+81 %) and DW of stems (+100 %) of T6776 plants were significantly higher compared to CNT plants. Recorded increase in FW of leaves, DW of leaves and DW of roots were not statistically significant (Table 1).

Effect on MT photosynthetic performance by T6776

Figure 2 reports the effects of *T*6776 on Φ_{PSII}. T6776 plants showed a significantly higher Φ_{PSII} at both 200 μmol m^{-2} s^{-1} and 300 μmol m^{-2} s^{-1} of PPFD compared with CNT throughout the analysed period. It is also worth noting that Φ_{PSII} showed no significant changes in T6776 plants from 14 to 28 DPI, whereas there was a slightly decrease in CNT plants measured at both 200 μmol m^{-2} s^{-1} (2.6 %) and 300 μmol m^{-2} s^{-1} (4.7 %) of PPFD.

At the end of the growth period (35 DPS and 28 DPI), Φ_{PSII} of T6776 plants was 4.5 % and 7 % higher than CNT at 200 μmol m^{-2} s^{-1} and 300 μmol m^{-2} s^{-1} of PPFD, respectively (Table 2). T6776 plants also showed a significantly higher Fv/Fm and a lower NPQ, at both light intensities, compared to CNT (Table 2). The higher values of both potential and actual PSII photochemistry in T6776 than CNT, were associated with significantly higher photosynthetic CO_2 assimilation rates (A) and stomatal conductance (g_s) values, whereas the

Table 1 Fresh and dry weight of leaves, stems, roots, of the total plant and shoot/root ratio of not inoculated (CNT) and inoculated with *Trichoderma harzianum* T6776 (T6776) tomato plants, 5 weeks after planting (28 DPI)

Treatment	Fresh weight					Dry weight				
	Leaves (g plant^{-1})	Stems (g plant^{-1})	Roots (g plant^{-1})	Total (g plant^{-1})	shoot/root ratio	Leaves (g plant^{-1})	Stems (g plant^{-1})	Roots (g plant^{-1})	Total (g plant^{-1})	shoot/root ratio
CNT	1±0.11a	0.28±0.01a	0.79±0.07a	2.1±0.17a	1.63±0.17a	0.11±0.009a	0.02±0.001a	0.02±0.002a	0.16±0.01a	6±0.37a
T6776	1.13±0.09a	0.45±0.01b	1.43±0.14b	3±0.23b	1.11±0.05b	0.14±0.018a	0.04±0.001b	0.03±0.004a	0.21±0.01b	5.80±0.44a
Percentage above control	13	60	81	46.66		27	100	39	31.86	

Values represent the mean ± SE of 3 replicates of 5 plants each (n=3). At different letters within the same column correspond values significantly different, according to Tukey's test (P<0.05). *CNT* control plants, *T6776* plants inoculated with *T. harzianum* T6776

intercellular CO_2 concentration (C_i) was not statistically different at either of the light intensities (Table 2).

The light response curves show the variation in photosynthetic CO_2 assimilation at increasing light intensities in CNT and T6776 (Fig. 3, upper panel). The maximum apparent quantum efficiency (A_{qe}) and the light saturated rate of photosynthesis at growth CO_2 (A_{max}) were determined from the initial slope of the curve with low light intensity and from the asymptote of photosynthesis with high light intensity, respectively. The results indicate higher A_{qe} and A_{max} values in T6776 (0.065±0.003 mol CO_2 mol photon^{-1} and 14.07±1.22 μmol $CO_2 m^{-2}$ s^{-1} respectively, n=3) than in CNT (0.048±0.006 mol CO_2 mol photon^{-1} and 11.07 ±0.72 μmol CO_2 m^{-2} s^{-1} respectively, n=3). The intercellular CO_2 concentration (C_i) decreased with increasing PPFD in both CNT and T7667 (Fig. 3, lower panel). Photosynthetic CO_2 assimilation determined at increasing PPFD was negatively related to C_i for both treatments, although the slope of the linear relationship was significantly higher in T6776 (P_{slope}<0.0001) than in CNT (see inset of lower panel in Fig. 3).

Effect on photosynthetic leaf pigment content by T6776

In order to evaluate whether T6776 affects pigment composition, the amounts of chl a, chl b, chl tot and x + c were determined on fully expanded leaves of 28 DPI plants. ANOVA analysis showed that *T*6776 significantly enhanced all pigment contents, with an increase ranging from 73.3 up to 76.5 %, compared to CNT (Table 3).

Effect of T6776 on hormone content in roots and xylem sap

SA, JA, IAA and ABA were determined in the xylem exudates and roots in order to compare the hormonal profile of T6776 and CNT plants (Fig. 4). Analysis of the hormonal content of xylem sap was performed in order to investigate the involvement of these hormones as root-to-shoot signalling. In roots, significant differences were found for IAA (P=0.031), JA (P=0.012) and SA (P=0.014) contents between treatments (Fig. 4a). IAA content was enhanced by about 18 % (IAA_{CNT}=29.3±0.24 ng g^{-1} FW; IAA_{T6776}=34.7± 0.44 ng g^{-1} FW) and JA content was strongly enhanced by about 70 % in T6776 compared to CNT plants (JA_{CNT}=19.8±0.45 ng g^{-1} FW; JA_{T6776}=33.4± 0.38 ng g^{-1} FW). SA content in roots of T6776 plants

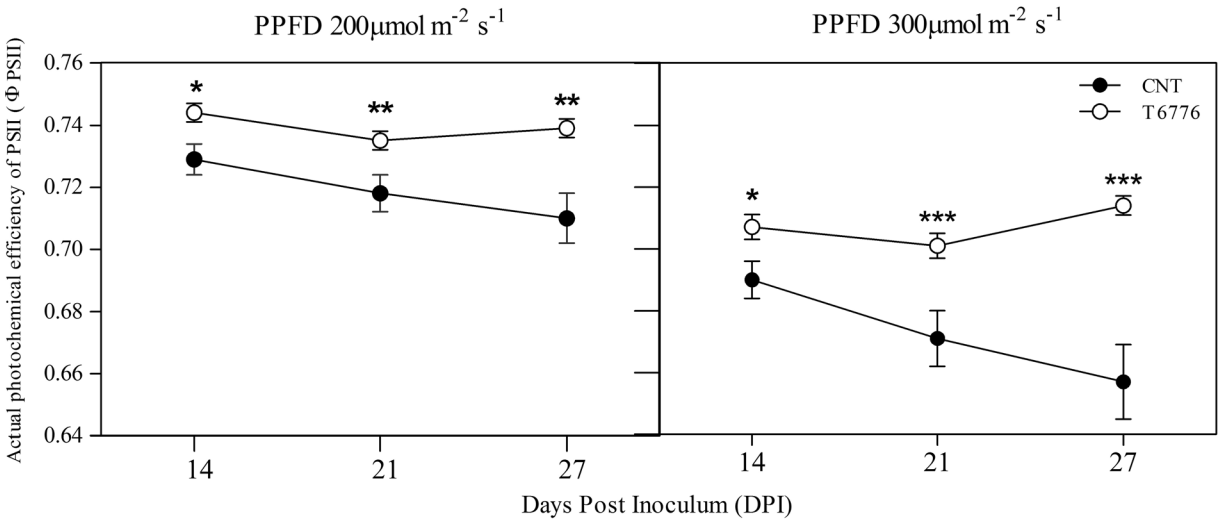

Fig. 2 Actual efficiency of PSII photochemistry in the light (Φ_{PSII}) of inoculated (T6776) and non inoculated plants (CNT), at growing light (PPFD 200 µmol m $^{-2}$ s $^{-1}$) and after 30 min of acclimation at 300 µmol m $^{-2}$ s $^{-1}$ of PPFD. Measurements were taken after 14, 21 and 28 DPI with T6776. Values represent the mean±SE of 3 replicates, 12 plants each (*n=36*) at 14 and 21 DPI, and mean ± SE of 3 replicates, 3 or 4 plants each (*n=10*) at 28 DPI. Data were submitted to one-way ANOVA and significant comparisons between treatments, performed by Tukey's test, are marked with * (*$P\leq0.05$; **$P\leq0.01$; ***$P\leq0.001$)

decreased by about 4 % compared with the concentration found in CNT plants (SA_{CNT}=727.2±1.32 ng g^{-1} FW; SA_{T6776}=695.8±0.96 ng g^{-1} FW). ABA concentration was not determined because it was under the MS detection threshold. As found in roots, significantly increased concentrations of IAA (IAA_{CNT}=43.4± 0.09 µM; IAA_{T6776}=356.9±12.72 µM; *P*=0.002) and JA (JA_{CNT}=337.6±8.40 µM; JA_{T6776}=518.3± 22.50 µM; *P*=0.002) were found in xylem exudates of T6776 plants compared with CNT (+823 % and +54 %, respectively), together with a significantly lower concentration of SA (−16 %; *P*=0.008) compared to CNT (SA_{CNT}=1000.6±0.10 µM; SA_{T6776}=843.6± 32.00 µM) (Fig. 4b).

Effect of T6776 on plant sugar partitioning

Glucose, fructose and sucrose contents were measured on fully expanded leaves, stems and roots of 28 DPI MT plants in order to evaluate differences in the production and allocation of photosynthetic products between treatments (Table 4). Additionally, the starch content was measured in roots. Analysis shows significant differences between treatments in the sugar partitioning within organs. A significant decrease of about 40 % (*P*= 0.011) was found in the glucose content of leaves of T6776 plants compared to CNT plants, while fructose and sucrose contents were similar between treatments. Sucrose content increased up to 99 % in stems of T6776 plants (*P*=0.049), while glucose and fructose stem contents were not statistically affected by the treatment. Sucrose, glucose and starch contents of roots of T6776 plants were significantly different from CNT plants (*P*= 0.008; *P*=0.013; *P*=0.026, respectively), while fructose content was not affected by the treatment. Sucrose, glucose and starch contents of the roots increased up to 35, 160 and 85 %, respectively.

Discussion

T6776 is a Micro-Tom roots endophyte

The beneficial effects of *Trichoderma spp*. in terms of growth promotion and increased tolerance against biotic and abiotic stresses are a consequence of the intimate dialog between the beneficial fungi and the plants established during root colonization (Harman et al. 2004a). Many studies have report on the ability of different strains of *Trichoderma spp*. to colonize the roots of different hosts, inducing different effects on the plant they interact with (Chacón et al. 2007; Bae et al. 2011; Brotman et al. 2013). The percentage of T6776-outgrowing mycelia from T6776 surface sterilized roots was statistically higher than in CNT plants, suggesting the ability of T6776 to colonize the roots of

Table 2 Photosynthetic parameters of not inoculated (CNT) and inoculated with *Trichoderma harzianum* T6776 (T6776) tomato plants, 5 weeks after planting (28 DPI). Measures were registered at 200 and 300 μmol $m^{-2}s^{-1}$ light intensity (PPFD)

Treatment	PPFD (200 μmol $m^{-2}s^{-1}$)						PPFD (300 μmol $m^{-2}s^{-1}$)				
	Fv/Fm[a]	Φ_{PSII}[b]	NPQ[c]	A^d (μmol $m^{-2}s^{-1}$)	g_s^e (mol $m^{-2}s^{-1}$)	C_i^f (μmol mol^{-1})	Φ_{PSII}[b]	NPQ[c]	A^d (μmol $m^{-2}s^{-1}$)	g_s^e (mol $m^{-2}s^{-1}$)	C_i^f (μmol mol^{-1})
CNT	0.808±0.003a	0.700±0.009a	0.290±0.030a	4.8±0.3a	0.103±0.016a	306±6a	0.661±0.015a	0.433±0.033a	5.6±0.3a	0.090±0.008a	283±5a
T6776	0.817±0.001b	0.737±0.003b	0.220±0.018b	6.2±0.3b	0.165±0.022b	320±5a	0.711±0.00 b	0.322±0.026b	7.5±0.5b	0.144±0.020b	296±5a

Values represent the mean ± SE of 8 replicates. At different letters within the same column correspond values significantly different, according to Tukey's test ($P<0.05$). *CNT* control plants, *T6776* plants inoculated with *T. harzianum* T6776, *PPFD* Photosyntetic photon flux density. [a] Maximum photochemical efficiency of Photosystem II (PSII); [b] Actual photochemical efficiency of PSII; [c] Non photochemical quenching; [d] Net CO_2 assimilation rate; [e] Stomatal conductance; [f] Internal CO_2 concentration

MT plants, inducing metabolic changes in MT physiology. The low level of contamination found in the CNT plants could be explained by the fact that, about two weeks after inoculation, T6776 sporulated on the surface of T6776 inoculated Rock-wool pots. Such spores can be the cause of the late spread of the fungus on some control plants. The small amount of infected CNT plants and the shorter time the fungus had to interact with it could have led to a very small underestimation of the recorded effects, which nevertheless does not invalidate the results.

T6776 stimulates growth rates and biomass production of MT plants

This study demonstrates the growth promotion activity of *T. harzianum* T6776 on Micro-Tom plants for the first time, highlighting the potential of this cultivar as a model plant to study the physiological basis of growth promotion in tomato. This cultivar is an emerging model for tomato research, since it has phenotypic features that fulfil the requirements of a model organism such as its convenient small size and the amenability to the large scale cultivation. In addition, its genome has been sequenced and compared with tomato 'Heinz 1706' (Kobayashi et al. 2014). The availability of several mutants in the MT background and a highly efficient genetic transformation make this cultivar very useful for plant microbe interaction studies (Meissner et al. 1997; Campos et al. 2010; Carvalho et al. 2011). In our experiments, inoculated MT plants were significantly higher compared to untreated plants, highlighting the higher developmental rate inducted by T6776 on this *cv.*. After 28 DPI, inoculated plants showed higher FW and DW values, confirming the results of previous studies on other cultivars (Gravel et al. 2007; Nzanza et al. 2012; Chowdappa et al. 2013).

T6776 enhances leaves pigment content of MT plants

Pigment content of fully expanded leaves was enhanced by T6776: chlorophyll a and b, total chlorophyll and carotenoid contents were enhanced up to 75 % in inoculated plants. Results concerning these parameters are controversial: Yedidia and colleagues (2001) reported an increased leaf chlorophyll content in cucumber plants treated with *T. harzianum* T-203, while no increase in pigment contents was reported in tomato seedling/*T. harzianum* T-22 interactions (Mastouri et al. 2012).

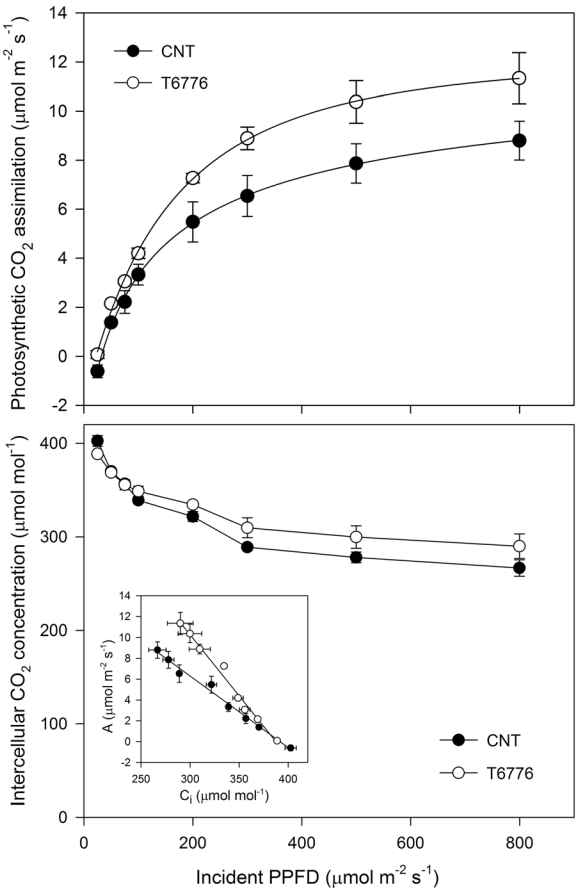

Fig. 3 Light response curves of photosynthetic CO_2 assimilation (A, upper panel) and intercellular CO_2 concentration (C_i, lower panel) against incident photosynthetic photon flux density (PPFD) in 5 week-old (28 DPI) inoculated (T6776) and not inoculated (CNT) plants. The inset of the lower panel shows the linear relationships between A and C_i determined at increasing PPFD values (CNT: y=26.93x – 0.07, R^2=0.988; T7667: y=45.50x – 0.12, R^2=0.985). Values are means ± SE (n=3)

Such different effects could be due to the specific abilities of strains recorded within this genus and in this species. The controversial results reported in literature could be explain by the fact that the *T. harzianum* species has been recently revised and shown as a species complex, including at least 14 species (Chaverri et al. 2015).

Photosynthetic activity was enhanced by T6776

T6776 plants showed a higher Φ_{PSII} than CNT at both light intensities throughout the measuring period. These differences became more significant in 5 week old plants and were associated with a higher NPQ in CNT plants than T6776, especially when NPQ was measured at a light intensity higher than that of growth. Taking into account that Φ_{PSII} gives a measurement of the rate of linear electron transport (Genty et al. 1989) and that NPQ is linearly related to heat dissipation (Bilger and Björkman 1990), our results suggest that the proportion of light absorbed by chlorophyll used in photochemistry was strongly increased by T6776. The light-limited initial slope of the photosynthetic light response curves confirms that the rate of photosynthetic electron transport was stimulated by T6776. Co-cultivation with T6776 also induced a greater potential of PSII photochemistry, as indicated by the higher Fv/Fm value in dark-adapted leaves of T6776 plants.

These results are partly supported by differences found in pigment composition between treatments, suggesting a potentiated photosynthetic apparatus in T6776 plants than in CNT. In agreement with this hypothesis, T6776 plants showed both a higher photosynthetic CO_2 assimilation rate (A) and stomatal conductance (g_s) in growth light conditions and a higher maximum photosynthetic CO_2 assimilation under saturating light intensity (A_{max}), when A is limited by the carboxylation capacity of Rubisco or by triose phosphate metabolism.

Despite the higher values of g_s, T6776 plants did not show any statistical differences in intercellular CO_2 concentration (Ci) in growth light conditions and

Table 3 Chlorophyll (a, b, total) and carotenoids contents of fully expanded leaves in 5 week old (28 DPI) not inoculated (CNT) and inoculated with *Trichoderma harzianum* T6776 (T6776) tomato plants

Treatment	Chl a	Chl b	Chl tot	Total carotenoids
CNT	0.7±0.12a	0.3±0.05a	0.98±0.17a	0.16±0.03a
T6776	1.2±0.04b	0.5±0.02b	1.72±0.06b	0.28±0.01b
Percentage above control	76.50	73.30	75.50	75

Values represent the mean ± SE of 9 replicates for treatment (3 samples for each replicates, 3 replicates for treatment, n=9). At different letters within the same column correspond values significantly different, according to Tukey's test (P<0.05). *CNT* control plants, *T6776* plants inoculated with *T. harzianum* T6776

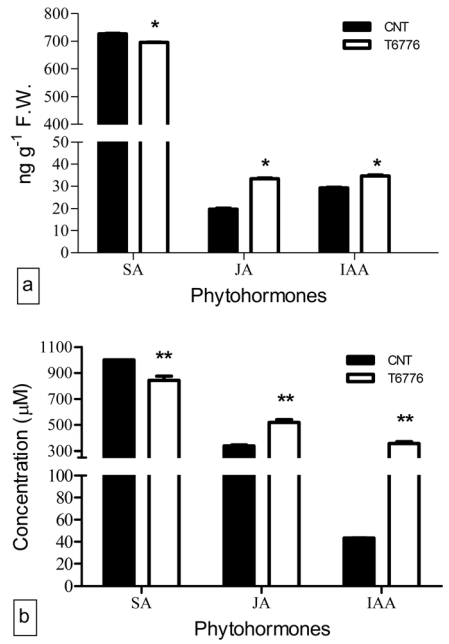

Fig. 4 Hormonal profiling (JA, SA and IAA) of root (**a**) and xylem exudates (**b**) of 5 week old (28 DPI) plants inoculated (T6776) or not inoculated (CNT) with T6776 determined by GC-MS. Concentrations are expressed in ng g^{-1} fresh weight (FW) for root samples and in µM for xylem exudates. Values are means ± SE ($n=6$). Data were submitted to one-way ANOVA and significant comparisons between treatments, performed by Tukey's test, are marked with * (*$P\leq0.05$; **$P\leq0.01$; ***$P\leq0.001$)

presented a higher slope of the relationship between A and Ci with increasing incident PPFD compared with CNT. This thus further support the hypothesis of an enhanced carboxylation efficiency induced by T6776. These results suggest that both stomatal and non-stomatal factors contribute to stimulating the photosynthetic activity in T6776 plants.

Previous works showed an improved photosynthetic rate as a result of the colonization of maize roots by *T. virens*, associated with increased steady-state levels of mRNA for the Rubisco small subunit and the oxygen-evolving enhancer 3-1 (Vargas et al. 2009). *T. harzianum* T-22 also increases the expression of photosynthetic related genes, such as Rubisco large subunit and PSII oxygen-evolving complex protein 2, in maize (Shoresh and Harman 2008). Mastouri et al. (2012) showed that T-22 induced an enhanced expression of genes associated with the ascorbate-glutathione cycle and an up-regulation of chloroplast ROS-scavenging enzymes in tomato plants. This thus, increased photosynthetic efficiency by reducing damage by the superoxide anion and other reactive species involved in photosynthesis. It is possible that the potentiated photosynthetic capabilities and light energy use efficiency in T6776 plants depend on an induction of both photosynthetic related genes and genes involved in the antioxidant pathways, as shown in T-22/tomato interaction.

Stimulation of soluble sugar partitioning in favour of roots by T6776

T6776 affects carbohydrate partitioning of MT, mainly in favour of roots, where increased concentrations of sucrose, glucose and starch were found, coupled with an

Table 4 Carbohydrate content at leaves, stems and roots level of 5 week old (28 DPI) not inoculated (CNT) and inoculated with *Trichoderma harzianum* T6776 (T6776) tomato plants

Treatment	Glucose	Fructose	Sucrose	Starch
	Leaves			
CNT	20.2±0.97a	6.3±0.56 a	42.5±0.004a	N.d.
T6776	12.1±1.50b	9.5±1.10a	41.8±0.600a	N.d.
	Stems			
CNT	19.5±2.45a	0.7±0.35a	4.4±1.10a	N.d.
T6776	19.5±2.45a	0.7±0.07a	8.0±1.30b	N.d.
	Roots			
CNT	3.6±1.20a	9.9±1.10a	26.3±0.87a	2.8±0.14a
T6776	9.37±0.63b	12.7±0.60a	35.2±1.61b	5.2±0.68 b

The content of glucose, fructose, sucrose and starch were expressed in µmol of hexose equivalent for 1 g of fresh weight (FW) of leaves, stems and roots tissue. Values (µmol hexose equiv. g-1 FW) represent the mean ± SE of 3 replicates for treatment ($n=3$). At different letters within the same column correspond values significantly different, according to Tukey's test ($P<0.05$). CNT: control plants; T6776: plants inoculated with *T. harzianum* T6776. N.d.: not determined

increased sucrose content in stems and a decreased glucose content in leaves. Sucrose is produced in source leaves and transported in heterotrophic tissues (sink organs), thus representing the main photosynthetic product that controls carbohydrate partitioning and signalling in plants. Differences found in carbohydrate allocations between T6776 and CNT plants show that plant carbohydrate partitioning was affected by the presence of T6776 in roots. T6776 alters the source-sink relationship of the plant, enhancing the movement of carbohydrates from leaves to sink colonized roots, similar to what happens with pathogens and during mycorrhizal symbiosis (Tejeda-Sartorius et al. 2008). These effects could be due to the increase expression of plant sucrose transporters and/or by the induction of apoplastic invertase, which is usually up-regulated upon pathogen infection and wounding (Benhamou et al. 1991; Ohyama et al. 1998). *T. virens* also has a plant-like sucrose transporter, and the degradation of plant derived sugar in the fungal cells plays a key role in the establishment of the interaction with maize plants and in the up-regulation of the photosynthetic genes (Vargas et al. 2009, 2011).

Micro-Tom plant hormones homeostasis is influenced by T6776

A dynamic *Trichoderma*-plant cross-talk model mediated by hormonal signalling has been already suggested (Hermosa et al. 2012) and several studies highlight the direct activation of JA and/or SA-related signalling pathways in *Trichoderma*-colonized plants (Contreras-Cornejo et al. 2011), which trigger plant defence responses (Nawrocka and Małolepsza 2013). Most of the studies on JA and SA highlighted *Trichoderma* treated plants defence responses to a pathogen challenge.

The present study shows that T6776 induces marked changes in the homeostasis of both growth and stress-related hormones in MT plants grown in optimal conditions and in the absence of a pathogen. A significant increase in IAA and JA contents was observed in T6776 plants, both in the roots and in the xylem saps, while SA content in the same tissues significantly decreased. The higher root content of IAA and its increased transport towards the aboveground part of the plants found here suggests that the promotional growth effect observed in T6776 plants is mediated by IAA, in accordance with previous studies (Gravel et al. 2007; Martínez-Medina et al. 2014). The JA root accumulation found here could be involved in the biocontrol activities against soil-borne pathogens reported for T6776 treated tomato plants (Sarrocco et al. 2013), as in *T. harzianum* treated melon against Fusarium wilt (Martínez-Medina et al. 2011).

The increased uptake of JA alarm signals in the xylem vessel suggests the important role of this hormone as a root-to-shoot signal in T6776 plants and a systemic increase in JA content in all part of the plants. This result is consistent with recent studies that reported a synthesis of JA in roots from where it is trans located to the shoot via xylem (Howe 2004; López-Ráez et al. 2010).

To the best of our knowledge, this is the first report concerning hormones transport through xylem vessels in a *Trichoderma*-interacting plant. Recent works highlight the key role of JA in plant defence systemic induction by *Trichoderma* spp., while the SA and ET involvement could be strain-specific (Martínez-Medina et al. 2013). An antagonistic interaction between SA and JA signalling pathways in the defence response has been reported (Pozo et al. 2004) which could explain the decreased level of SA found in both roots and xylem vessels in T6776 plants. There is evidence that a strong JA shoot accumulation is correlated with induced systemic resistance against air borne pathogens in *Arabidopsis* and melon plants colonized by *T. virens* or *T. atroviride* (Contreras-Cornejo et al. 2011), *T. harzianum* (Martínez-Medina et al. 2011) or *T. longibrachiatum* (Martinez et al. 2001). The relationship between the enhanced defence status and a high level of JA has also been shown during mycorrhizal-plant interaction (Wasternack and Hause 2002). Conversely, recent studies have not reported any increase in JA systemic accumulation in defence responses of tomato and melon inoculated with *T. asperellum* and with several strains of *Trichoderma* spp., respectively (Fernández et al. 2014; Martínez-Medina et al. 2014). The physiological implication of JA accumulation and its transport upon pathogen infection in T6776-MT interaction needs further examination. Investigations concerning the induction of defence-related genes are therefore needed, since preliminary results suggest that T6776 enhances MT defence responses against the air-borne pathogen *A. solani* (Fiorini, unpublished data). Recently, the role of JA and SA in the *Arabidopsis* root colonization by two root endophytes (Lahrmann et al. 2015) and by *T. harzianum* (Alonso-Ramírez et al. 2014) has been explored. Lahrmann and colleagues. (2015) report JA accumulation and decreasing SA concentration in the root of

A. thaliana due to colonization by *Piriformospora indica* and *Sebacina vermifera*, thus supporting data collected in our work. Conversely, Alonso-Ramírez and colleagues (2014) found that a significant up regulation of genes involved both in JA and SA biosynthesis (*Lox1* and *ICS1*, respectively), was triggered in *A. thaliana* roots 72 h after *T. harzianum* inoculation. Using the *A. thaliana* SA impaired mutant *sid2*, the authors show that *sid2* does not limit *T. harzianum* colonization in the roots, highlighting the key role of SA in preventing *T. harzianum* from entering the vascular system of the roots (Alonso-Ramírez et al. 2014). The discordance with the data presented here concerning SA accumulation could be due to the different timing of the measurements. Our data were collected four weeks after inoculation with T6776, while the data in Alonso-Ramírez et al.(2014) were collected in the early stages of root colonization, 72 h after inoculation.

Besides its role in defence and root colonization, JA has a function in multiple developmental and growth processes, including photosynthesis gene modulation and sugar partitioning (Reinbothe et al. 1993a, b; Babst et al. 2005). Its role in sugar partitioning was supported by the finding that the application of exogenous JA accelerates photosynthates export from leaves towards stems and roots in poplar (Babst et al. 2005). JA's influence on sugar partitioning has also been shown during the mycorrhizal colonization of tomato plants (Tejeda-Sartorius et al. 2008). JA might be an important compatibility factor, which regulates root colonization in *Trichoderma*-interacting plants, as well as for the mutualistic symbiosis (Hause and Schaarschmidt 2009) and for sebacinoid fungi (Lahrmann et al. 2015). All this evidences strongly supports our results where a higher level of JA in roots and in xylem vessels matches with improved photosynthetic efficiency, altered sugar partitioning and root colonization in the T6776-MT system.

This paper has highlighted that tomato *cv.* Micro-Tom is an effective means to study the interaction of a beneficial fungus with plants. We also demonstrated, for the first time, that the GC-MS analysis of tomato xylem sap highlights the complex relationship between root production and the transport of important plant hormones in plants interacting with a beneficial fungus. Phenotypic and biochemical data were obtained on the same plant/fungus system at the same time, thus making their connection more fruitful by avoiding the uncertainties of comparing data obtained on different cultivars or with different fungus isolates/species. This study shows the complex connection between increased hormone accumulation and transport, altered sugar partitioning and enhanced photosynthetic efficiency induced by T6776, and how growth promotion is the result of the combination of these drastic changes in MT plants. The results presented here enhance current knowledge on the mechanisms that regulate the beneficial effects resulting from *Trichoderma* inoculation, and highlight for the first time the role of JA in establishing symbiotic interaction, in addition to its role in defence.

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