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A Three-Way Interaction System for Understanding the Ability of *Trichoderma* spp. to Trigger Defenses in Tomato Challenged by *Phytophthora nicotianae* †

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Abstract: This study evaluated the early activation of plant-defense related genes during a three-way interaction plant-antagonist-pathogen in the model system tomato-*Trichoderma-Phytophthora nicotianae*. Thirty-day-old tomato seedlings were treated at the root system with a suspension of germinated conidia of two selected strains of *T. asperellum* and *T. atroviride* and then inoculated with zoospores of *P. nicotianae*. The defense mechanisms activated by tomato plants upon the simultaneous colonization of the root system by *Trichoderma* spp. and *P. nicotianae* were evaluated 72 h post-inoculation by analysing the transcriptomic profiles of genes involved in the pathways of salicylic acid (i.e., pathogenesis-related proteins - *PR1b1* and *PR-P2*-encoding genes), jasmonic acid (i.e., lipoxygenases enzymes—*TomLoxC* and *TomLoxA*-encoding genes) and the tomato plant defensin protein (i.e., *SlyDF2*-encoding gene). Results showed that *PR1b1* was more strongly up-regulated in the three-way system including *T. asperellum*, while the gene *PR-P2* was up-regulated in the system including *T. atroviride*. *TomLoxA* was significantly up-regulated only in the three-way system including *T. asperellum*, while *TomLoxC* was up-regulated in no one of the analyzed three-way systems. Finally, the gene *SlyDF2* was significantly up-regulated in tomato seedlings from both three-way systems.

Keywords: *Trichoderma asperellum*; *Trichoderma atroviride*; transcriptomic profiles; plant-defense related genes; gene up-regulation

1. Introduction

The European Directive 2009/128/EC, which establishes a framework for Community action to pursue the sustainable use of pesticides, prescribes the adoption of measures aimed at reducing their use. The National Action Plans (NAPs) represent the instrument for each Member State to implement the European Directive. NAPs are finalized to setting quantitative objectives, targets, measures, timetables and indicators to reduce risks and impacts of pesticide use on human health and the environment and to encourage the development and introduction of integrated pest management strategies and alternative approaches or techniques devoted to reduce dependency on the use of pesticides. In the last decades the use of biological control agents (BCAs) of plant pathogens has

became an effective alternative to conventional practices based on the use of chemicals for the management of plant diseases [1–3].

Among BCAs, selected strains of fungi belonging to *Trichoderma* genus showed a high effectiveness in the control of fungal and oomycete plant pathogens [3–7]. The success of *Trichoderma* strains as BCAs is due to their direct antagonistic activity toward pathogens [4] and to their efficiency in the elicitation of plant defense by the triggering of mechanisms of both systemic acquired resistance (SAR) and induced systemic resistance (ISR), whose activation is mediated by the synthesis of salicylic acid and jasmonic acid/ethylene, respectively [2,3,8–10].

In the last years the research focused the attention on the investigation of the role of *Trichoderma* spp. in the elicitation of SAR and ISR mechanisms in model experimental three-way systems including plant, *Trichoderma* and pathogen [11]. Together with maize, cucumber and pepper, tomato represents one of the most studied model organisms used in three-way model systems including *Trichoderma* spp. [2,3,12–15]. The sequencing of tomato genome [16] together with the susceptibility of this crop to numerous diseases [17] made this plant a suitable model for transcriptomic based-researches aimed at elucidating the mechanism by which *Trichoderma* spp. stimulate plant defense mechanisms to counteract infections by fungi and oomycetes. A very recent study provided preliminary information on the role of *Trichoderma* spp. in activating genetic pathways of plant defense mechanisms at an advanced stage of root infection in the three way systems tomato-*T. asperellum*-*P. nicotianae* and tomato-*T. atroviride*-*P. nicotianae* (Figure 1) [3].

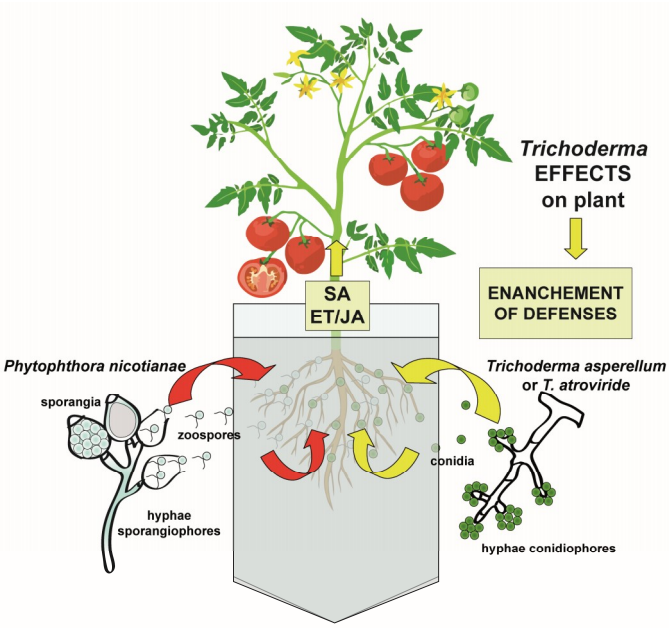

Figure 1. Proposed model for the three-way system plant-pathogen-antagonist showing how *Trichoderma* species modulate the molecular signaling in the challenge between the oomycete pathogen *Phytophthora nicotianae* and the host plant tomato.

However, it should be determined if the elicitation of tomato defenses by *Trichoderma* spp. is an ability of specific strains or if it is conserved in the population of a species; furthermore, the early activation of the defense response in tomato plants under the simultaneous *Trichoderma* spp. colonization and *P. nicotianae* root infection has not yet been investigated. To gain a better insight of transcriptomic mechanisms involved in the complex three-way plant-antagonist-pathogen interaction, this study describes how two recently selected strains of *T. asperellum* and *T. atroviride* modulated salicylic acid, jasmonic acid and antifungal defensin genetic pathways of tomato plants in an early stage of the infection process by the root pathogen *P. nicotianae*, in the system tomato-*Trichoderma*-*Phytophthora nicotianae*.

2. Experiments

2.1. Selection and Culture of Test Microrganisms

T. asperellum strain T_asp_1, *T. atroviride* strain T_atr_6 and *P. nicotianae* isolate Ph_nic [3] were selected from the collection of the Molecular Plant Pathology laboratory, Di3A, University of Catania.

For the experiment, *Trichoderma* spp. and *P. nicotianae* strains were preliminarily cultured on Potato Dextros Agar (PDA) for 7 days at 25 °C and on V8-agar Petri dishes for one week at 28 °C in the dark, respectively.

2.2. Plant Material

Tomato test seedlings were cultured in accordance with La Spada et al. [3]: tomato seeds (*S. lycopersicum* var. Cuor di bue-Vilmorin Italia S.R.L.) were sterilized in 2% NaClO for 20 min, rinsed in sterile distilled water and sowed in an alveolar tray containing sterile vermiculite soaked in a nutrient solution (NS) prepared with fertilizer 20-20-20 (Asso di Fiori-Cifo, S. Giorgio di Piano, Bologna, Italy) (0.1634 g/L), $MgSO_4 \times 7H_2O$ (0.15 g/L), FeNa-EDTA (40 mg/L). Trays were kept for 3 days in the dark at 23 °C and 80% relative humidity; then, seedlings were transferred to a photoperiodic lighting (16 h of light and 8 h of dark) and kept at the same temperature conditions and relative humidity for 30 days. 30 mL of NS were weekly provided to renew the content of mineral salts; tomato plantlets were also watered twice a week. Seedlings were then transferred into plastic tubes containing 30 mL of NS.

2.3. Trichoderma Colonization and Phytophthora nicotianae Infection Assays

Once in the plastic tubes, tomato seedlings were colonized by *T. asperellum* or *T. atroviride* test strains as well as infected by *P. nicotianae* isolate Ph_nic according with the method reported in La Spada et al. [3]. For each *Trichoderma* species, the colonization of the root system was established by treating the tomato seedlings with 300 µL of a dispersion of germinated conidia (100 conidia/mL) prepared as it follows: 100 mL of a synthetic medium, consisting of the NS amended with 15 g/L of sucrose, was autoclaved and then inoculated with 1 mL of conidial dispersion (10^6 conidia/mL) obtained from 7-day-old cultures grown on PDA medium; flasks were then shaken at 150 rpm for 24 h at 25 °C to allow spore germination.

After 48 h of incubation, each seedling colonized by *T. asperellum* or *T. atroviride* was inoculated with zoospores of *P. nicotianae* isolate Ph_nic (concentration: 100 zoospores/mL). *P. nicotianae* inoculum was prepared as it follows: mycelial plugs from a 7-day-old culture of the pathogen grown on V8-agar were flooded with 20 mL of sterile distilled water and incubated at 25 °C for 48 h under a constant fluorescent light; zoospores were released in sterile distilled water by placing mycelial plugs at 6 °C for 1 h and then for another hour at 25 °C. Zoospore concentration was measured by using a hemocytometer. Controls were inoculated with sterile distilled water.

2.4. Experimental Design

Overall, the scheme of the experiment consisted of the treatments reported in Table 1. Each treatment included 5 biological replicates. All the seedlings from each treatment were collected 72 h post-inoculation; stem and roots from each plant were then ground to a fine powder with liquid nitrogen; obtained powders were stored at −80 °C until RNA extraction. All the microrganisms used in the test were re-isolated and then sequenced, from additional seedlings from respective treatments.

2.5. RNA Isolation, cDNA Synthesis and Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted by using RNeasy Plant Mini Kit (Qiagen) from 100 mg of powder from both stem and roots of tomato seedlings, following manufacturer instructions and treated with TURBO DNA-free™ Kit. RNA concentration was adjusted to 200 ng/µL and its quality was verified by an RNA electrophoresis gel in TAE agarose performed in accordance with Masek et al. [18].

The cDNA synthesis was performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) following the manufacturer's instructions.

Each qRT-PCR was performed in a total volume of 20 µL by mixing 10 ng of cDNA with 1 µL of 10 µM of each primer and 10 µL of PowerUp™ SYBR™ Green Master Mix (2X) (Applied Biosystems). qRT-PCR experiments were carried out in triplicate. The thermocycling conditions were 2 min at 50 °C (UDG activation), 2 min at 95 °C (Dual-Lock™ DNA polymerase) followed by 40 cycles of two steps: 95 °C for 15 s (Denaturation) and 59 or 60 °C (annealing/extension) for 1 min. The amplified target genes were *PR1b1*, *PR-P2*, *TomLoxA*, *TomLoxC* [2] and *SlyDF2* [19]; the actin-7-like *LOC101262163* [2] was used as housekeeping. For each gene, primer pair sequences and annealing temperatures were in accordance with La Spada et al. [3].

Table 1. Scheme of the treatments included in the experiment.

Treatment ID	*T. asperellum* Strain T_asp_1	*T. atroviride* Strain T_atr_6	*P. nicotianae* Isolate Ph_nic
1 (control)	[1] -	-	-
2	-	-	[2] X
3	-	X	X
4	X	-	X

[1] - or [2] X represent the un-received or received treatment, respectively. Δ

2.6. Gene Expression Profile

The quantification of gene expression was carried out by using the $2^{-\Delta\Delta Ct}$ method [20] where $\Delta\Delta Ct = (Ct \text{ of target gene} - Ct \text{ of reference gene})_{sample} - (Ct \text{ of target gene} - Ct \text{ of reference gene})_{calibrator}$ and Ct is the threshold cycle of each transcript, defined as the point at which the amount of amplified target reaches a fixed threshold above the background fluorescence. The calibrator sample was represented of five biological replicates of untreated tomato seedlings control samples (i.e., treatment 1 in Table 1).

Data were analyzed by one-way ANOVA and Dunnett's multiple comparisons test using R software. Differences at $p \leq 0.1$ were considered significant.

3. Results

A generalized up-regulation was observed for all the analyzed genes in all the treatments compared to the control. Both genes involved in the pathway of the salicylic acid, namely *PR1b1* and *PR-P2*, showed a significant up-regulation in all the treatments (Table 1); the relative expression levels of *PR1b1* ranged around 4.10 (treatment 2), 2.50 (treatment 3) and 4.70 (treatment 4) times more than that of plants from the calibrator (i.e., treatment 1, Table 1), while it was ca. 23.8 (treatment 2), 15.8 (treatment 3) and 14.00 (treatment 4) times more than that of plants from the calibrator for the *PR-P2*-encoding gene. Similarly to previous genes, *SlyDF2* was significantly up-regulated in plants from all the treatments, with fold changes ca. 7.30 (treatment 2), 4.30 (treatment 3) and 5.70 (treatment 4) times more than the calibrator (Figure 2).

Compared to the other genes, the up-regulation of the genes encoding for lypoxygenases, namely *TomLoxA* and *TomLoxC*, was weakly activated. *TomLoxA* was significantly up-regulated exclusively in the treatments 2 (i.e., seedlings inoculated with *P. nicotianae*) and 4 (i.e., seedlings treated with *T. asperellum* and inoculated with *P. nicotianae*), while *TomLoxC* was significantly transcribed only in seedlings from treatment 2 (Figure 2).

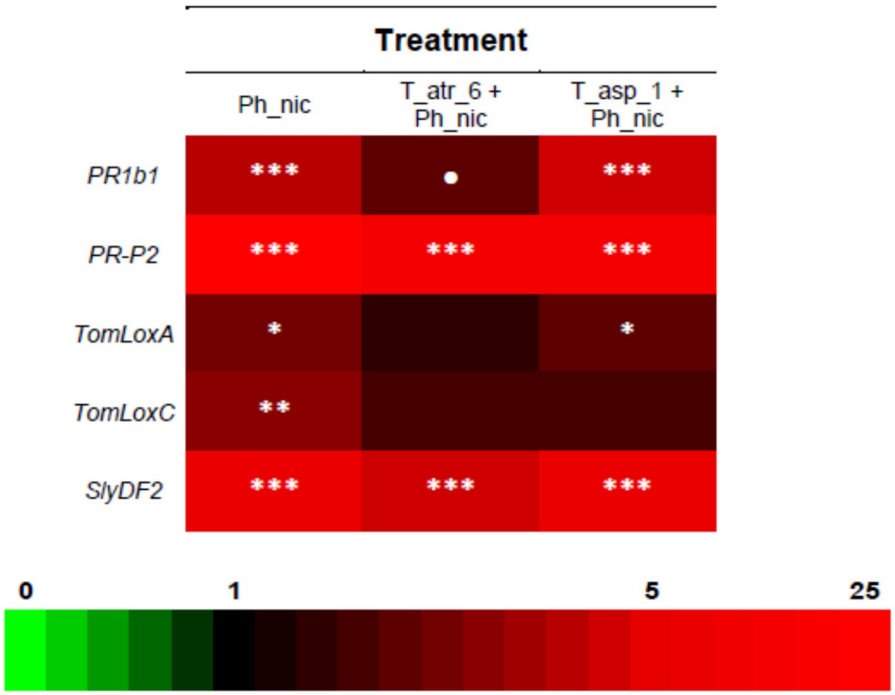

Figure 2. Differences in the expression levels of *PR1b1*, *PR-P2*, *TomLoxA*, *TomLoxC* and *SlyDF2*-encoding genes from 72-h-old *Trichoderma*-treated *Solanum lycopersicum* cv. Cuor di bue seedlings inoculated or non-inoculated with *Phytophthora nicotianae*. The heatmap illustrates fold change in expression ($2^{-\Delta\Delta Ctt}$). Different shades represent induced or repressed gene expression. Cells containing symbols are statistically different according to Dunnett's test ($p < 0.1$, $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$), compared to their calibrator.

4. Discussion

The present study described the response of tomato plants cv. Cuor di Bue, in terms of activation of genetic pathways related with plant defenses, during a three way interaction tomato-*Trichoderma* spp.-*Phytophthora nicotianae* by the application of two recently selected strains of *T. asperellum* (T_asp_1) and *T. atroviride* (T_atr_6) and the pathogen *P. nicotianae*, isolate Ph_nic [3]. Previous studies widely investigated the role of *Trichoderma* species in the elicitation of plant defense mechanisms [15,21–23], including three way systems plant-*Trichoderma* spp.-pathogen [2,3,14]. However, there is a lack of information about the early activation of the defense response in tomato plants under the simultaneous *Trichoderma* colonization and *P. nicotianae* infection. To fill this gap of knowledge, in this study the expression profile of the genes involved in the pathways of salicylic (*PR1b1* and *PR-P2*) and jasmonic acids (*TomLoxA* and *TomLoxC*) [2], and that of the antifungal defensin *SlyDF2* [19] have been evaluated 72 h post-inoculation in *T. asperellum*- or *T. atroviride*-treated tomato seedlings inoculated with *P. nicotianae* and compared with that of seedlings that received only the pathogen *P. nicotianae*.

Results obtained here showed that, 72 h post-treatment, a generalized trend of up-regulations was observed for all the evaluated genes in tomato seedlings from both the three-way systems. Both genes involved in the genetic pathway of the salicylic acid, namely *PR1b1* and *PR-P2*, were significantly up-regulated in seedlings from both the three-way systems, with a stronger up-regulation for *PR-P2* compared to *PR1b1*. In detail, *PR1b1* was more strongly up-regulated in the three-way system including *T. asperellum* strain T_asp_1 (treatment 4), while the gene *PR-P2* in one including *T. atroviride* strain T_atr_6 (treatment 3). With reference to the genes involved in the pathway of the jasmonic acid, namely *TomLoxA* and *TomLoxC*, they both were up-regulated on a smaller scale compared with *PR1b1* and *PR-P2*. *TomLoxA* was significantly up-regulated only in the

three-way system including *T. asperellum* strain T_asp_1 (treatment 4), while *TomLoxC* was not significantly up-regulated in any of the analyzed three-way systems. Finally, similarly to *PR1b1* and *PR-P2*, the gene *SlyDF2* encoding for an antifungal defensin was statistically significantly up-regulated in tomato seedlings from both the three-way systems. The transcriptional response of the defensive pathways of tomato plants in a three-way system including *Trichoderma* spp. and a plant pathogen, was previously described under *Botrytis cinerea* [2] or *P. nicotianae* infections [3]. Tucci et al. [2] evidenced that at the early stages of the three-way interaction, the tomato transcriptional profile of the genes *PR1b1*, *PR-P2*, *TomLoxA* and *TomLoxC* can range from up- to down-regulations depending on several aspects, including the tested tomato cultivar, the *Trichoderma* selected species as well as the tested antagonistic strain. La Spada et al. [3] observed that, among two different three-way systems, namely tomato-*P. nicotianae*-*T. asperellum* strain IMI393899 [24,25] or -*T. atroviride* strain TS, 7 days post-inoculation of the pathogen the gene *PR1b1* was significantly up regulated only in the system including *T. asperellum*, *PR-P2* in both three-way systems, while *TomLoxA*- and *TomLoxC*-encoding genes in no one of the systems. In the same study, La Spada et al. [3] also observed a marked up-regulation of the *SlyDF2*-encoding gene in plants from both the tested three-way systems. Consistently with the above-cited studies [2,3], results obtained here support the hypothesis that the effects due to the colonization of roots by *Trichoderma* spp. occur mainly during the first phases of the three-way interaction and, as reported in La Spada et al. [3], run out after a short time.

Previous literature supported the hypothesis that when plants are challenged with a pathogen after the establishment of the interaction with *Trichoderma* spp., they are primed to react more strongly, increasing defense gene expression sooner and to higher levels than in untreated plants [15,21,22]. However, in the present study, the transcription of all the evaluated genes was more strongly activated in plants that received only the inoculum rather than in *Trichoderma*-pre-colonized and inoculated ones. This result is in agreement with those obtained in other similar pathogen-plant systems. A decreasing trend in the expression of PR-encoding genes at 48 h post-inoculation with *B. cinerea* was reported from different lines of tomato pre-treated with *T. atroviride* strain P1 or *T. harzianum* strain T22 [2]. Additionally, the level of the *CTR1*-encoding gene, which is involved in the signaling pathway of the jasmonic acid, was significantly higher in the roots of cucumber plants inoculated with only the *Pseudomonas syringae* pv. *lachrymans* rather than in plants inoculated and pre-colonized by *T. asperellum* T203 [15]. Furthermore, La Spada et al. [3] observed that, 7 days post-inoculation with *P. nicotianae*, the expression level of the *SlyDF2* gene in tomato plants pre-colonized by *T. asperellum* strain IMI393899 or *T. atroviride* strain TS was lower than in plants that received only the inoculum of the pathogen. Results obtained in this study support the hypothesis that the promotion of plant defenses by *Trichoderma* spp. is a complex mechanism affected by a variability of responses that could depend both on tested plant species and inoculated pathogen [3].

5. Conclusions

In conclusion, this study indicates the tomato defense genetic pathways related with salicylic acid, jasmonic acid and antifungal defensins in the three way interaction system tomato-*Trichoderma* spp.-*P. nicotianae* are activated since the first stages of the infection process. Moreover, results support the hypothesis that the ability to elicit a defense response in tomato plants challenged with a root pathogen, like *P. nicotianae*, is a conserved feature within the same species of *Trichoderma*.

Author Contributions: S.O.C. and F.L.S. conceived and designed the experiments; F.L.S., C.S., M.R. performed the experiments; F.L.S. and A.P. analyzed the data; A.P. and S.O.C. contributed reagents/materials/analysis tools; F.L.S. wrote the paper; S.O.C. supervised the manuscript. All authors have read and agreed to publish this version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript: MDPI: Multidisciplinary Digital Publishing Institute; DOAJ: Directory of open access journals; TLA: Three letter acronym; LD: linear dichroism.

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