Physiological and biochemical response of tomato plants treated with *Trichoderma harzianum* T-22 and infected by *Cucumber mosaic virus*

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Abstract

The study of the host-pathogen-antagonist interaction from a biochemical and molecular point of view is of key importance for understanding the dynamics of infectious processes, and can be useful for the development of new strategies to control phytopathogens, particularly viruses, against which chemical treatments have no effect. Herein, we demonstrate the activity of the fungus *Trichoderma harzianum* strain T-22 (T22) against *Cucumber mosaic virus* (CMV) in tomato plants (*Solanum lycopersicum* var. *cerasiforme*). Plants treated with T22 and inoculated with CMV did not show the typical symptoms induced by CMV, such as stunting or necrosis, and maintained high values of net photosynthesis and stomatal conductance. Furthermore, T22 also had the ability to control CMV infection in tomato plants by inhibiting CMV, as assessed by the absence of the CMV RNA-dependent RNA polymerase gene (*RdRp*) in the oldest plants. In conclusion, our data indicate that the T22-based strategy is a largely practicable way to pursue the goal of an effective control measure against CMV.

Keywords: biocontrol agent, fruit and root development, gas exchange, plant growth-promoting activity

INTRODUCTION

The filamentous ascomycetous fungi *Trichoderma* spp. are among the most abundant and culturable fungi found in many soil types. They are able to colonize plant roots and plant debris. Fungi of this genus are genetically diverse and show a number of different activities between strains (Sofo et al., 2013). *Trichoderma* spp. are agriculturally and industrially important, being the major source of many commercial enzymes and biofungicides. Many *Trichoderma* species (e.g., *Trichoderma harzianum*, *Trichoderma viride*) have been used to antagonize the growth of plant-pathogenic fungi, and thus act as biocontrol agents (BCAs). For this reason, more than 60% of all registered biofungicides used for plant disease control are *Trichoderma*-based (Sofo et al., 2013).

The mechanism of the *Trichoderma*-plant-pathogen interaction is very complex, and includes not only mycoparasitism (a phenomenon wherein one fungus directly kills and obtain nutrients from other fungi), but also competition for nutrients, release of extracellular hydrolytic enzymes, antagonism against nematodes, colonization of the rhizosphere and phyllosphere, production of secondary metabolites that are toxic to plant pathogens, promotion of plant growth and root development, and induction of systemic resistance against different pathogens (Vitti et al., 2015). Particularly, *T. harzianum* strain T-22 (T22) is the active ingredient of various commercial biocontrol products. It works as a deterrent, protecting the root system from attack by pathogenic fungi like *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia* (Sofo et al., 2013). This biofungicide protects the plant by establishing itself in the rhizosphere. T-22 can grow along the entire length of the root system, along which it establishes a barrier against pathogen attack. As long as the root

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system remains active in its growth, T22 continues to grow, feeding on released plant waste products. In this way, it removes the nutrients that pathogens use to feed (Sofo et al., 2012).

Traditional chemical treatments have no effect to protect plants from virus infection (Vitti et al., 2015). Thus, it is important to analyze new approaches for defending plants from viral disease. Considering that the study on the antiviral effect of biocontrol agents and the pathogenic and molecular aspects of plant-virus-antagonist interactions is a necessary step for both basic and applied research, the aims of this study were as follows: a) to optimize the use of T22 for the defense of tomato plants against *Cucumber mosaic virus* (CMV), and b) to understand the molecular basis of the biocontrol action of T22 against CMV in tomato plants.

MATERIALS AND METHODS

T22, the antagonist microorganism used in this study, was utilized as a granule formulation (Trianum G, Koppert, Berkel en Rodenrijs, The Netherlands). CMV strain Fny was propagated in *Nicotiana tabacum* 'Xanthi' plants and purified according to Vitti et al. (2015). CMV genes were obtained from full-length cDNA copies of CMV genomic RNA1, RNA2 and RNA3 (Scottish Crop Research Institute, Dundee, UK).

Seeds of *Solanum lycopersicum* var. *cerasiforme* were sterilized using 1% sodium hypochlorite solution for 1 min and then rinsed with sterile distilled water, before imbibition on moist filter paper at 4°C for 24 h in the dark. Seeds were germinated on water-dampened filter paper in a sterile Petri dish at 26°C. One day after germination, seedlings were transferred to sterilized soil-filled pots (four pots per treatment at a density of one plantlet per pot). Throughout the experiment, plants were kept in a growth chamber with a 16-h photoperiod, at 26/23°C (day/night), and watered with Hoagland solution.

As shown schematically in Figure 1, tomato plants were treated with T22 and/or inoculated with CMV, according to the following six conditions: control plants untreated and healthy (PA); plants treated with T22 only (PB); plants inoculated with CMV only (PC); plants first treated with T22 and, after 7 days, inoculated with CMV (PD); plants simultaneously treated and inoculated with T22 and CMV (PE); and plants first inoculated with CMV and, after 7 days, treated with T22 (PF). Trianum G was mixed with the soil on which the plants were grown at 750 g m^{-3}. An amount of 10 µg purified CMV was used to mechanically inoculate tomato plants at the four-leaf stage. Tomato fruits were harvested from the bottom branch of 3-month-old plants.

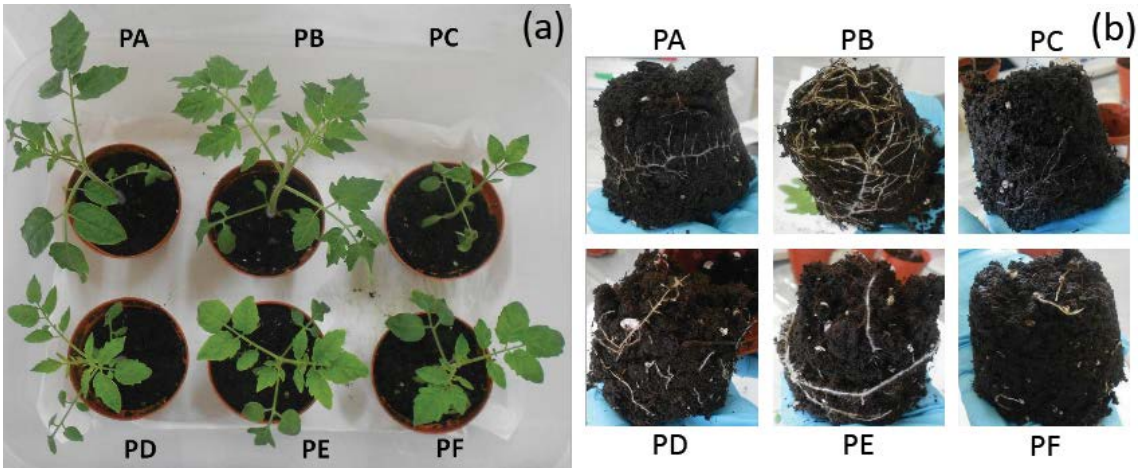

Figure 1. Shoots (a) and roots (b) of 1-month-old tomato plants. In each panel, a representative plant is shown. PA, Control tomato plant; PB, plant treated with only T22; PC, plant inoculated with CMV; PD, plant treated with T22 and, a week later, inoculated with CMV; PE, plant simultaneously treated and inoculated with T22 and CMV; PF, plant first inoculated with CMV and, a week later, treated with T22.

Measurements of net photosynthesis (A) and stomatal conductance (g_s) were carried out with 2- and 3-month-old plants on apical mature leaves, using a LI-6400 portable photosynthesis system and a 6400-40 LED light source (LI-COR Biosciences; Lincoln, NE, USA), operating at ambient CO_2 concentration. Gas exchange analyses were carried out between 12:00 and 14:00 h (solar time) under saturating light conditions (photosynthetically active radiation, PAR = 1500 µmol photons m^{-2} s^{-1}).

Total RNA from leaf tissue (100 mg) was extracted with TRIzol® reagent (Invitrogen, Milan, Italy). In order to assess the presence of CMV in plant tissues, RT-PCR analysis was prepared according to Vitti et al. (2015). The PCR fragments were fractionated on a 1.2% agarose gel and stained with SYBR Safe™ DNA gel stain (Invitrogen).

RESULTS AND DISCUSSION

T22 showed the ability to control CMV infection on tomato cherry plants by modulating viral symptoms during the entire life cycle of the plants and also by inhibiting CMV, as assessed by the presence of the CMV RNA-dependent RNA polymerase (*RdRp*) gene (513 bp). This gene was detected by RT-PCR analysis in 1-month-old plants, but it was absent in 3-month-old plants (Figure 2).

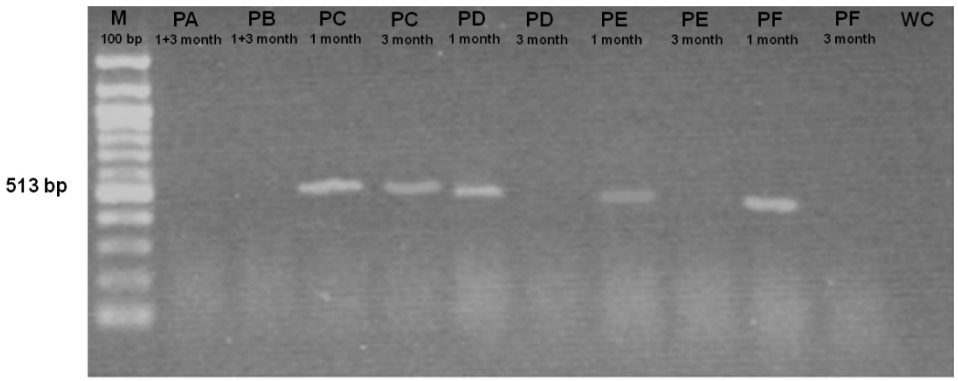

Figure 2. Detection of a DNA fragment (513 bp) of the CMV *RdRp* gene in all groups of tomato plants by RT-PCR. Legend of plant treatments as in Figure 1. M, 100 bp DNA Ladder (BioLabs); WC, water control.

It was found that plants treated with T22 and inoculated with CMV did not show the typical symptoms induced by CMV on tomato, such as stunting or necrosis (Figure 1a) or inhibition of root development (Figures 1b and 3a). As shown in Figure 3, plants treated only with T22 (PB) showed the largest fruit size and root development (Figure 3).

In contrast, control plants inoculated with CMV alone (PC) showed the smallest fruits, with delayed ripening (Figure 3b), accompanied by the lowest root development (Figure 3a). Plants treated with T22 and inoculated with CMV (PD, PE and PF) were similar to the controls (PA) considering both root development and fruit size (Figure 3).

Our data highlighted that T22 improved net photosynthesis: values of A were the same or significantly higher in all plants treated with T22 and inoculated with CMV (PD, PE and PF) than in PA, regardless of plant age (Figure 4a). This physiological behavior could be due to the enhanced sink activity of roots during the plant-T22 interaction increasing the rate of photosynthesis, according to Vargas et al. (2009) and Kaschuk et al. (2009). In 2-month-old PC plants, g_s was very low (-40%, compared with PA and PB). After 3 months, a general increase in g_s was observed in all treatments, with the exception of PF. The decreased g_s in PC plants could be a form of plant defense against invaders, especially at the early stage of infection, according to Alazem and Lin (2015).

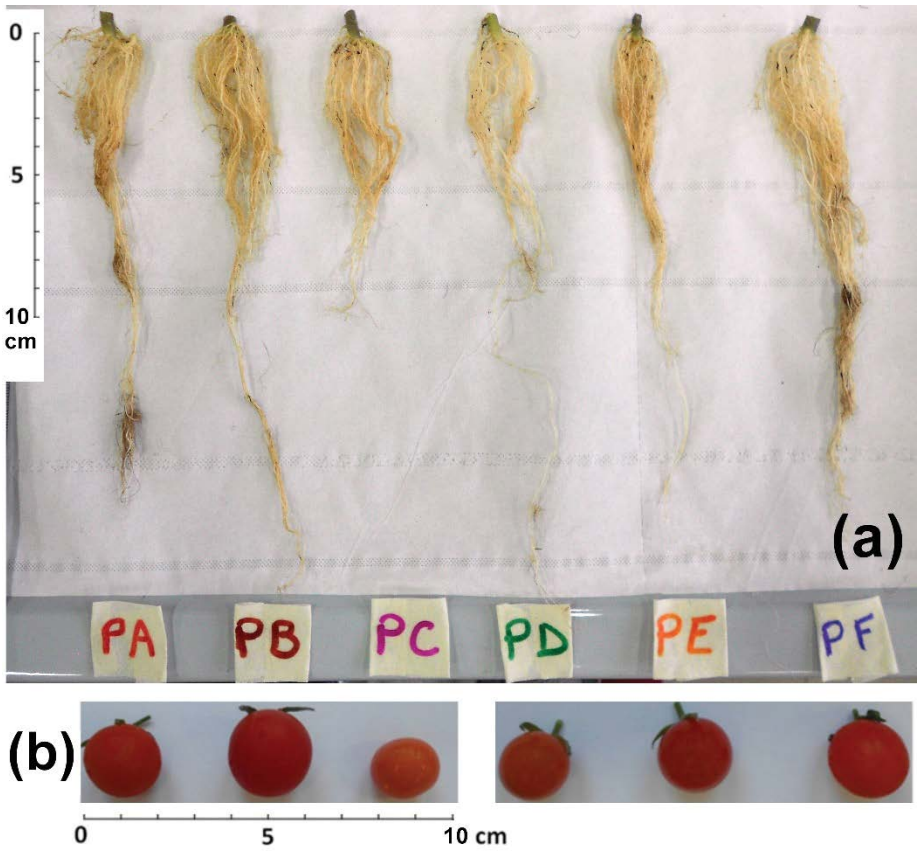

Figure 3. Roots (a) and fruits (b) of 3-month-old tomato plants. In each panel, a representative root or fruit is shown. Legend of plant treatments is as in Figure 1.

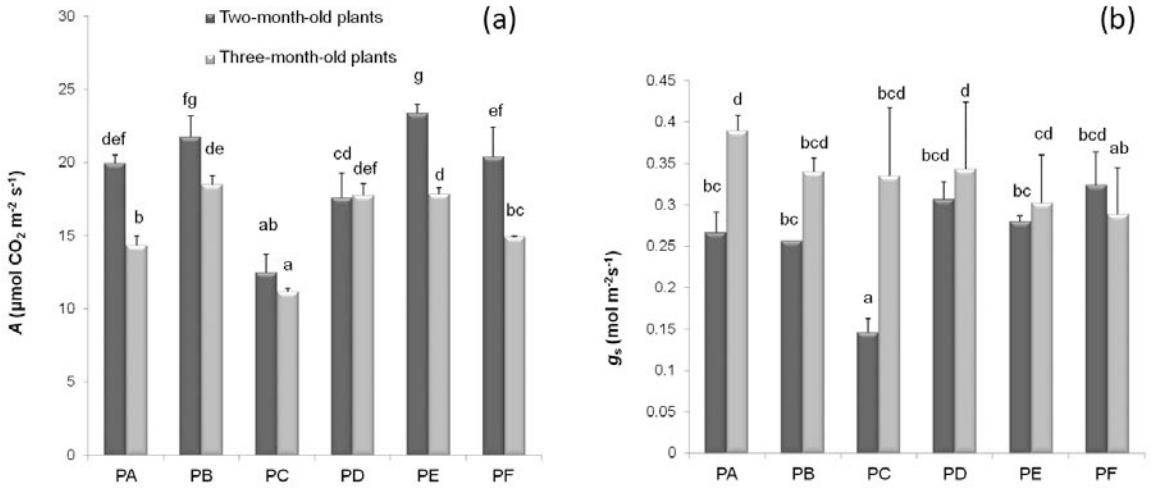

Figure 4. Net photosynthesis (a) and stomatal conductance (b) (±standard deviation) in 2- and 3-month-old tomato plants. Legend of plant treatments is as in Figure 1. Comparison among means was determined by Fisher's least significance (LSD) test. Different letters indicate significant differences at $p \leq 0.05$.

CONCLUSIONS

In recent years, the need for a gradual and irreversible reduction in the use of chemical tools in agriculture has emerged, specifically in the control of plant diseases. The innovation

of this research lies in the possibility of a novel approach to studying the three-way molecular cross-talk between plants, virus and fungi, thanks to which a new system based on the use of a fungal antagonist could be applied to improve the response and the protection of crops against viruses.

The results obtained here could open an avenue of new applications, in both agriculture and biotechnology, that exploit the ability of these fungi to change plant metabolism and resistance to viruses. The demand for innovative management strategies against viruses is urgent and of primary importance for tomato cultivation. In modern agro-industry, fungi such as T22 could offer many beneficial roles. In addition to their biocontrol characteristics, T22 also exhibits plant growth-promoting activity, acting as a powerful biostimulant. The utilization of T22 as an anti-CMV agent in tomato could lead to a reduction in the use of pesticides and fertilizers in sustainable agricultural production, with consequent benefits for the environment.

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