

Beneficial effects of *Trichoderma harzianum* T-22 in tomato seedlings infected by *Cucumber mosaic virus* (CMV)

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Received: 4 April 2014 / Accepted: 26 September 2014 / Published online: 10 October 2014
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Abstract The study of the biochemical and molecular mechanisms deriving from the host-pathogen-antagonist interaction is essential to understand 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. In this work, we demonstrate the ability of the rhizospheric fungus *Trichoderma harzianum* strain T-22 (T22) to induce defense responses in tomato (*Solanum lycopersicum* var. *cerasiforme*) against *Cucumber mosaic virus* (CMV, family Bromoviridae, genus *Cucumovirus*) strain Fny. A granule formulation containing T22 was used for treating the plants before, simultaneously or after the CMV inoculation, in order to study the molecular and biochemical aspects of the interaction between T22 and tomato against the virus. Reactive oxygen species (ROS) and the genes encoding for ROS scavenging enzymes were investigated. Histochemical analysis revealed a different increase in the

superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) content in plants infected by CMV alone or in the presence of T22, confirming the involvement of ROS in plant defense responses. Gene expression analysis suggested a definite improvement in oxidative stress when plants were treated with T22 after inoculation with CMV. In conclusion, our data indicate that *Trichoderma harzianum* T-22 stimulates the induction of tomato defense responses against CMV, an action that implies the involvement of ROS, pointing towards its use as a treatment rather than as a preventive measure.

Keywords *Cucumber mosaic virus* (CMV) · *Trichoderma harzianum* T-22 (T22) · Biocontrol agent (BCA) · Reactive oxygen species (ROS) · Antioxidant enzymes · Tomato (*Solanum lycopersicum* var. *cerasiforme*)

Introduction

Biological control is a safe method to reduce plant disease incidence without collateral damages to the environment and to human health induced by synthetic chemicals (Tucci et al. 2011). Some microorganisms, such as fungi belonging to the genus *Trichoderma*, are used as biocontrol agents (BCAs) to antagonize plant pathogens through a series of mechanisms including competition for nutrients and space, fungistasis, antibiosis and/or modification of the rhizosphere

Handling Editor: Jesus Mercado Blanco.

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(Benítez et al. 2004). *Trichoderma* spp. are agriculturally important also for their beneficial effects on plant growth and development, and for their capability to induce plant defense responses against pathogens, damage provoked by insects, and abiotic stress (Yedidia et al. 1999; Harman et al. 2004; Woo and Lorito 2006). For this reason, they are a major source of many biofungicides and biofertilizers (Verma et al. 2007; Kaewchai et al. 2009). In particular, the strain T-22 of *T. harzianum* represents the active ingredient of registered products widely employed in plant disease control.

Trichoderma spp. and/or their secondary metabolites have shown their potential in the control of plant diseases, not only against fungal pathogens (Vinale et al. 2009; Akrami et al. 2011) and bacteria (Segarra et al. 2009), but also against viruses (Luo et al. 2010). This results in the induction of resistance mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) in plants (Benítez et al. 2004; Harman et al. 2004). Plant's defense against pathogens is regulated through a complex network of signal transduction pathways involving several molecules such as reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA) and ethylene (Et) and cross-talk between them (Kunkel and Brooks 2002). Pathogenesis-related (PR) genes, a series of marker genes for activation of SA, JA, and Et signaling, are also involved in these defense-transduction pathways (Bouchez et al. 2007). Specific coat protein (CP) epitopes of *Tobacco mosaic virus* (TMV) seems to be required for an early ROS induction during the interaction between TMV and tobacco plant (Allan et al. 2001). In addition, Bacsó et al. (2011) showed that accumulation of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) contributes to the development of resistance to TMV. A crucial role for phytohormones, especially JA, SA and Et, was demonstrated in *Penicillium simplicissimum* GP17-2-induced systemic resistance in *Arabidopsis* and tobacco against *Cucumber mosaic virus* (Elsharkawy et al. 2012).

Cucumber mosaic virus (CMV, family Bromoviridae, genus *Cucumovirus*) has the widest host range of any RNA virus, making it one of the most economically important plant viruses to deal with. It infects more than 1,200 plant species in 100 families (Edwards and Christie 1991), and has been intensively

studied as an interesting model from a physicochemical point of view (Nuzzaci et al. 2009). CMV causes a wide range of symptoms, including yellow mottling, distortion and stunting. Viral 2b protein encoded by the genomic RNA 2 is an important virulence determinant of CMV and is also responsible for the inhibition of SA and JA and thus plant defense pathways (Mochizuki and Ohki 2012). Recently, it was demonstrated that a direct interaction between the *Arabidopsis* catalase 3 and the 2b protein of CMV strain Lily induces necrotic symptoms (Inaba et al. 2011) due to a decreased antioxidative catalase activity, that in turn led to H_2O_2 accumulation in the cell and eventually to cell death.

To date, the effects of *Trichoderma* spp. in the induction of plant defense against CMV are poorly known (Elsharkawy et al. 2013) and the biochemical and molecular mechanism involved in this kind of three-way cross-talk between the plant, virus, and antagonist agent has still to be well elucidated. In such a scenario, the present work contributes to develop an innovative strategy against CMV infection in tomato plants, based on the activity of *Trichoderma harzianum*, strain T-22. In addition, we were able to analyze and improve the knowledge on the possible underlying mechanisms involved in plant-pathogen-antagonist interactions.

Materials and methods

Trichoderma spp. and CMV sources

Trichoderma harzianum strain T-22 (T22) was the antagonist microorganism used in this study. It was utilized as a granule formulation (Trianum G, Kopert, Berkel en Rodenrijs, The Netherlands).

Cucumber mosaic virus strain Fny (CMV-Fny) was propagated in *Nicotiana tabacum* cv Xanthi plants and purified as described by Lot et al. (1972). CMV-Fny genes were obtained from full-length cDNA copies of CMV-Fny genomic RNA1, RNA2 and RNA3 (the gift of Peter Palukaitis, Scottish Crop Research Institute, Dundee, UK).

Plant material and experimental design

Seeds of *Solanum lycopersicum* var. *cerasiforme* were sterilized using 1 % Na-hypochlorite solution for

1 min and then rinsed with sterile dH₂O, before imbibition on moist filter paper at 4 °C for 24 h in the dark. Seeds 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. 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 (2.53 mM KNO₃, 0.75 mM Ca(NO₃)₂·4H₂O, 0.50 mM NH₄H₂PO₄, 0.50 mM MgSO₄·7H₂O, 4.10 mM FeSO₄·7H₂O, 2.03 mM Na₂-EDTA, 11.58 mM H₃BO₃, 2.28 mM MnCl₂·4H₂O, 0.08 mM CuSO₄·5H₂O, 0.15 mM H₂MoO₄·H₂O, 0.40 mM ZnSO₄·7H₂O).

Treatments with T22 were performed by incorporating Trianium G (Koppert, Berkel en Rodenrijs, The Netherlands) granules in the substrate used for planting (750 g m⁻³), according to the application and dose suggested by the company. Ten µg of purified CMV-Fny was used to mechanically inoculate tomato plants at the four-leaf stage. Plants were treated with T22 and/or inoculated with CMV, according to the following six conditions (16 plants for each condition): control plants untreated and healthy; plants only treated with T22; plants only inoculated with CMV; plants first treated with T22 and after seven days inoculated with CMV; plants simultaneously treated and inoculated with T22 and CMV; plants first inoculated with CMV and after one week treated with T22. Fourteen days after CMV inoculation and when the plants were five months old, leaves were collected and used for the following analyses.

Histochemical detection of O₂^{•-} and H₂O₂

Histochemical staining of O₂^{•-} and H₂O₂ were both performed according to Xia et al. (2009) with some modifications. Briefly, leaf discs (1.5 cm in diameter) were vacuum infiltrated for 1 h in 25 mM K-HEPES (pH 7.8) containing nitroblue tetrazolium (NBT), 0.1 mg ml⁻¹ for O₂^{•-} staining. The same procedure was carried out for H₂O₂ staining, where vacuum infiltration of the leaf discs was performed in ultrapure water containing 3,3'-diaminobenzidine (DAB), 1 mg ml⁻¹ (SIGMAFASTTM DAB tablet, Sigma Chemical Co., Milan, Italy). Subsequently, the tissues were incubated in the dark at 25 °C for 2 and 24 h for O₂^{•-} and H₂O₂ staining, respectively. Thereafter, the

buffer was eliminated and the leaves were washed in 80 % ethanol for 10 min at 70 °C. The leaves were mounted in lactic acid/phenol/water (1:1:1 v/v) and finally analyzed by Axioskop light microscopy (Karl Zeiss, Germany) at 20× magnification. In all cases, leaf discs were vacuum infiltrated in ultrapure water as a negative control. The experiment was conducted four times.

Total RNA extraction

Total RNA from leaf tissues was extracted by TRIzol[®] Reagent (Invitrogen, Milan, Italy). Tissues (100 mg) derived from four individual plants randomly chosen among the 16 plants of each condition. Four biological replicates (*n* = 4) were used. Samples were ground with mortar and pestle in 1 ml of sterile RNase-free water. To 300 µl of crude extract, 1 ml of TRIzol[®] Reagent was added. The sample was homogenized and the following procedure for the dissociation of nucleoprotein complexes, phase separation, RNA precipitation, RNA washing and RNA re-dissolution were carried out as described by the manufacturer. The concentration and purity of the resulting total RNA was checked with the NanoDrop[®] ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Isogen Life Science, IJsselstein, the Netherlands).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

In order to assess the presence of CMV in the plant tissues, 500 ng of total RNA extracted from tomato seedlings were reverse-transcribed and amplified in a single tube using the SuperScriptTM III One-Step RT-PCR System with Platinum[®] Taq DNA Polymerase (Invitrogen, Milan, Italy). The RT-PCR reaction mixture (final volume of 50 µl) was prepared according to Vitti et al. (2013), using an annealing temperature of 55 °C. The following couples of primers were used: P_{RevRep} (5'-TAACCTCCCAGTTCTCACCGT-3'), complementary to position 1,895–1,915 of the CMV-Fny RNA-dependent RNA polymerase (RdRp) gene, and P_{ForRep} (5'-CCATCACCTTAGCTTCATGT-3'), homologous to the position 1,403–1,423 of the CMV-Fny RdRp gene, according to Grieco et al. (2000). The PCR fragments were fractionated on a 1.2 % agarose gel and stained with SYBR SafeTM

Table 1 Gene-specific primers used in quantitative RT-PCR

| Gene name | Accession number | Primer sequence | Tm (°C) | Amplicon size (pb) |
|------------------------------|------------------|---------------------------------|---------|--------------------|
| <i>Actin (ACT)</i> | BT013524 | 5'-GTGTTTCCTAGTATTGTTGGTCG-3' | 54.2 | 176 |
| | | 5'-TGATGCCAGATCTTCTCCAT-3' | 53.7 | |
| <i>Ubiquitin 3 (UBI)</i> | X58253 | 5'-TCGTAAGGAGTGCCCTAATGCTGA-3' | 61.1 | 119 |
| | | 5'-CAATCGCCTCCAGCCTTGGTTGTAA-3' | 60.9 | |
| <i>Uridylate kinase (UK)</i> | AK322232 | 5'-TGGTAAGGGCACCCAATGTGCTAA-3' | 59.7 | 114 |
| | | 5'-ATCATCGTCCCATCTCGGAACCA-3' | 59.9 | |
| <i>Mn-SOD</i> | XM_004240820 | 5'-AGATGAACGCAGAAGGTGCT-3' | 57.2 | 216 |
| | | 5'-GCGTGTCCCAAACGTCTAT-3' | 54.6 | |
| <i>Cu-Zn-SOD</i> | X14040 | 5'-GTCCATGCCCTTGGTGATAC-3' | 53.4 | 178 |
| | | 5'-GAGGAATCTGCTTGTGCGTA-3' | 54.4 | |
| <i>CAT2</i> | NM_001247257 | 5'-AGCCGGTGGGAAGATTAGTT-3' | 55.0 | 216 |
| | | 5'-GATGAGCACACTTGGAGCA-3' | 55.4 | |
| <i>APX</i> | AY974805 | 5'-GGACCTGATGTTCCCTTTC-3' | 52.7 | 209 |
| | | 5'-CAAGGTCCCTCAAACCAGA-3' | 53.4 | |
| <i>Coi-1</i> | NM_001247535 | 5'-GTGATTTTCGGCTGGTTTTG-3' | 64.2 | 247 |
| | | 5'-GGACATCCTTTAGAGAACTCCAG-3' | 61.9 | |

DNA gel stain (Invitrogen). The experiment was conducted four times.

Real-time reverse transcription PCR (qRT-PCR)

Priming of the cDNA reaction from the RNA template was carried out starting from 1 µg of total RNA extracted. The reaction was performed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK), in a final volume of 15 µl, by using the random hexadeoxynucleotides pd(N)6, according to the manufacturer's instructions. Primer pairs were designed for real-time PCR amplification of sequences belonging to antioxidative and PR genes (Table 1). In addition, primer efficiency was tested on dilutions of the biological replicates ($n = 4$), of which one sample was obtained by pooling each condition together. Dilutions of the samples were prepared as follows: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128. Using the resulting Ct's of the dilutions, a calibration line was obtained to calculate primer efficiency ($E = 10^{(-1/\text{slope})}$) (Table 2).

Real-Time PCR was performed in duplicate for each sample in an optical 96-well plate with a 7,500 Fast Real-Time PCR system (Applied Biosystems, Lennik, Belgium). Reactions were prepared in a total

Table 2 Primers efficiency

| Primer | PCR efficiency (80–120 %) | Equation | R ² Coefficient |
|------------------|---------------------------|-------------------------|----------------------------|
| <i>ACT</i> | 88.63 % | $y = -3.3623x + 24.370$ | R ² = 0.9851 |
| <i>UBI</i> | 99.03 % | $y = -3.3453x + 24.397$ | R ² = 0.9864 |
| <i>UK</i> | 98.34 % | $y = -3.6283x + 28.007$ | R ² = 0.9474 |
| <i>Cu/Zn-SOD</i> | 109.63 % | $y = -3.1109x + 27.133$ | R ² = 0.9953 |
| <i>Mn-SOD</i> | 86.69 % | $y = -3.6884x + 26.708$ | R ² = 0.9155 |
| <i>CAT</i> | 85.77 % | $y = -3.7178x + 25.511$ | R ² = 0.9960 |
| <i>APX</i> | 101.42 % | $y = -3.2883x + 28.162$ | R ² = 0.9723 |
| <i>Coi-1</i> | 116.46 % | $y = -2.9818x + 30.341$ | R ² = 0.9082 |

volume of 10 µl containing 5 µl SYBR® Green Master Mix (Applied Biosystems), 300 nM of a gene specific forward and reverse primer (Table 1) and 2 µl of the 1:5 diluted cDNA template. Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity.

Using geNorm software to investigate the stability of possible reference genes, the following genes were chosen to normalize gene expression data: actin (*ACT*), ubiquitin 3 (*UBI*) and uridylyate kinase (*UK*) (Mascia et al. 2010). Relative expression for genes of interest in each sample was calculated as $2^{-\Delta Cq}$, and normalized by the geometric average of $2^{-\Delta Cq}$ values for three reference genes per sample.

Statistical analysis

Statistical analysis of real-time PCR data was performed with the R software version 3.0.1 (R Development Core Team 2011). After testing for normality (Shapiro–Wilk) and homoscedasticity (Fligner–Killeen), significant differences for gene expression analysis were determined by one-way ANOVA. When the overall *p* value was lower than 0.05, the Tukey post-hoc test was performed to correct for multiple comparison at a significance level of $p < 0.05$.

Results

In the experimental set-up, healthy control plants were neither infected with CMV, nor treated with the T22 strain (PA in figures). A set of control plants was treated with T22 alone (PB) or infected with CMV alone (PC) to obtain information on the interaction between the fungus or between CMV and the tomato plant, respectively. Whether the T22 strain can be used in a preventive way or as a treatment, it was co-inoculated before (PD), simultaneously (PE) or after (PF) the CMV infection. Plant parameters were screened in one-month-old (14 days after CMV infection) and five-months-old plants (flowering and fruits ripening period).

Phenotypical observations

Representative tomato plants grown under different conditions after five months of observation are depicted in Fig. 1. According to a preliminary evaluation of T22-induced growth promotion effect and its influence on viral disease (data not shown), plants treated with T22 only (PB) showed an improvement in growth at both time points (one and five months) as compared to the control plants (PA). This was observed in shoots (Fig. 1a) and roots (Fig. 1c) that increased both in number and in thickness. The

augmentation in root thickness was observed in all T22-treated one-month-old plants. On the other hand, a higher number in roots was observed for T22-treated five-months-old plants (Fig. 2c) in comparison to the healthy control plants (PA), above all in PB or in plants treated with T22 after the CMV inoculation (PF). A difference in shoot shape was notable in five-months-old plants treated with T22 having a more stretched appearance whereas the control plants illustrated a richer, more condensed form. Plants inoculated with CMV alone (PC) showed thinner roots (Fig. 1c), and stunting, mild mosaic in younger leaves, and chlorosis in the leaves of five-months-old plants (Fig. 1a). These plants did not survive one month further, showing the typical necrosis induced by CMV-Fny on tomato. Among the plants inoculated with CMV and also treated with T22, those that were first inoculated with CMV and then treated with T22 (PF) had a green leaf colour similar to that of control plants (PA), while for the other two co-inoculation treatments (PD and PE) plants showed a light yellowing of leaf tissues (Fig. 1a). Tomato fruits were harvested from five-months-old plants. They showed a greater size when the plants were treated only with T22 (PB) and, on the contrary, a reduction in size when inoculated with only CMV (PC) (Fig. 1b). In this last case, the fruits also showed several chlorotic spots, compared to the control plant (PA) and a delayed ripening. Finally, tomatoes from plants treated with T22 and inoculated with CMV showed a mild reduction in size and a loss of their typical red colour, except tomatoes harvested from plants first inoculated with CMV and then treated with T22 (PF), that were similar to the controls (PA) (Fig. 1b).

Histochemical detection of $O_2^{\bullet-}$ and H_2O_2

The results of $O_2^{\bullet-}$ and H_2O_2 staining in leaf discs derived from one-month-old plants are shown in Fig. 2. For all experimental conditions, the water controls resulted negative to the stain-specific detection. Plants treated with only T22 (PB) showed a mild increase in both $O_2^{\bullet-}$ (staining with NBT) and H_2O_2 (using DAB) as compared to the healthy control plants. However, the plants inoculated with only CMV (PC) demonstrated a strong augmentation in NBT and DAB staining, and thus in $O_2^{\bullet-}$ and H_2O_2 content, respectively. A mild augmentation of both compounds

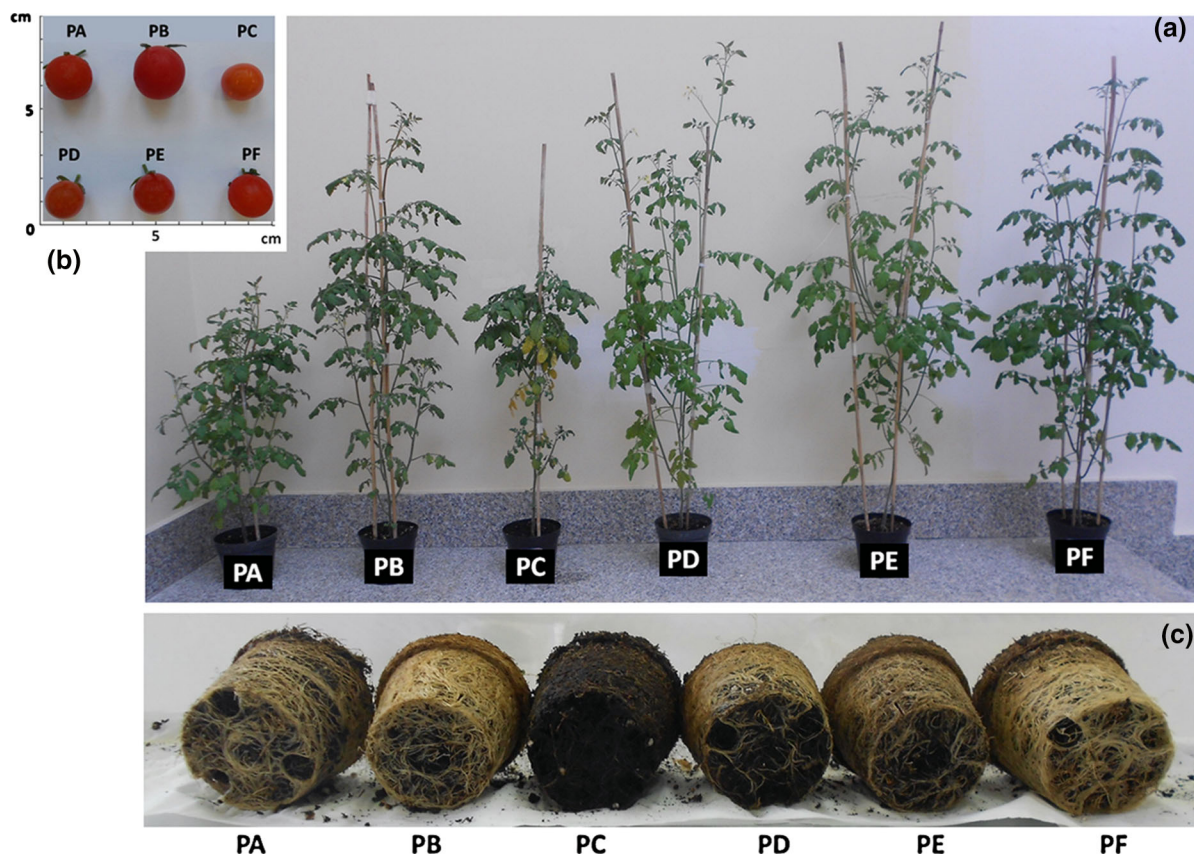


Fig. 1 Five-month-old tomato plants. In each panel, a representative plant is shown. **a** Entire plants. **b** Fruits. **c** Roots. *PA* healthy control tomato plant, *PB* plant treated with only T22, *PC* plant inoculated with CMV, *PD*: plant treated with T22 and, a

week later, inoculated with CMV. *PE* plant simultaneously treated and inoculated with T22 and CMV, *PF* plant first inoculated with CsMV and, a week later, treated with T22

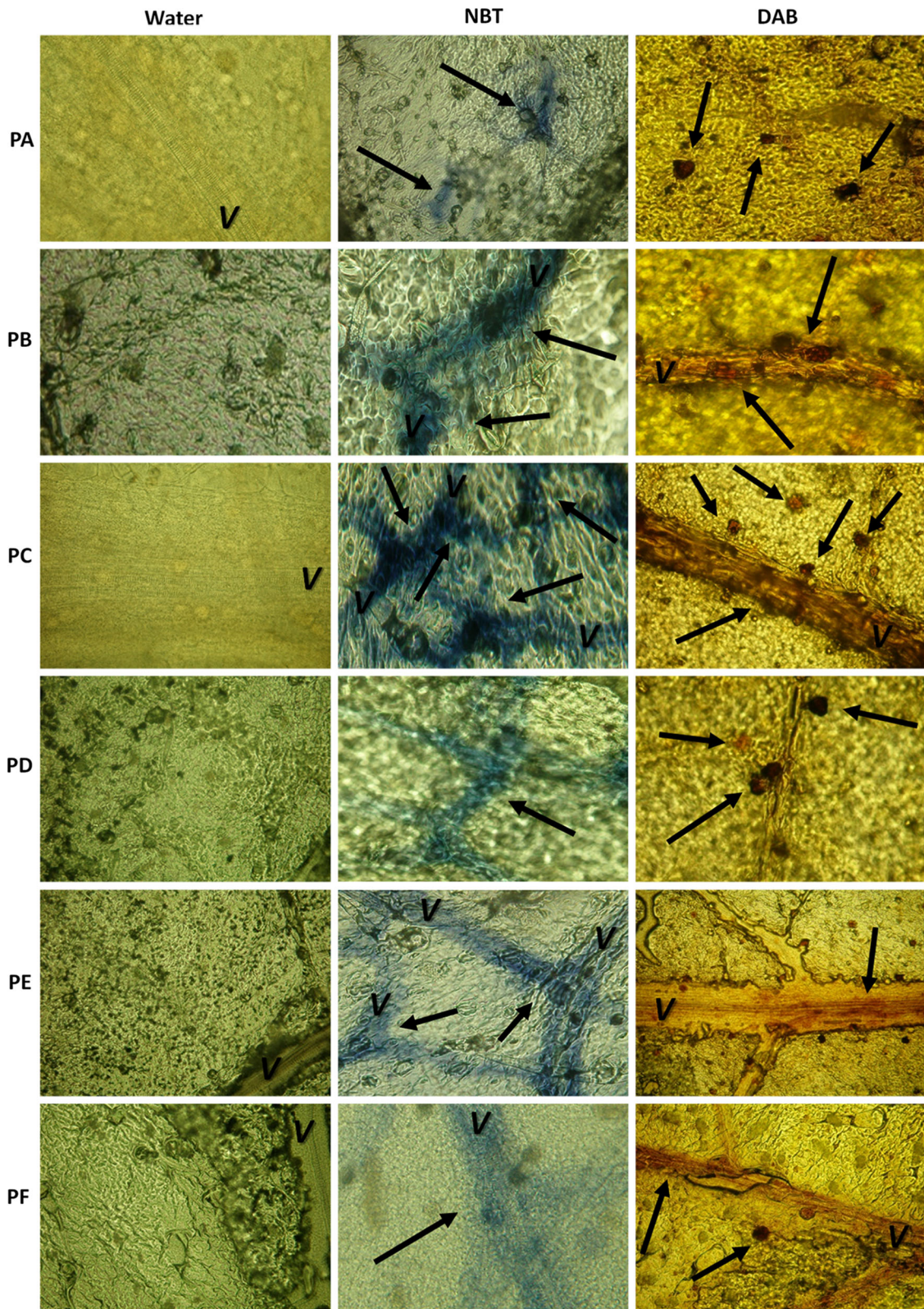
in T22-treated and CMV-inoculated plants was found, compared to the healthy controls. This was mostly visible regarding $O_2^{\bullet -}$ content when T22 and CMV were not simultaneously given (*PD* and *PF*). Interestingly, all staining was mainly confined to leaf nerves, but some precipitates of H_2O_2 accumulation were also detected outside of them, in particular for plants first treated with T22 and then inoculated with CMV (*PD*).

Verification of the presence of CMV in tomato seedlings

RT-PCR analysis from nucleic acid extracts of leaves infected systemically, collected 14 days post-inoculation with CMV, showed the presence of the CMV-Fny RNA-dependent RNA polymerase (*RdRp*) gene (513 bp) in one-month-old plants (Fig. 3). Noteworthy was that in the five-months-old plants only those

inoculated with CMV alone revealed the presence of the *RdRp* gene (Fig. 3). Thus, after five months the *RdRp* gene of CMV was not found in plants treated with T22 and inoculated with CMV. Control plants, as well as the plants only exposed to T22 did not show the presence of the *RdRp* gene.

Fig. 2 Histochemical staining of $O_2^{\bullet -}$ and H_2O_2 in tomato leaf discs (Micrograph at $\times 20$ magnification). The first column is the water control to exclude background noise. In the second column, the staining of $O_2^{\bullet -}$ using NBT is shown, while the third column represents the staining of H_2O_2 using DAB. *PA* healthy 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 inoculated with CMV and, a week later, treated with T22. In the presence of $O_2^{\bullet -}$ or H_2O_2 , deposits by NBT or oxidized DAB inside leaf veins and/or in the treated tissues are indicated by black arrows. V vein



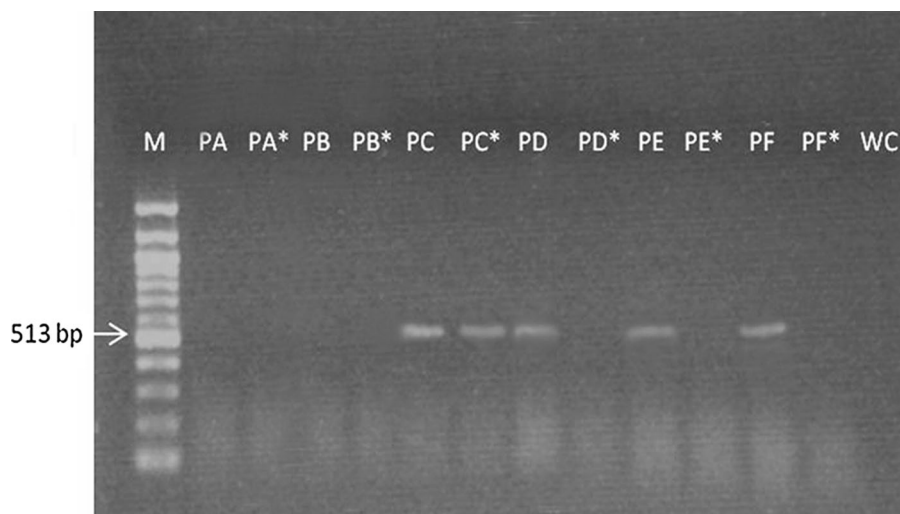


Fig. 3 Detection of DNA fragment (513 bp) of the CMV *RdRp* gene in all groups of tomato plants by RT-PCR. M 100 bp DNA Ladder (BioLabs); PA and PA* healthy control tomato plants at one and five months, respectively; PB and PB* plants treated with only T22 at one and five months, respectively; PC and PC* plants treated with CMV at one and five months, respectively;

PD and PD* plants treated with T22 and, a week later, inoculated with CMV at one and five months, respectively; PE and PE* plants simultaneously treated and inoculated with T22 and CMV at one and five months, respectively; PF and PF*: plants inoculated with CMV and, a week later, treated with T22 at one and five months, respectively; WC water control

Quantitative-PCR analysis

To evaluate the mechanisms involved in the action of T22 in CMV-infected tomato plants, transcript levels of the genes implicated in plant defense were analysed. More specifically, the expression of the genes encoding for antioxidant enzymes such as Cu/Zn superoxide dismutase (*Cu/Zn-SOD*), Mn superoxide dismutase (*Mn-SOD*), catalase (*CAT*), ascorbate peroxidase (*APX*) and the pathogenesis-related coronatine insensitive 1 (*Coi-1*) were considered. Gene expression was analyzed in leaves of one- and five-months-old tomato plants (Fig. 4).

When plants were exposed to T22 or CMV alone, no significant difference in mRNA transcript levels was observed as compared to healthy control plants. Comparing the transcript abundance of the antioxidative genes in co-inoculated plants, it becomes clear that in one-month-old plants the expression of *Cu/Zn-SOD* (O_2^- -scavenging) and *APX* (H_2O_2 -scavenging) is affected (Figs. 4a, d). This behaviour shifts towards *Mn-SOD* and *CAT*, for $O_2^{\bullet-}$ and H_2O_2 -scavenging, respectively, for five-months-old plants (Figs. 4b, c). Overall, transcript levels of these antioxidative genes were significantly elevated in plants simultaneously treated with the T22 and inoculated with CMV (PE), compared to control plants. Also treatment with T22

prior to CMV infection (PD) resulted in an increase in *Cu/Zn-SOD* and *APX* transcript levels at one and five months of plant age, respectively (Fig. 4a). Plants obtained from T22 inoculation after CMV infection (PF) showed no significant difference with respect to the control plants. No significant differences were observed concerning the pathogenesis-related *Coi-1* gene (Fig. 4e).

Discussion

Trichoderma harzianum strain T-22 (T22) is particularly important for agronomic purposes, as it is able to colonize the roots of most plant species across a wide range of soil types, with beneficial effects for plant growth and disease resistance (Tucci et al. 2011; Sofó et al. 2004). Furthermore, it has been successfully used for the biological control of many plant pathogens through chemotrophic mycoparasitic interactions with fungal or bacterial organisms as a target (Vitale et al. 2012; Sofó et al. 2004). On the other hand, to date, studies concerning the use of T22 to control plant viral diseases are unknown.

In the present study T22 showed the ability to control CMV infection on tomato plants by modulating the viral symptoms during the entire life cycle of

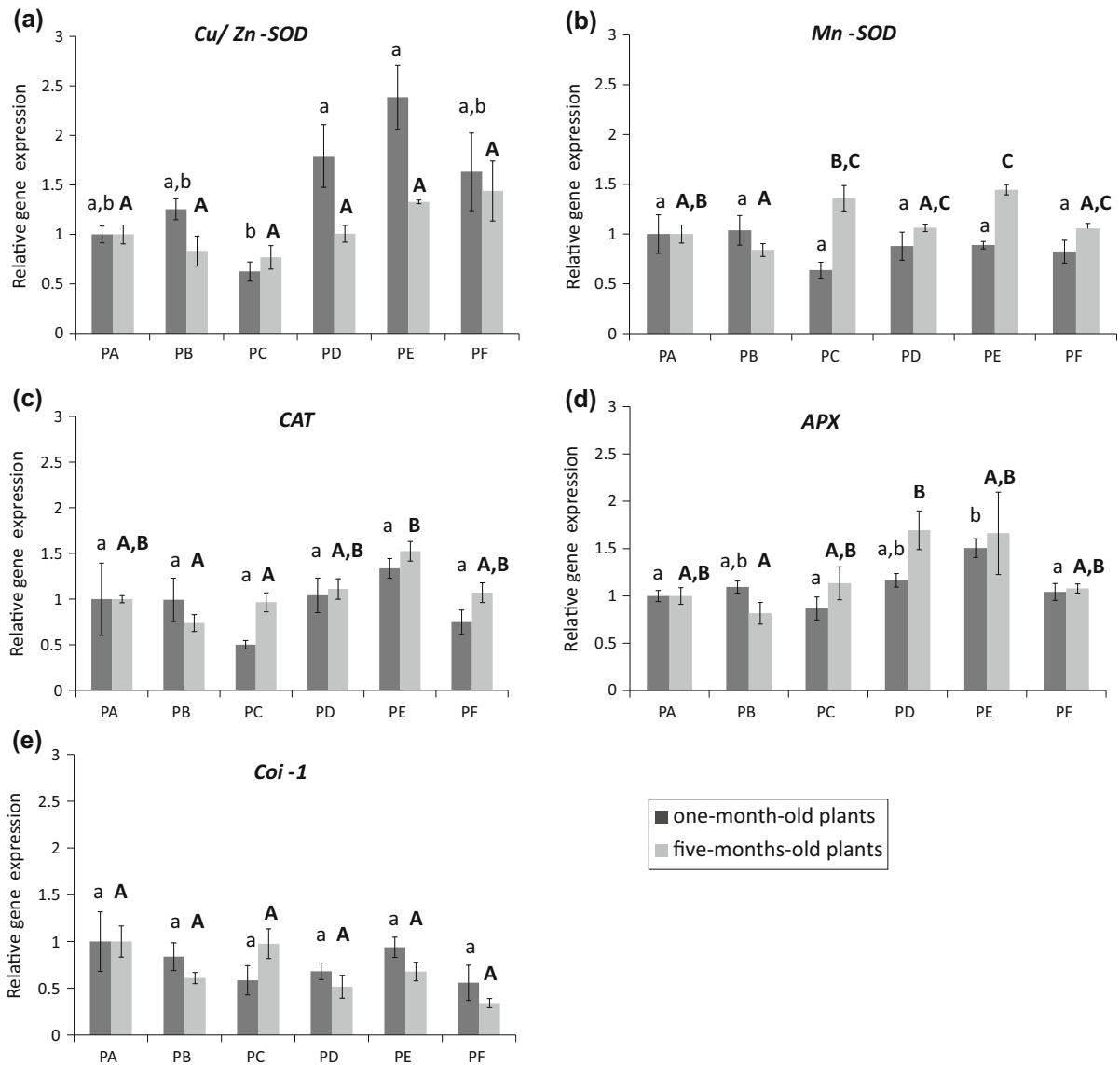


Fig. 4 Gene expression analysis in one- and five-month-old tomato leaves. **a** Copper-Zinc superoxide dismutase (*Cu/Zn-SOD*), **b** Manganese superoxide dismutase (*Mn-SOD*), **c** catalase (*CAT*), **d** ascorbate peroxidase (*APX*), **e** coronatine insensitive 1 (*Coi-1*). *PA* healthy control tomato 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 inoculated with CMV and, a week later, treated with T22. Mean values ($n = 4$) \pm SD with different letters (small letters and capital, bold letters for one- and five-months-old plant,

respectively) indicating statistical significant differences between conditions, according to one-way ANOVA combined with Tukey post-hoc test at $p \leq 0.05$. Statistical outcome for one month: *Cu/Zn-SOD* ($F_{5,18} = 5.2423$; $p = 0.00487$), *MnSOD* ($F_{5,18} = 1.167$; $p = 0.3675$), *CAT* ($F_{5,18} = 2.1175$; $p = 0.116$), *APX* ($F_{5,18} = 5.5102$; $p = 0.003882$), *Coi-1* ($F_{5,18} = 1.3142$; $p = 0.3073$). Statistical outcome for five-months: *Cu/Zn-SOD* ($F_{5,18} = 3.2483$; $p = 0.04386$), *MnSOD* ($F_{5,18} = 8.7846$; $p = 0.001056$), *CAT* ($F_{5,18} = 6.1314$; $p = 0.004808$), *APX* ($F_{5,18} = 3.5026$; $p = 0.03492$), *Coi-1* ($F_{5,18} = 3.5304$; $p = 0.03407$)

the plants (Fig. 1), and also by inhibiting the presence of the *RdRp* gene in five-months-old plants (Fig. 3). It was found that plants treated with T22 and inoculated

with CMV did not show the typical symptoms induced by CMV-Fny on tomato, such as stunting or necrosis, during flowering and fruits ripening (Fig. 1). In

contrast to plants only infected with CMV, all those treated with T22 showed, in appearance, an improvement in plant growth, in shoots as well as in roots, confirming the potential beneficial effect induced by this fungus on different lines of tomato, as reported by Tucci et al. (2011).

Luo et al. (2010) demonstrated that trichokonins, antimicrobial peptaibols isolated from *Trichoderma pseudokoningii* SMF2, can induce tobacco systemic resistance against *Tobacco mosaic virus* (TMV) via the activation of multiple plant defense pathways based on the involvement of reactive oxygen intermediate (ROS)-mediated signaling pathways, and of salicylic acid (SA)-, ethylene (Et)-, jasmonic acid (JA)-mediated defense pathway marker genes. The results of the present study support the hypothesis of an involvement of ROS in plant defense against a viral disease when *Trichoderma*, and not only its peptaibols, is applied. Indeed, plants only infected with CMV (PC) showed a high $O_2^{\bullet-}$ and H_2O_2 content when systemically infected leaves were stained with NBT and DAB, respectively. On the contrary, the accumulation of both compounds drastically decreased when T22 was used (Fig. 2), indicating that ROS are implicated in tomato defense mechanisms upon CMV infection.

As reported by Whitham et al. (2006), virus replication and movement, and the development of symptoms, as well as plant defense responses, depend on specific host plant-virus molecular interactions. In particular, Inaba et al. (2011) demonstrated that CMV is able to induce necrosis in the host plant through a specific binding established between protein 2b of CMV and a host factor essential to promote the cellular H_2O_2 scavenging. We found that plants inoculated with CMV alone (PC), after 14 days from the infection, that is generally the acute phase in terms of maximum symptoms expression, showed a strong augmentation in $O_2^{\bullet-}$ and H_2O_2 content (Fig. 2). Therefore, it can be hypothesized that the interaction between CMV and tomato plants results in an oxidative burst and hence elevated ROS production, which becomes toxic for the plants (Sharma et al. 2012). Indeed, at five months of age, these plants showed necrosis in all tissues, which was also observed by Inaba et al. (2011). On the other hand, during the CMV-tomato-T22 interaction, the biochemical three-way cross-talk was able to change the

ROS signal. This indicated that ROS are implicated as secondary messengers of the host's defense responses against the viral pathogen, mediated by the fungal biocontrol agent, as reported by Orozco-Cardenas et al. (2001). This is confirmed by the gene expression levels of the antioxidant enzymes in our study. Indeed, plants only infected by CMV (PC) always showed significantly different transcript levels of *SOD*, *CAT* and *APX* with respect to those found in plants where T22 was used (PD, PE and PF) (Fig. 4). *SOD*, *APX*, and *CAT* have to act together in order to avoid toxic ROS levels in the tissues (Apel and Hirt 2004). Enzymatic ROS scavenging via *SOD* activity represents the first line of defense against ROS, by the dismutation of $O_2^{\bullet-}$ to H_2O_2 . Increased activity of *SOD* is often correlated with increased tolerance of the plant against environmental stresses and has been reported to result in enhanced oxidative stress tolerance in plants (Kunkel and Brooks 2002). The significantly higher relative expressions in plants first treated with T22 and then inoculated with CMV (PD), and also in plants simultaneously treated and inoculated (PE), indicates that the enzyme Cu/Zn-SOD is properly functioning and scavenging $O_2^{\bullet-}$ in these plants (Fig. 4a). Therefore, this result confirms that T22 has a beneficial effect on the damage caused by CMV. However, the expression of *Mn-SOD* in five-month-old tomato plants was also elevated in CMV inoculated plants (PC) (Figs. 4a, b), which might be due to early induced senescence in these plants upon viral infection and hence mitochondria-related oxidative stress (Sharma et al. 2012).

Since long time, it is recognized that *CAT*, detoxifying H_2O_2 , is indispensable for plant oxidative biotic stress tolerance (Willekens et al. 1997). The reaction rate of *CATs* is high though the affinity for H_2O_2 is much higher in *APXs*. Therefore, *CATs* are more involved in detoxification of H_2O_2 than the regulation as a signaling molecule (Cuypers et al. 2011). This explains the absence of significant differences in the relative gene expression of *CAT*, in contrast to those of *APX*, in one-old-month plants (Figs. 4c, d). Considering that gene expression levels in plants first inoculated with CMV and then treated with T22 (PF) were never significantly different to that of the healthy and T22-treated control plants, this is an important indication on the fact that this combination could guarantee the best control against CMV.

Resistance mechanisms comparable to the hypersensitive response (HR), systematic acquired resistance (SAR), and induced systematic resistance (ISR), the latter elicited by a JA/Et-dependent pathways, are the base of the plant defense induced by *Trichoderma* spp. against numerous pathogens (Harman et al. 2004). Hermosa et al. (2012) assert that the expression of defense-related genes of the JA/Et and/or SA pathways may overlap because of the dynamics in the *Trichoderma*–plant cross-talk. The latter depends on the colonized plant and its developmental stage, the *Trichoderma* strain and its concentration and timing used for the plant-fungus interaction. *Trichoderma asperellum* used for the colonization of *Arabidopsis* roots produced a clear ISR (Yoshioka et al. 2012). The same species was exploited against CMV by using barley grain inoculum or culture filtrate. In the first case, *Trichoderma asperellum* SKT-1 induced SAR, while ISR was elicited in the second case (Elsharkawy et al. 2013). *Coi-1* gene is considered a marker for defense responses mediated by the phytohormone JA (Xie et al. 1998; Kazan and Manners 2008), and thus ISR, while it has an inhibitory effect on the phytohormone SA and thus SAR (Kunkel and Brooks 2002). In fact, *Coi-1* negatively modulated the activation of SAR through SA signaling in *Arabidopsis thaliana* infected by the strain Y of CMV (Takahashi et al. 2004). Studies on jasmonate-insensitive *Coi-1* mutants of tomato and *Arabidopsis* showed that COI1 protein accomplishes a similar function in JA signal transduction in tomato and *Arabidopsis* (Li et al. 2004). In the present study, the *Coi-1* gene was analyzed, but no significant differences in its relative expression levels were observed (Fig. 4e). However, *Coi-1* expression tended to be suppressed in all plants treated with T22, as compared to CMV-infected (PC) plants, at five months of age. This could indicate a SAR-related response but further investigation is required to confirm these findings.

In conclusion, data produced in this work demonstrate that *Trichoderma harzianum* T-22 stimulates the induction of defense responses against CMV-Fny in *Solanum lycopersicum* var. *cerasiforme* by the clear involvement of ROS. Furthermore, this study improves the knowledge on the molecular and biochemical aspects of the plant-virus-biocontrol agent interactions in combination with the dynamics of application. In this way, a new system based on the use of T22 as a microbial antagonist could be made

available for the protection of tomato against CMV disease, which can be extended to other plant species.

Acknowledgments This work was supported by a grant from University of Basilicata, Potenza, Italy. We thank Prof. Ippolito Camele (University of Basilicata) for microscopy analyses in the histochemical experiments.

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