

Antonella Vastola *Editor*

The Sustainability of Agro-Food and Natural Resource Systems in the Mediterranean Basin



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Sustainable Agricultural Practices in Disease Defence of Traditional Crops in Southern Italy: The Case Study of Tomato Cherry Protected by *Trichoderma harzianum* T-22 Against *Cucumber Mosaic Virus* (CMV)

Antonella Vitti, Adriano Sofo, Antonio Scopa, and Maria Nuzzaci

Abstract Nowadays, crop production is at risk due to global warming, especially in Mediterranean areas where the increase of air temperature and/or reduction of precipitation is relevant. Climate changes that are occurring can severely prejudice plant defensive mechanisms during host-pathogen interactions by modifying growth and physiology of the host plant. In particular, viral diseases cause serious economic losses destroying crops and reducing agronomic productivity, and, in some cases such as tomato crops, they become the limiting factor production of both open field and under greenhouse cultivation systems. This is because plant viruses are obligate parasites and require living tissue for their multiplication and spread. Therefore, they are able to interfere with plant metabolism and compete for host plant resources, so determining a decrease of plant growth and productivity. Severe outbreaks of *Cucumber mosaic virus* (CMV) and other viruses caused disruption of tomato plants in the Mediterranean region and in Southern Italy since the 1970s. In such a scenario, it is necessary to introduce new strategies for controlling plant pathogens and parasites in order to help maintain ecosystems and to boost sustainable agriculture. The aim of this work is to give an up-to-date overview on the recent breakthroughs in the use of microorganisms on plants for improving crop yields, quality and plant tolerance against pathogens. In particular, here we report a case study regarding an innovative strategy to control a viral disease (CMV) in tomato, based on the use of rhizosphere microorganism (*Trichoderma harzianum*, strain T-22) as an antagonist biocontrol agent (BCA).

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1 Introduction

In the Mediterranean basin, horticultural crops have a great economic relevance. If we think of the Mediterranean diet, which is considered as one of the healthiest amongst world cuisines, above all since November 2010, when it was inscribed on the representative list of the intangible cultural heritage of humanity of UNESCO, tomato (*Solanum lycopersicum*) is certainly the vegetable most widely consumed. In Italy, in particular in the South, tomato is not only the first vegetable employed for fresh consumption, but it also represents the principal ingredient of many dishes, and it is above all used cooked to prepare sauces. The importance of tomato consists in its nutraceutical properties, due to the presence of an antioxidant substances mixture, such as lycopene, ascorbic acid, phenolic compounds, flavonoids and vitamin E. For this reason, nowadays tomato is cultivated both in open field and under greenhouse conditions in order to be always available for both fresh consumption and industrial processing.

Unfortunately, crop production is at risk due to global warming, especially in areas where the increase of air temperature and/or reduction of precipitation is relevant. In addition, climate changes can prejudice plant defensive mechanisms and increase the risk of illness, through growth and physiology alteration of the host plant and also by modifying host-pathogen interactions. In particular, viral diseases cause serious economic losses destroying crops and reducing agronomic productivity. In many Mediterranean coastal areas, several viral infections have become the limiting factor in the tomato production of both open field and under greenhouse cultivation systems. For example, in Italy, Spain, Portugal and Greece, the cultivations are at risk due to *Tomato spotted wilt virus* (TSWV) infections (Pappu et al. 2009). Another important example is *Tomato yellow leaf curl virus* (TYLCV), which caused serious economic problems in the eastern Mediterranean basin in the 1970s, and it is still now a threat (Lapidot et al. 2014). In late summer 2000, more than 30 ha of Greek tomato greenhouses (Avgelis et al. 2001) were affected and the disease incidence by TYLCV, in 2001, in most cases, was 80–90 %, or even 100 % (Dovas et al. 2002). In the same the 1970s, *Cucumber mosaic virus* (CMV) severe outbreaks caused disruption and death of tomato plants in the Mediterranean region (Gallitelli et al. 1991). Tomato necrosis epidemic occurred in the eastern coastal area of Spain in the late 1980s and early 1990s (García-Arenal et al. 2000). During this period, in Southern Italy (Puglia, Basilicata and Campania regions), some high-quality varieties of tomato, i.e. San Marzano, were severely affected by the strong CMV epidemic (Valanzuolo et al. 1999).

Indeed, plant viruses are obligate parasites because they require living tissue for their multiplication and spread, interfering with plant metabolism and/or competing for host plant resources, and all this is translated as decreasing of plant growth and productivity. The ability of viruses to significantly interfere with physiological processes of plants is closely related to a range of symptoms caused by an abnormal growth, as stunting, galls, enations and tissue distortions. In particular, CMV is the plant virus with the largest host range of all RNA viruses; therefore, its spreading on crop plants may cause serious economic damages. It infects more than 1,200 plant

species in 100 families (Edwardson and Christie 1991) and has been widely studied because it represents an interesting model from a physico-chemical point of view, as it causes a wide range of symptoms, especially yellow mottling, distortion and plant stunting (Nuzzaci et al. 2009; Whitham et al. 2006).

In such a scenario, the present work contributed to elucidate the importance in the use of sustainable agricultural practices in disease defence. In addition, here we report a case study regarding an innovative strategy to control a viral disease (CMV) in tomato cherry, based on the use of rhizosphere microorganism (*Trichoderma harzianum*, strain T-22) as an antagonist biocontrol agent (BCA).

2 Sustainable Agricultural Practices in Disease Defence

During the last decade, the studies on alternative environmental friendly technologies have received a strong impulse and have proposed a wide range of options, including agronomical, physical and biological control means. Recently, it was growing the idea that the plants have enormous self-defence potentiality, and this would allow a natural disease control with positive effects on environmental and human health safeguard (Sofa et al. 2014).

Many factors, both biotic (pathogens, insects, nematodes) and abiotic (e.g. wounds, pollutants, thermal, water and nutritional imbalances, environmental contaminants) are causes of plant stress. Plants can react to these stressors through a series of constitutive and/or inductive mechanisms which result in the elimination or the limitation of the negative effects induced by the adverse factors. The studies on these biochemical mechanisms allow to individuate control strategies against plant pathogens and parasites, based on the exploitation of the natural mechanisms of plant defence. One of this type of mechanism, already documented by Ross (1961), is known as systemic acquired resistance (SAR). It is effective against a wide range of pathogens and its action differs in relation to the inducer agent. Actually, the SAR represents a valid opportunity in plant natural protection, and, therefore, the research activities are oriented to the use of biocontrol agents as inducers of SAR in agronomically important species against some of their most severe pathogens (Sofa et al. 2014). In fact, research data accumulated in the past few years have produced a completely novel understanding of the way by which bacteria and fungi interact not only with other microbes but especially with plants and soil components. This has opened an avenue of new applications, both in agriculture and biotechnology, that exploit the ability of some microorganisms to change plant metabolism and resistance to biotic and abiotic stresses (Woo et al. 2006). Generally, as a response to diseases, plants may compensate with a broad range of cellular processes by up- or down-regulating certain genes; changing the levels of substances implicated in plant defence pathway; increasing the levels of reactive oxygen species (ROS); activating specific transcription factors, defence-regulated genes and heat shock proteins; and enhancing the transport of macromolecules, enzymes and phytohormones involved in defence signalling pathways [e.g. salicylic acid (SA); jasmonic acid (JA); ethylene (ET); auxins, such as indole-3-

acetic acid (IAA); cytokinins (CKs); abscisic acid (ABA); gibberellic acid (GA)] (Bari and Jones 2009; Vitti et al. 2013). On the other hand, all physiological process changes of plants as response to pathogens negatively affect the crops' yield with a loss of billions of euros each year not only for direct productivity decrease but also for the consequent managing of the pests. In addition, the use of traditional methods such as chemical pesticides, herbicides or fertilizer is not an eco-friendly approach, and their continued employment resulted on contamination of water, atmosphere pollution and the release of harmful residues in soils (Naher et al. 2014).

A safe method to reduce plant disease incidence without collateral damages to the environment and to human health induced by synthetic chemicals is the biological control (Tucci et al. 2011). In such a way, it is possible to manage pests by means of a sustainable approach where biocontrol agents can be used either alone or with other chemicals in an integrated practice of disease defence, according to European legislation Directives establish. The use of microorganisms for controlling plant pathogens has been shown to be very efficacious for some fungi of the genus *Glomus*, *Streptomyces*, *Trichoderma* and some species of bacteria (e.g., *Agrobacterium radiobacter* and *Bacillus subtilis*). In particular, some of these fungi interact with other fungi in a mechanism called mycoparasitism, wherein one fungus directly kills and obtains nutrients from other fungi. Mycoparasitism is one of the most important biocontrol mechanisms of *Trichoderma* spp. (Mukherjee 2011), which is considered the most versatile amongst all biocontrol agents and, for this reason, has long been used for managing plant pathogenic fungi (Vinale et al. 2009; Weindling 1934; Wells 1988). It was demonstrated that some fungal diseases can be also prevented when plants are treated with the conidial suspensions of *Trichoderma* spp. (Harman et al. 2004a). Fungi belonging to the genus *Trichoderma* are used as biocontrol agents to antagonize plant pathogens through a series of mechanisms including, in addition to mycoparasitism, competition for nutrients and space, fungistasis, antibiosis and/or modification of the rhizosphere (Benítez et al. 2004). *Trichoderma* spp. are some of the most abundant fungi found in many soil types and are able to colonise plant roots and plant debris (Harman et al. 2004a). They are agriculturally important also for their beneficial effects on plant growth and development and for their capability to induce plant defence responses against pathogens, damage provoked by insects and abiotic stress (Yedidia et al. 1999; Harman et al. 2004a; Woo and Lorito 2006). For this reason, more than 60 % of all registered products used for plant disease control are *Trichoderma*-based and 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* (here called T22) represents the active ingredient of registered products widely employed in plant disease control. It is known that T22, by working as a deterrent, protects the roots from the assault of pathogens fungi (e.g. *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia*). Establishing itself in the rhizosphere, T22 can grow on the root system, along which it establishes a barrier against pathogens. The action of T22 is not to produce something toxic to the pathogen but to induce the plant to change its physiology and metabolism to ameliorate its resistance to that disease (Harman et al. 2008). It was

demonstrated that T22 improves growth in maize plants, increasing root formation (size and area of main and secondary roots) and, at the same time, rising crop yields, drought tolerance and resistance to compacted soils (Harman 2000; Harman et al. 2004b). This improvement in growth was probably due to direct effects on plants because of a better solubilization of soil nutrients or by a direct enhancing plant uptake of nutrients linked to the presence of T22 in the agroecosystems (Yedidia et al. 2001). The beneficial effects of T22 application depend on the treated plant genotype, as recently demonstrated by Tucci et al. (2011) on tomato plants.

3 The Case Study of Tomato Cherry Protected by *Trichoderma harzianum*T-22 Against CMV

In the context of plant defence by biotic stresses, understanding biochemical and molecular mechanisms deriving from the host-pathogen-*Trichoderma* interaction is without doubt essential for investigating the dynamics of infectious processes. This knowledge can be very useful for the development of new approaches for controlling phytopathogens, particularly viruses, against which chemical treatments have no effect (Vitti et al. 2015b). Thanks to recent studies, new strategies have been based on the use of peptaibols, a class of linear peptides biosynthesized by many species of *Trichoderma* (Daniel and Filho 2007). For example, it was 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 defence pathways based on an elicitor-like cellular response: production enhanced in tobacco plants of superoxide anion radical and peroxide; production enhanced of enzymes involved tobacco resistance, as peroxidase (POD); up-regulation of antioxidative enzyme genes, known to be associated with the ROS intermediate-mediated signalling pathway; and of SA-, ET- and JA-mediated defence pathway marker genes (Luo et al. 2010). This finding implies the antiviral potential of peptaibols, supporting the hypothesis to using them as biocontrol antiviral agents. Therefore, *Trichoderma* spp., already used as BCAs against bacterial (Segarra et al. 2009) and fungal phytopathogens (Vinale et al. 2009; Akrami et al. 2011), it was hypothesized could be advantageously used also in the control of virus diseases. *Trichoderma* spp. and/or their secondary metabolites were able to induce resistance mechanisms, similar to the hypersensitive response (HR), SAR and induced systemic resistance (ISR) in plants (Benítez et al. 2004; Harman et al. 2004a), regulated through a complex network of signal transduction pathways involving not only the above-mentioned molecules, such as ROS, SA, JA and ET but also the crosstalk between them (Kunkel and Brooks 2002) and the so-called pathogenesis-related (PR) genes, a series of marker genes for the activation of SA, JA and ET signalling, involved in these defence transduction pathways (Bouchez et al. 2007). At this regard, Hermosa

et al. (2012) assert that the expression of defence-related genes of the JA/ET and/or SA pathways may overlap just because of the dynamics in the *Trichoderma*-plant crosstalk.

To date, the effects of *Trichoderma* spp. in the induction of plant defence against CMV were poorly known. Only studies conducted by Elsharkawy et al. (2013) demonstrated that *Arabidopsis* plants were exploited against CMV by using *Trichoderma asperellum* SKT-1. In particular, when the researchers used barley grain inoculum, the fungus induced SAR, while ISR was elicited when *T. asperellum* was utilized as culture filtrate. On the other hand, the biochemical and molecular mechanism involved in this kind of three-way crosstalk between the plant, virus and antagonist agent has still to be well elucidated.

In such a scenario, our work represents the starting point to improve the knowledge on the possible underlying mechanisms involved in plant-pathogen-antagonist interactions and, at the same time, to develop an innovative strategy against CMV infection in tomato plants, based on the activity of a biocontrol agent (Vitti et al. 2015a).

Trichoderma harzianum strain T-22 (T22) was the antagonist microorganism used in this study. It was utilized as a granule formulation (Trianum G, Koppert, Berkel en Rodenrijs, the Netherlands). *Cucumber mosaic virus* strain Fny (CMV-Fny) was propagated in tobacco plants, purified as described by Lot et al. (1972), so that the purified CMV-Fny was used to mechanically inoculate tomato plants (*Solanum lycopersicum* var. *cerasiforme*).

As shown schematically in Fig. 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 only treated with T22 (PB); plants only inoculated with CMV (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 1 week treated with T22 (PF).

During the entire cycle of plant's life, symptom observations were monitored. Fourteen days after CMV inoculation (that is when the plants were at 1 month of age) and when the plants were 5 months old, leaves were collected and used for the following analyses: histochemical staining of O_2^- and H_2O_2 in leaf discs, in order to study the involvement of ROS and total RNA extraction from leaf tissues followed by reverse transcription polymerase chain reaction (RT-PCR) analysis, for the verification of the presence of CMV in tomato seedlings, or by real-time reverse transcription PCR (qRT-PCR), in order to analyse the transcript levels of the genes implicated in plant defence, such as genes encoding for antioxidant enzymes and for pathogenesis-related protein (Vitti et al. 2015a). In addition, here we report the yield evaluation for each experimental condition, determined since plants started to produce flowers and fruits (3 months of age) and until plants were 5 months old.

T22 showed the ability to control CMV infection on tomato cherry plants by modulating the viral symptoms during the entire life cycle of the plants and also by inhibiting the presence of CMV in 5-month-old plants. Furthermore, an involvement of ROS in plant defence against a viral disease when *Trichoderma* is applied

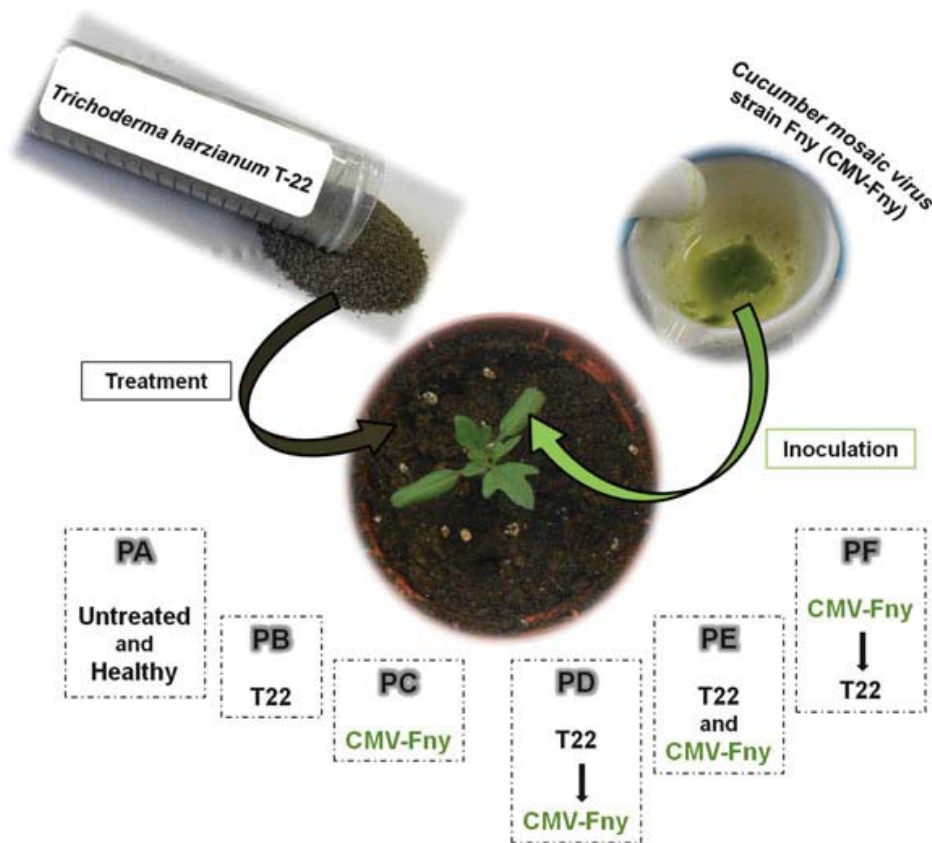


Fig. 1 Scheme of the experimental set-up. Treatments with *Trichoderma harzianum* T-22 (T22) were performed by incorporating Triam G (Koppert, Berkel en Rodenrijs, the Netherlands) granules in the substrate used for planting (750 gm^{-3}), according to the application and dose suggested by the company. Ten micrograms of purified *Cucumber mosaic virus* strain Fny (CMV-Fny) was used to mechanically inoculate tomato cherry plants at the four-leaf stage. Plants were treated with T22 and/or inoculated with CMV in order to gain the six conditions PA, PB, PC, PD, PE and PF, as reported in the text

was demonstrated. In fact, 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. Conversely, during the CMV-tomato-T22 interaction, ROS are implicated as secondary messengers of the host's defence responses against the viral pathogen, mediated by the fungal biocontrol agent. In addition, an indication on the fact that a particular combination whereby plants first inoculated with CMV and then treated with T22 could guarantee the best control against CMV has been speculated. Finally, results obtained could also indicate an SAR-related response by the tomato plants against CMV attack, but further investigation is required to confirm these findings (Vitti et al. 2015a).

Tomato fruits were harvested from bottom branch of 3-month-old plants. As reported in Fig. 2, plants treated only with T22 (PB) showed the best size fruit and also the best root development, as expected. On the contrary, the control plants inoculated with CMV alone (PC) showed the smallest fruits, with delayed ripening, accompanied by the worst root development. Plants treated with T22 and inoculated with CMV (PD, PE and PF) were similar to the controls (PA), considering both size fruit and root development.

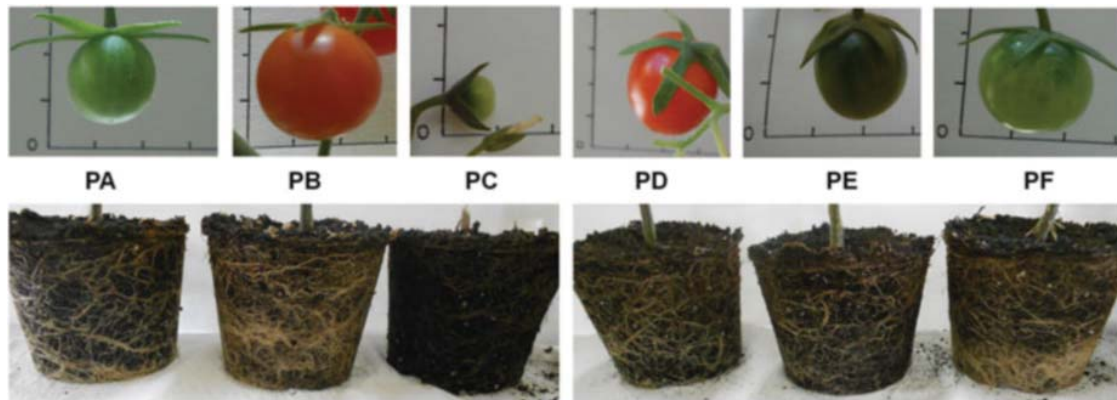


Fig. 2 Three-month-old tomato cherry plants. In each panel, a representative fruit (*above*) and roots (*below*) are shown. *PA* healthy control tomato plant; *PB* plant treated with only T22; *PC* plant inoculated with CMV; *PD* plant treated with T22 and, a week later, inoculated with CMV. *PE* plant simultaneously treated and inoculated with T22 and CMV; *PF* plant first inoculated with CMV and, a week later, treated with T22. Scale units for both abscissa and ordinate are of 1 cm

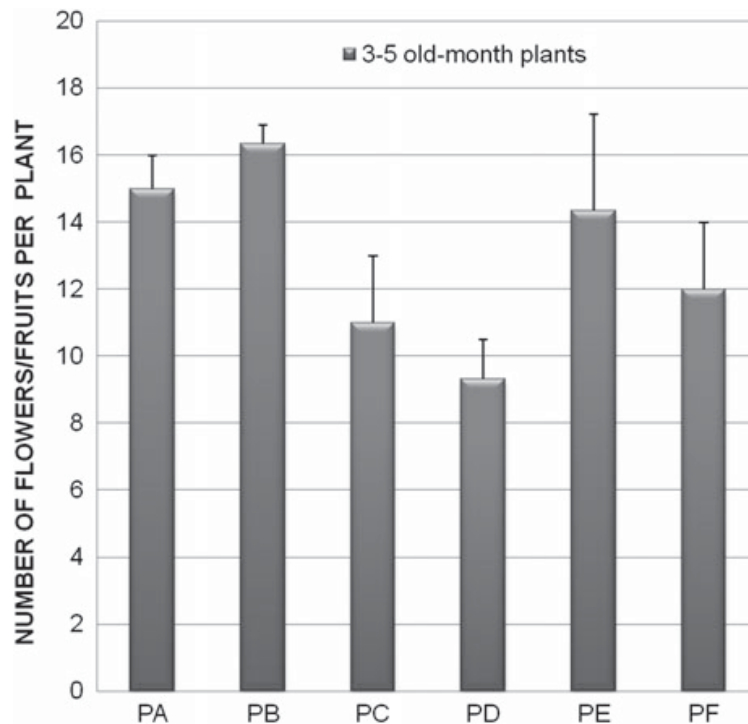


Fig. 3 Evaluation of the effect induced by T22 treatment on yield, determined as number of flowers and fruits per plant, observed from 3 to 5 months of plant's age. *PA* healthy control tomato plant; *PB* plant treated with only T22; *PC* plant inoculated with CMV; *PD* plant treated with T22 and, a 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. Each bar indicates the mean value \pm SD of 16 plants observed for each condition

As clearly showed in Fig. 3, in terms of yield, considered as production of flowers and fruits in 3–5-month-old plants, those treated only with T22 (*PB*) showed the highest values, as expected. Conversely, plants inoculated with

CMV alone (PC) not showed the lowest yield, as we expected. This is because the number of fruits was not low, but they resulted in an important reduction in size, as Fig. 2 shows, accompanied by chlorotic/necrotic spots when they were ripe, confirming the observations previously made by Vitti et al. (2015a). Instead, as it is possible to see in Fig. 3, plants treated with T22 and also inoculated with CMV, in particular in the case of co-inoculation/treatment (PE), showed an increase in yield respect to that inoculated with only CMV (PC), except for plants first treated with T22 and then inoculated with CMV (PD).

4 Conclusion

In conclusion, data produced in the case study here reported demonstrate that *Trichoderma harzianum*T-22 stimulates the induction of defence responses against CMV-Fny in *Solanum lycopersicum* var. cerasiforme, by the clear involvement of ROS, as well as an enhancement in yields and root development. Furthermore, the knowledge on the molecular and biochemical aspects of the plant-virus-biocontrol agent interactions, in combination with the dynamics of application, has been improved. 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 also extended to other plant species. Furthermore, a routine utilization of T22 in the agricultural practices in disease defence could surely bring to a reduction of the use of fertilizers and fungicides in agricultural production, with consequent benefits for the environment. Today, more than ever, this is necessary to help maintain ecosystems and to develop sustainable agriculture.

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