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Trichoderma and its secondary metabolites improve yield and quality of grapes

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ABSTRACT

Trichoderma is one of the most studied and applied fungal biocontrol agents. The benefits of these microorganisms to the plant include: suppression of pathogens, growth promotion, enhanced nutrient availability and induction of resistance. The biological activity is related to the variety of metabolites that they produce. These metabolites have been found to directly inhibit the pathogens, increase disease resistance and enhance plant growth.

In this study, we have examined the effect of two *Trichoderma* strains and their secondary metabolites on *Vitis vinifera* in terms of induction of disease resistance, plant growth promotion and increase of polyphenols or antioxidant activity in the grapes. Applications of *T. harzianum* M10 or *T. atroviride* P1, as well as their respective major secondary metabolites, harzianic acid (HA) and 6-pentyl-α-pyrone (6PP), have been conducted in greenhouse by foliar spray or drenching. The treatments suppressed the development of powdery mildew caused by *Uncinula necator*. In a field experiment, a spore suspension of *T. harzianum* strain T22 or a 6PP solution was applied until fruit harvest. The results indicated that both *T. harzianum* T22 and 6PP are able to improve crop yield and increase the total amount of polyphenols and antioxidant activity in the grapes. The effects of the isolated natural compounds were comparable with those obtained by using the living fungus.

1. Introduction

The traditional methods used to protect agricultural crops from various pathogens have been based mainly on the use of chemical pesticides. The Italian National Institute of Statistic (ISTAT) reported that in Italy the total usage of fungicides in 2010 has been 67.7 millions of kilograms, of which the 27.5% (18.6 millions of kilograms) have been used for vineyards treatments. Among the total volume of fungicides applied on grapes, about the 75% were based on sulphur active ingredients, used for the control of powdery mildew.

Beside the cost of synthetic pesticides, environmental impact and the risk for animal and human health has to be considered. Furthermore, the repeated use of such chemicals has been noted to cause the development of microbial pathogens resistant to pesticides. Consequently, in recent years there has been an increasing interest in the use of new tools based on biocontrol agents (BCAs) or their metabolites for disease control. Some of the most studied biocontrol agents are fungi belonging to the genus *Trichoderma,* used as a biopesticides and biofertilizers to protect plants and enhance vegetative growth (Harman et al., 2004; Lorito et al., 2010; Woo et al., 2014). The advantages related to the application of these fungi in agriculture include: suppression of pathogens by using a variety of mechanisms (competition, antibiosis and direct mycoparasitism), plant growth promotion and induction of disease resistance (Harman et al., 2004; Shoresh et al., 2010). One factor that contributes to the beneficial biological activity of some *Trichoderma* species is related to the wide variety of secondary metabolites that they produce (Reino et al., 2008; Sivasithamparam and Ghisalberti, 1998; Vinale et al., 2014a,b). These natural compounds may be involved in antibiosis, functioning synergistically with other compounds as inhibitors of the growth and development of pathogenic microbes (Sivasithamparam and Ghisalberti, 1998; Reino et al., 2008). In

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addition they may play a positive role in the interaction with the plant, by inducing systemic resistance and promoting plant growth (Vinale et al., 2008a,b; 2012). Today, there are many agricultural products based on *Trichoderma* that are commercially marketed all over the world (Woo et al., 2014). Formulations containing the living fungus present some limitations for their use in the field such as: unreliable biocontrol effects when locations, environmental conditions, or target crops are changed; the reduced ability of some strains to colonize different soils and plant roots at effective levels; the dose-effect response for disease control is often not directly proportional; the viability of the fungal inoculum is subjected to good storage conditions; and finally the products function effectively only if used as preventative treatments (Woo et al., 2014). However, using the compounds, naturally produced by *Trichoderma*, which are actively involved in the different mechanisms of biological control, may overcome many of the problems compared to the use of the living microorganism. The aim of this study was to investigate if the efficacy of applying living *Trichoderma* BCAs already used as an alternative to synthetic pesticides, can be improved, or substituted by single or combined treatments with the bioactive secondary metabolites produced by the same beneficial microbe. The metabolites considered in this study are 6-pentyl-α-pyrone (6PP), a food grade volatile pyrone, and harzianic acid (HA), a tetramic acid derivative with iron binding activity (Vinale et al., 2013).

2. Materials and methods

2.1. Fungal material

T. harzianum strain M10 (M10) and *T. atroviride* strain P1 (P1), were used for the *in-vivo* experiments and for the production of the secondary metabolites (microbe collection of the Department of Agricultural Sciences, Biocontrol Laboratory, University of Naples, Federico II). For the field experiment was used the highly-effective *T. harzianum* strain T22 (*Trichoderma harzianum* Rifai, anamorph ATCC® 20847™).

The fungi were maintained on Potato Dextrose Agar (PDA, HiMedia Mumbai, India) medium covered with sterilized mineral oil (Sigma Aldrich, St. Louis, MO.).

The *Trichoderma* propagules needed to conduce the *in-vivo* and *in-field* experiments, were produced by solid-state fermentation on rice bran (500 g) inoculated with the *Trichoderma* spore suspension (1×10^6 spores/ml) and incubated at 25 °C. After 7 days the spores were collected washing rice bran with sterile water.

2.2. Isolation and characterization of Trichoderma spp. secondary metabolites

The two effective *Trichoderma* strains M10 and P1 were used to produce the bioactive molecules. Mycelia were inoculated to 5 L conical flasks containing 1 L of sterile potato dextrose broth (PDB, HiMedia Mumbai, India). Stationary cultures of each strain were grown for 30 days at 25 °C. The cultures were vacuum-filtered through filter paper (Whatman No. 4, Brentford, UK), and the filtrate (2 L) was extracted exhaustively with ethyl acetate (EtOAc). Organic fractions were dried with Na_2SO_4 and the solvent evaporated in vacuum at 35 °C. The yellow oil residue obtained from P1 was subjected to flash column chromatography (50 g Si gel 0,2–0,5 mm Merck-EMD Darmstadt Germany), by eluting with a gradient of petroleum ether: EtOAc (9:1 to 4:6) to obtain 80 mg 6-pentyl-α-pyrone (6PP). Recovered fractions were analyzed by thin-layer chromatography (TLC Si gel 60 F_{254} Merck-EMD Darmstadt Germany; mobile phase: petroleum ether: EtOAc; 8:2); and fractions with similar profiles were combined. The red residue obtained from M10 was dissolved in $CHCl_3$ and extracted three-times with NaOH 2 M. Harzianic acid (HA) then precipitated with HCl 2 M. The solid was recovered (135 mg), solubilised and subjected to RP-18 vacuum chromatography (20 g Si gel RP-18, 40–63 μm Sigma Aldrich, St. Louis, MO), eluting with a gradient of methanol (MeOH):H_2O:CH_3CN (0,5:9:0,5 to 10:0:0). After separation approximately 45 mg of pure HA was collected. The compounds were detected on TLC using UV radiation (254 or 366 nm) and/or by dipping the plates in a 5% (w/v) ethanol solution of 2 M H_2SO_4 and heating at 110 °C for 10 min. Purified metabolites were characterized by NMR analysis recorded with a Bruker AM 400 spectrometer operating at 400 (^{1}H) MHz using residual and deuterated solvent peaks as a reference standard and/or by LC/MS qTOF analysis recorded with an Agilent system (6400).

All the chemicals used are from Sigma Aldrich St. Louis, MO, unless specified differently.

2.3. In vivo powdery mildew control assay on Vitis vinifera

One year old plants of *Vitis vinifera* cv. Sangiovese were planted in pots (14 cm of diameter 500 ml) containing a peat and soil mixture (1:1 v:v). Plants were grown for 2 months, from April to June, in greenhouse (25° ± 5 °C; 70% ±10% RH; with natural photoperiod). Treatments were conducted by foliar spray with a solution of pure 6PP or HA at 10 μM and 1 μM, or with spore suspensions of P1 or M10 (applied at 10^8 spore/litre). After 2 months disease incidence and severity of powdery mildew infection caused by *U. necator* were evaluated. Disease severity was determined on a 0–4 scale, where 0 was healthy and 1–4 diseased (1 = <10% of the leaf surface covered with powdery mildew; 2 = 11–25% of the leaf surface covered with powdery mildew; 3 = 26–50% of the leaf surface covered with powdery mildew; 4 = >50% of the leaf surface covered with powdery mildew) considering the mean calculated on the first 10 leaves for each plant. Plants naturally infected by *U. necator* were introduced in the glasshouse 30 days earlier and shaken over the whole plants. All the *in-vivo* biocontrol experiments have been repeated at least three times.

2.4. Field experiments

The experimental field of *V. vinifera* cv. Sangiovese (4 years old plants) was laid out in 9 rows (3 rows per treatment in random order) composed of 12 plants per row. A 1 μM solution of 6PP and a spore suspension of T22 (10^8 spore/litre) were applied by drenching (5 L per row). Controls consisted in water treated plants. Treatments were applied every 14 days, starting one month after plants sprouted, and finished with fruit harvest, for a total number of 10 treatments. The field trial has been repeated two times.

2.5. Analysis of polyphenols and antioxidant activity

Polyphenols were extracted from fruits by homogenizing for 1 min in 20 ml of extraction solution containing methanol/water/formic acid (60:37:3 v/v/v) 5 g of whole grapes. The homogenate was centrifuged for 5 min at 5000 rpm, and the supernatant was collected, evaporated to dryness by using a SpeedVac concentrator (ThermoSavant, Holbrook, NY, USA) with no radiant heat and then re-suspended in extraction solution. The quantity of total polyphenols in the extracts was determined according to the Folin–Ciocalteau method (Fogliano et al., 1999). Gallic acid was used as standard and results were expressed as gallic acid equivalents (GAE) (mg GAE/100 g of seeds or skin dry matter - DM). The absorbance was measured in triplicate for each sample using a UV–Vis spectrophotometer (Lambda 25, PerkinElmer, Italy) at 765 nm.

The antioxidant capacity was measured using the ABTS/HRP colorimetric assay. The method is based on the capacity of a sample to scavenge the ABTS radical cations ($ABTS^{+\bullet}$), which are reactive to many antioxidants such as phenols and causes a colour change that is detected by spectrophotometry. The experimental samples are compared to a standard antioxidant (Trolox) in a dose-response curve (Cho et al., 2004; Re et al., 1999).

2.6. Data analysis

Data of disease incidence, grape yield, polyphenols and antioxidant activity were analysed by R statistical software, using Agricola package for ANOVA and Least Significant Difference test according to the *p-value* reported in each graph. The data analysis in the present paper are referred only to the results of the last year experiments since the results of each repetition had similar trends.

3. Results

3.1. Characterization of Trichoderma spp. secondary metabolites

The ^1H NMR spectra of the secondary metabolites isolated from P1 and M10 indicated that the major compounds purified from the two *Trichoderma* strains had the same signals of 6PP and HA, respectively, as those observed in the literature and identical to the standards available in our laboratories (Collins and Halim, 1972; Sawa et al., 1994; Vinale et al., 2009). LC − MS analysis showed that HA was detected as $[M+H]^+$ (*m/z* 366), $[M+Na]^+$ (*m/z* 388), $[M+K]^+$ (*m/z* 404) and $[M_2 + Na]^+$ (*m/z* 753.3) while 6PP as [M+H]+ (*m/z* 167) and $[M+Na]^+$ (*m/z* 189).

3.2. In vivo disease control of powdery mildew on Vitis vinifera

In vivo experiment for disease control on grape plants showed that the two secondary metabolites treatments (6PP and HA) inhibited the disease development compared to the control similarly to the applications of the two *Trichoderma* strains. Control plants showed typical disease signs caused by *U. necator* consisting of a white mildew growing on the upper side of leaves with a disease severity of 3,06 (mean value).

Application of HA and 6PP showed a significant reduction of disease development (Fig. 1-A; Fig. 1-B).

Spraying 10 μM solution of HA significantly reduced the powdery mildew development by 60% on leaves but produced typical phytotoxicity symptoms (necrosis and irregular leaves edge) whereas the 1 μM HA solution sprayed resulted in a reduction of 20% of disease incidence compared to the controls without negative effects on plant. Drenching applications of HA at 10 μM and 1 μM reduced the disease severity respectively of about 23% and 13% with no negative effects on plants (Fig. 1-A).

Spray application of 6PP at 10 μM and 1 μM resulted in the reduction of disease severity respectively of about 28% and 32% whereas reduction obtained by drenching was about 25% and 15% (Fig. 1-B).

Plants treated with propagules of M10 and P1 generally showed a higher level of pathogen control (Fig. 2) compared to the treatments with the secondary metabolites.

Applications of these *Trichoderma* strains reduced disease development at the same level by drenching or spraying, thus indicating that the fungus is able to induce systemic resistance in grape as well as the applications of their secondary metabolites.

3.3. Yield increase effect on Vitis vinifera

Field experiments on *V. vinifera* were carried out with

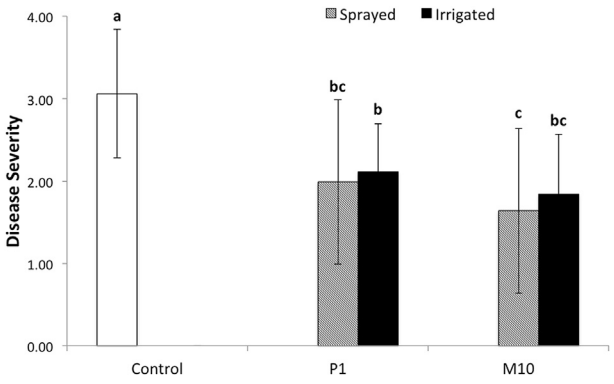

Fig. 2. Disease control of a sprayed or a drenched spore suspension of *T. atroviride* P1 and *T. harzianum* M10. Control: H_2O treated. Bars indicate disease severity levels on grape leaves affected by powdery mildew. Different letters on the bars indicate significative differences according to LSD test ($p \leq 0{,}01$).

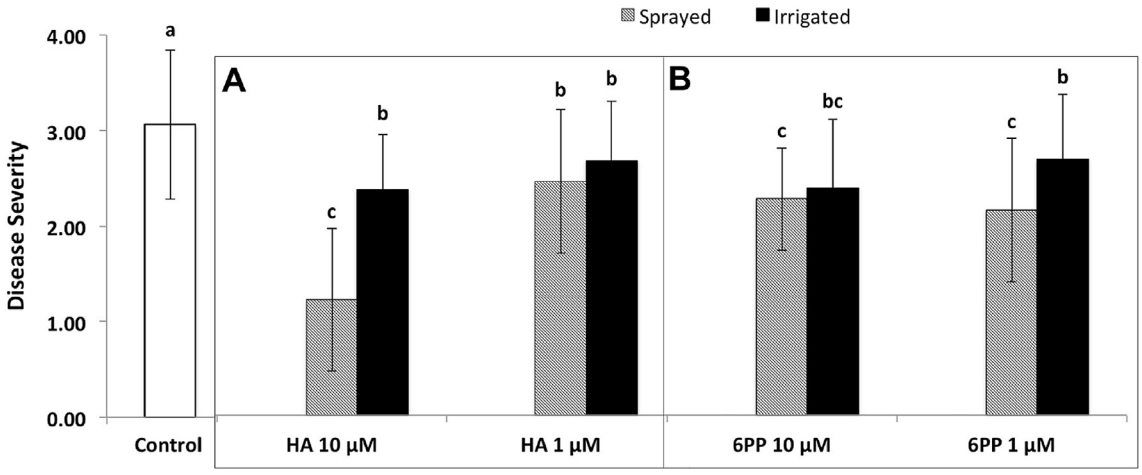

Fig. 1. Disease control by the *Trichoderma* secondary metabolites. A: Harzianic acid (HA) sprayed or drenched solutions applied at two different concentrations (10 μM and 1 μM). B: 6-pentyl-α−pyrone solution (6PP) sprayed or drenched solutions applied at two different concentrations (10 μM and 1 μM). Control: H_2O treated. Bars indicate disease severity levels on grape leaves affected by powdery mildew. Different letters on the bars indicate significative differences among the control and each metabolite (HA or 6PP) according to LSD test ($p \leq 0.01$).

treatments based on the *Trichoderma* metabolite 6PP, in comparison to the use of T22. In order to evaluate the growth promotion effects of 6PP and *Trichoderma* in the yield, no fertilizers were added to grape plants.

T22 and 6PP increased yield in terms of weight (kg) of grape product, respectively of 63% and 97%, as compared to the untreated controls (Fig. 3).

3.4. Effect on grape quality

In order to evaluate the effect of 6PP and T22 on the quality of grape fruits, total polyphenol content and antioxidant activity (ABTS assay) were measured.

The antioxidant activity increased with the treatments of T22 and 6PP respectively by 48.7% and 60.3% compared to control treatments (Fig. 4).

The polyphenol content increased in harvested fruits of plants treated with either T22 or 6PP, with no significative differences between the two applications. Results are reported in Fig. 5 as mg equivalents of gallic acid.

4. Discussion

The first part of the present work was aimed at evaluating the effectiveness of the two purified *Trichoderma* secondary metabolites 6PP and HA on the control of powdery mildew, one of the most important foliar diseases of grape, compared to the applications of the living *Trichoderma* strains that produce the two natural compounds (respectively P1 and M10). The results showed that the purified molecules were able to reduce the disease severity on grape leaves, regardless to the application methods (sprayed or drenched) as well as those obtained by treatments with *Trichoderma*. The reduction of the symptoms, achieved when treatments were applied to the soil (far from the pathogen challenge site), indicates that the disease control effect is due the induction in the plants of the systemic resistance. This ability of *Trichoderma* is widely described and it is recognized as one of the most important mechanisms my which these fungi protect plants (Woo et al., 2006; Shoresh et al., 2010; Pieterse et al., 2014).

Trichoderma establishes an intimate relation with the host plants and induces several changes in the plant defence-related metabolic pathways, including the expression of pathogenesis related proteins (PR proteins) or the upregulation of phenylalanine ammonia-lyase (PAL) that is involved in the productions of phytoalexins (Shoresh et al., 2010). Among the tools that *Trichoderma* utilizes to interact with plants there is the production of many different molecules that are recognized as microbe-associated molecular patterns (MAMPs) such as low molecular weight secondary metabolites, extracellular enzymes and hydrophobic proteins (Seidl et al., 2006; Djonovic et al., 2007; Vargas et al., 2008; Lorito et al., 2010; Ruocco et al., 2015). Vinale et al. (2008b; 2014a,b) demonstrated that different *Trichoderma* secondary metabolites, including 6PP, are involved in the induction of plant systemic resistance as they cause a reduction of disease symptoms and the overexpression of defense genes. However, differences found in terms of level of disease control between the effect of *Trichoderma* and its secondary metabolites could be attributed to the fact that the living microbe may use more than one mechanism to attach the pathogen including combination of enzymes and secondary metabolites that act synergistically (Harman et al., 2004; Lorito et al., 2010).

The experiments conducted in the greenhouse showed also a significant growth promotion effect on grape plants treated with either *Trichoderma* strains propagules or their purified secondary metabolites (data not shown). Based on this evidences, we tested the *Trichoderma* secondary metabolite 6 PP (produced in a

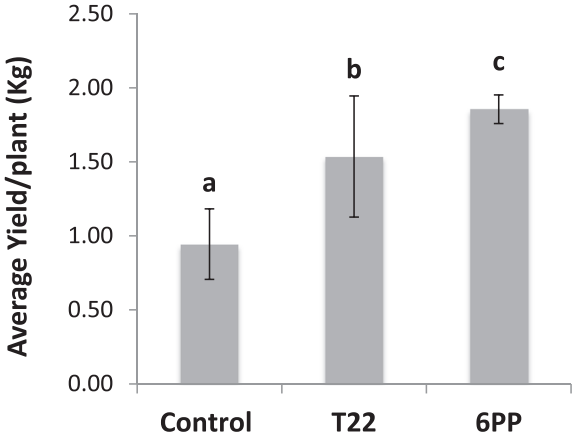

Fig. 3. Effect of *T. harzianum* strain T22 (T22) and 6-pentyl-α-pyrone (6PP) treatments on grape yield. Control: H_2O treated. Bars indicated the mean of yield per plant in terms of Kg of grapes produced. Different letters on the bars indicate significative differences according to LSD test ($p \leq 0,05$).

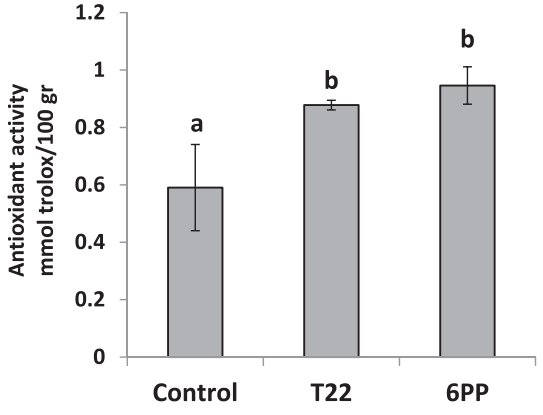

Fig. 4. Effect of *T.harzianum* strain T22 (T22) and 6-pentyl-α-pyrone (6PP) based treatments on grape fruits antioxidant activity. Control: H_2O treated. Bars indicated the mean on 100 gr of products, expressed as mmol equivalents of Trolox. Different letters on the bars indicate significative differences according to LSD test ($p \leq 0,05$).

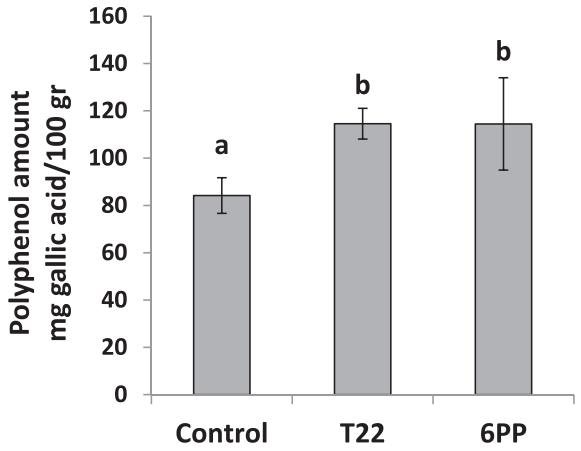

Fig. 5. Effect of *T. harzianum* strain T22 (T22) and 6-pentyl-α-pyrone (6PP) based treatments on grape fruits total polyphenols amount. Control: H_2O treated. Bars indicated the mean of polyphenols amount on 100 gr of products expressed as mg equivalents of gallic acid. Different letters on the bars indicate significative differences according to LSD test ($p \leq 0,05$).

relatively large amount by several *Trichoderma* spp.) on grape plants grown in field conditions with the aim to evaluate the effectiveness of the purified compound in terms of agronomic performances such as yield and quality of fruits. We also compared the use of 6PP to the effectiveness of T22, one of the most studied and commercially successful *Trichoderma* strain in agricultural applications (Harman, 2000).

The results obtained show that both *Trichoderma* and 6PP treatments produce a substantial and comparable increase of grape yield, especially when plants are grown without fertilization.

It's well known that *Trichoderma* strains can improve the plant fitness in both axenic and natural soils especially in suboptimal growth conditions in the field, where the fungus has a direct positive influence on plant growth other than alleviate the effects of biotic or abiotic stresses that may naturally occur (Harman et al., 2004: Shoresh et al., 2010). Enhancement of yield on corn has been reported in several commercial and academic trials and it has been considered as a direct effect of an increased root and foliar systems (Harman, 2000). The plant growth promotion induced by *Trichoderma* can be explained by an upregulation of photosynthesis related proteins and a higher photosynthetic efficiency (Shoresh et al., 2010) Some *Trichoderma* strains may also enhance the plant nutrient uptake mechanism (Harman et al., 2004), and increase the plant nitrogen use efficiency (Harman, 2000; Shoresh et al., 2010). Furthermore, it already has been described the role on the growth promotion of plants of *Trichoderma* secondary metabolites (Vinale et al., 2008b, 2012; 2013). In particular, Vinale et al. (2008a,b) demonstrated that 6PP has an auxin-like mechanism of action with a concentration-related plant growth promotion effect. Garnica-Vergara et al. (2016) confirmed this hypothesis demonstrating that the growth of lateral roots in *Arabidopsis* plants was stimulated by low concentration of 6 PP and they correlated this phenotypic response to an overexpression of genes involved in the auxin signaling.

Beside the yield enhancement, analysis on fruits performed in this study demonstrated that *Trichoderma* and 6PP treatments increased total polyphenols amount and a consequent augmentation of antioxidant capacity.

The plant phenolic compounds are a wide range of substances, produced by the phenylpropanoid metabolism (Dixon and Paiva, 1995). The key factor for the synthesis of this class of molecules is the phenylalanine ammonia lyase (PAL), that is the first enzyme of this biosynthetic pathway (Dixon and Paiva, 1995). A large number of these low molecular weight compounds, defined as phytoalexins, are rapidly accumulated in plants as response to different stresses, biotic, caused by pathogens or insects attack, or abiotic, caused by wounds, exposure to UV light, water or nutritional deficits (Hammerschmidt, 1999; Grayer and Kokubun, 2001). Yedidia et al. (2003) demonstrated that cucumber plants treated with *Trichoderma asperellum* increased total amount of polyphenols and treated plants engaged by the pathogen *Pseudomonas syringe* pv. *Lachrymans* increased the total amount of polyphenols with antimicrobial activity. Our results confirmed the ability of the living fungus to enhance the accumulation in the fruits of this class of compounds, thus activating or priming induced systemic resistance mechanisms, and that a similar effect is obtained by the application of 6PP. The augmentation of these substances in grapes is of particular interest since they are a qualitative parameter with a direct effect on grape and wine in terms of aroma and shelf life (Robinson et al., 2014).

To the best of our knowledge, the present work is the first that reports the effects of *Trichoderma* secondary metabolites on grape plants in field conditions, thus demonstrating that a 6PP treatment produces similar positive results as the living fungus in terms of yield and fruit quality enhancement. Therefore, our results suggest new possibilities of using natural compounds produced by beneficial microbes in grape production, also by eliminating some of the constrains associated with the application of living biological control agents.

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