





Article

Effect of Selected *Trichoderma* Strains and Metabolites on Olive Drupes

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Abstract: Beneficial fungal strains of the genus *Trichoderma* are used as biofungicides and plant growth promoters. *Trichoderma* strains promote the activation of plant defense mechanisms of action, including the production of phenolic metabolites. In this work, we analyzed the effects of selected *Trichoderma* strains (*T. asperellum* KV906, *T. virens* GV41, and *T. harzianum* strains TH1, M10, and T22) and their metabolites (harzianic acid and 6-pentyl- α -pyrone) on drupes of young olive trees (4-year-old) cv. *Carolea*. This study used the untargeted analysis of drupe metabolome, carried out by LC–MS Q-TOF, to evaluate the phenolics profiles and target metabolomics approach to detect oleuropein and luteolin. The untargeted approach showed significant differences in the number and type of phenolic compounds in olive drupes after *Trichoderma* applications (by root dipping and drench soil irrigation method) compared to control. The levels of oleuropein (secoiridoid) and luteolin (flavonoid) varied according to the strain or metabolite applied, and in some cases, were less abundant in treated plants than in the control. In general, flavonoids' levels were influenced more than secoiridoid production. The dissimilar aptitudes of the biological treatments could depend on the selective competence to cooperate with the enzymes involved in producing the secondary metabolites to defend plants by environmental stresses. Our results suggest that using selected fungi of the genus *Trichoderma* and their metabolites could contribute to selecting the nutraceutical properties of the olive drupe. The use of the metabolites would bring further advantages linked to the dosage in culture and storage.

Keywords: secondary metabolites; phenolic compounds; LC–MS Q-TOF; *Olea europaea*; *Trichoderma*; metabolomics



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1. Introduction

Since ancient times olive trees (*Olea europaea* L.) have been cultivated throughout the Mediterranean area for their fruits and oil production. Each country has its local cultivars because the human selection and pedoclimatic conditions have resulted in genetic variations [1–4]. In Italy, many trees sprout spontaneously, and oil and table cultivars are grown mainly in Calabria, Apulia, Sicily, and Campania, where centuries-old trees and archaeological finds document their presence from old times [5]. Extravirgin olive oil (EVOO) is obtained from crushing the olive drupe and separating olive oil by pressure, centrifugation, and percolation (selective filtration process) [6]. EVOO is present in all variants of the Mediterranean diet. The latter is a healthy diet adopted by the Italian and Greek population in the 1960s [7], reducing the risk of cardiovascular disease, cancer, type 2 diabetes, and

cognitive disorders [8]. It is characterized by high consumption of vegetables, fruit, salads, bread, whole grains, legumes/beans, nuts, seeds, moderate use of wine, and EVOO as the primary source of fat [8]. The EVOO has protective effects on human health due to the high content of monounsaturated fatty acids (MUFAs) and secondary bioactive molecules, including phenolic compounds, tocopherols, phytosterols, and carotenoids [9]. The phenolic compounds in EVOO range from 50 to 800 mg/kg [10,11]. They consist of phenolic alcohols (e.g., tyrosol and hydroxytyrosol), phenolic acids (e.g., vanillic, caffeic, coumaric, protocatechuic, ferulic, and p-hydroxybenzoic), flavones (e.g., apigenin, apigenin-7-O-glucoside, luteolin, luteolin-7-O-glucoside), lignans (e.g., pinoresinol and acetoxypinoresinol), and secoiridoids (e.g., oleacein, oleuropein, oleocanthal, and p-HPEA-EA) [12] responsible for EVOO bitterness, pungency, fragrance, and antioxidative properties [13]. Fruit's maturation, cultivar varieties, pedoclimate condition [14], and the type of oil extraction processes affect the phenolic quality and concentration [15]. The oleuropein, tyrosol, and hydroxytyrosol (the main phenolic compounds in EVOO) [12] have antioxidant, anti-inflammatory, immunomodulatory, and neuroprotective activities [16]. The EVOO's polyphenols protect blood lipids against oxidative stress [17,18]. Therefore, the EVOO's phenolics are used in supplements for the prevention of chronic degenerative diseases such as cardiovascular diseases and cancer [19], in antiaging cosmetics [20,21], in the food industry as flavorings or preservatives [22–24], and in functional foods preparations [25,26]. Olive plants make the phenolics in response to abiotic stress and pathogen attack [27–32]. Dini et al. in 2020 and 2021 investigated the effects of some *Trichoderma* strain applied to olive trees to evaluate a selective phenols production [33,34]. Nowadays, fungi belonging to the genus *Trichoderma* are commonly used in agriculture as biocontrol agents (they inhibit soils and air diseases) and plant growth promoters [35,36]. They also enhance the abiotic stress tolerance (e.g., salinity, drought), yields production, nutritional uptake, leaf area, root system growth, and activate protective mechanisms against oxidative injury [37,38]. Some *Trichoderma* strains produce secondary metabolites such as 6-pentyl- α -pyrone (volatile antibiotics), heptelidic acid, and peptaibols to help in metal transport, symbiosis, differentiation, and competition with another organism [39,40], and phenols against oxidative damage [41–43]. Phenols decrease cardiovascular pathologies, hypoglycemia, hypotension, and hypocholesterolemia and prevent angiogenesis, inflammation [44], and cancer [45].

In the present work, we report the effect of selected *Trichoderma* strains (e.g., *T. asperellum* KV906, *T. harzianum* strains TH1, M10, and T22; and *T. virens* GV41) and their metabolites (e.g., harzianic acid (HA), and 6-pentyl- α -pyrone (6PP)) upon in vivo application, on weight and phenol metabolites of the olive drupes in consideration of the commodity and nutraceutical importance of their potential effects.

2. Materials and Methods

2.1. Microbial Strains

Five *Trichoderma* strains (*T. asperellum* strain KV906, *T. harzianum* strains TH1, M10, and T22; and *T. virens* strain GV41) were used in this work. Strains were provided by Department of Agricultural Sciences of the University of Naples Federico II, after cultivation on previously described conditions [46].

A hemocytometer (Neubauer-improved, BRAND GMBH + CO KG, Wertheim, Germany) was used to establish the concentration of the spore suspensions.

2.2. *Trichoderma* Bioactive Metabolites

We used 6PP and HA in this work. The former was extracted from *T. atroviride* strain P1 [47] and the latter from *T. harzianum* strain M10 [48]. Metabolite solutions used for treatments were obtained by resuspending HA and 6PP in distilled water and ethyl acetate 0.01% (v/v) to facilitate the process. Once a clear solution was made, ethyl acetate was evaporated under nitrogen flow.

2.3. Plant Material

Four-year-old olive trees (*Olea europaea* L cv. *Carolea*.) were used for experimental purposes. The plants were grown into plastic pots (50 cm diameter × 40 cm high) located in a field at the University of Naples Federico II-Department of Agricultural Sciences (Portici, Italy). Each pot contained one plant and 50 L of universal soil (granulated pumice, peat, and coconut fiber). Once a week, the plants were watered to field capacity. No nutrients were added.

2.4. Experimental Design

Eight treatments were performed (including water control), using five *Trichoderma* strains and two metabolites. The field trial was performed in a randomized block design. *Trichoderma* metabolite solutions (1×10^{-6} M), or spore suspensions (1×10^6 sp mL⁻¹) were inoculated through root system exposure at the time of transplant (10 min, 1 L plant⁻¹) by root dip, and every 30 days (400 mL plant⁻¹) by soil irrigation (six applications in total). Each treatment was performed on 15 plants (five plants in each replicate and three biological replicates per treatment). Drupes were collected and weighed with an electronic digital scale (Precisa Instruments AG, model XB220A, Dietikon, Switzerland). Finally, drupes samples were stored at -80 °C until extraction of metabolites.

2.5. Chemicals

Solvents (methanol, water, formic acid, acetonitrile) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). ESI-TOF tune mix was bought from Agilent Technologies (Agilent Technologies, Santa Clara, CA, USA).

2.6. Olive Metabolites Extraction

Talhaoui et al. method [49], with some modifications, was used to extract the phenolic compounds. Briefly, drupes were freeze-dried and crushed. 200 mg of powder were extracted twice in an ultrasonic bath for 10 min (Model 6.51200 H, Dakshin, India), using 5 mL of a solution of methanol/water (50/50, v/v) and then centrifuged (Hettich GmbH and Co., Tuttlingen, Germany) at 4000 rpm, 4 °C for 10 min. The supernatants were collected, dried in a speed-vac (Savant SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA) and dissolved in 2 mL of a solution of methanol/water (50/50, v/v). Finally, the extracts obtained were filtered (Millipore 0.45 µm) and stored in the dark (at -80 °C) until use.

2.7. Phenolics' Isolation, Identification, and Quantification

2.7.1. Metabolites' Analysis

The analysis of metabolites was performed on an Agilent HP 1260 Infinity Series liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD system (Agilent Technologies, Santa Clara, CA, USA) and a Q-TOF mass spectrometer model G6540B (Agilent Technologies, Santa Clara, CA, USA).

Chromatographic Conditions

An InfinityLab Poroshell 120 EC-C18 column (2.1 mm × 100 mm, 2.7 µm) (Agilent Technologies, Santa Clara, CA, USA), at controlled temperature (25 °C) was used as stationary phase. Two eluents, phase A (0.1% (v/v) formic acid in water) and phase B (0.1% (v/v) formic acid in acetonitrile), were employed as mobile phase. The gradient was set as follows: 0 min, 95% A; 4 min, 91% A; 7 min, 88% A; 8 min, 85% A; 9 min, 84% A; 14 min, 80% A; 15 min, 78% A; 18 min, 72% A; 19 min, 70% A; 20 min, 69% A; 21.50 min, 68% A; 23 min, 66% A; 24 min, 65% A; 25.5 min, 60% A; 27 min, 50% A; 30 min, 0% A; 35 min, 0% A; and 37 min, 95% A. Flow rate was 0.5 mL min^{-w}.

Spectroscopic and Spectrometric Conditions

The UV spectra were recorded every 0.4 s, with a resolution of 2 nm, from 190 to 750 nm by DAD (Agilent Technologies, Santa Clara, CA, USA).

The MS system was equipped with a dual ESI (electrospray ionization) source and operated in negative mode as reported by Tafuri et al. [50]. All the parameters were controlled by the Agilent MassHunter Data Acquisition Software, version B.05.01. Mass spectra were recorded in the mass range 100–1600 m/z (3 scans per second). Hexakis (^1H , ^1H , ^3H -tetrafluoropentoxy)-phosphazene ($\text{C}_{18}\text{H}_{18}\text{O}_6\text{N}_3\text{P}_3\text{F}_{24}$ at m/z 922.009798, $2\ \mu\text{mol L}^{-9}$) (Sigma-Aldrich, St. Louis, MO, USA), and purine ($\text{C}_5\text{H}_4\text{N}_4$ at m/z 121.050873, $10\ \mu\text{mol L}^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA) were injected in the source ($0.060\ \text{mL min}^{-e}$) to perform the lock mass correction in real-time. The capillary was set at 4000 V, cone 1 (skimmer 1) at 45 V, fragmentor at 180 V. Gas temperature was $350\ ^\circ\text{C}$, and the nebulizer was at 45 psi. The injection volume was $5\ \mu\text{L}$. For each treatment, three biological samples were analyzed in triplicate.

Mass Profiler Professional (Agilent Technologies, MPP v 13.1.1, Santa Clara, CA, USA) was used for molecular feature normalization, alignment, compound identification, and statistical analysis. MPP normalization and alignment parameters were: minimum number of ions (2); abundance filter (>5000 counts); intercept (0.4 min), alignment RT window, and slope (0%); intercept (2 mDa), alignment mass window, and slope (20 ppm). Only masses occurring in two of three samples were accepted. Masses found in blank runs from filtered masses were used to remove background noise. The ion chromatogram (EIC) was extracted with ± 20 ppm single ion expansion with MassHunter software v B.06.00 (Agilent Technologies, Santa Clara, CA, USA).

2.7.2. Phenolics' Identification

The phenolics were identified by Mass Hunter Qualitative Analysis Software version B.06.00 (Agilent Technologies, Santa Clara, CA, USA). Identification was achieved by comparison with in-house databases (comprising data from METLIN library) and with existing literature data. Empirical formulas, calculated by isotope model (common organic molecules, ppm limit = 10, limit charge state to a maximum of 2, and use ^+H or ^-H , or sodium and potassium adducts) were given for unidentified compounds. Experimental retention time, monoisotopic mass, and UV max of standards confirmed the identification.

2.7.3. Phenolics' Quantification

The quantification of phenolics was performed by using oleuropein and luteolin commercial standards purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol: water (50:50, v/v) to make standard calibration curves (Table S1).

2.8. Statistical Analysis

Statistical analysis was performed by SPSS V24 statistic software (IBM Corporation, Armonk, NY, USA). One-way ANOVA analyzed the data of SPAD index. Significant differences among treatments were compared using Fisher's least significant difference (LSD) post hoc tests and S–N–K (Student–Newman–Keuls) (with 0.05 level of significance). The Student's t -test (with a 0.05 level of significance) determined significant differences.

3. Results

3.1. Effect of Treatments on the Olive Trees Drupes' Weight

In the first year of production, the average weight of the drupes for each treatment was evaluated. All treatments, except *T. harzianum* M10 and *T. asperellum* KV906, positively affected the drupes' average weight compared to the control (Table 1).

Table 1. Effects of treatments with *Trichoderma* spp. strains T22, M10, GV41, TH1, KV906 or its metabolites 6-pentyl- α -pyrone (6PP) and harzianic acid (HA) on average weights of the drupes collected from the experimental field. The control (CTRL) was not treated with biostimulants.

Treatment	Drupe's Average Weight (g)
GV41	8.10 ^b
M10	5.66 ^a
T22	7.61 ^b
TH1	6.63 ^b
KV906	5.90 ^a
6PP	6.12 ^b
HA	9.40 ^b
CTRL	5.85 ^a

Different letters within each column indicate significant differences ($p < 0.05$).

3.2. Characterization of Olive Drupe Metabolome

Thirteen phenolic compounds were identified based on total ion chromatogram (TIC), mass/UV–VIS, spectra, and literature data. Identification parameters (retention time, UV maximum absorption, experimental and calculated monoisotopic masses, molecular formula) are reported in Table 2.

Table 2. Phenolics' identification parameters.

Compound	RT (min)	UV Max (nm)	Experimental Mass	Mass Theoretical	Formula
Secoiridoids					
Oleuropein aglycone	10.90	235; 271	378.1569	378.13	C ₁₆ H ₂₆ O ₁₆
Oleuropein isomer a	19.10	240; 280	540.1840	540.18	C ₂₅ H ₃₂ O ₁₃
Oleuropein isomer b	20.10	235; 280	540.1848	540.184	C ₂₅ H ₃₂ O ₁₃
2''-Methoxyoleuropein	15.81	236; 280	570.1942	570.19	C ₂₆ H ₃₄ O ₁₄
Ligstroside	20.52	230; 280	524.1900	524.19	C ₂₅ H ₃₂ O ₁₂
Flavonoids					
Luteolin	20.80	255; 286	286.0488	286.05	C ₁₅ H ₃₅ O ₁₄
Luteolin rutinoside	11.90	248; 267	594.1589	594.16	C ₂₇ H ₃₀ O ₁₅
Luteolin di-glucoside	12.25	248; 267; 335	610.1537	610.15	C ₂₇ H ₃₀ O ₁₆
Rutin	14.60	253	610.1539	610.15	C ₂₇ H ₃₀ O ₁₆
Simple phenols					
Hydroxytyrosol-glucoside	4.75	230; 280	316.1160	316.12	C ₁₄ H ₂₀ O ₈
Verbascoside	14.55	234; 329	624.2064	624.20	C ₂₉ H ₃₆ O ₁₅
Oleosides					
Oleoside methyl ester	6.33	235	404.1321	404.13	C ₁₇ H ₂₄ O ₁₁
Secologanoside	7.45	234	390.1151	390.12	C ₁₆ H ₂₂ O ₁₁

3.3. Untargeted Metabolomics Analyses of Phenolics in Olive Drupes

Untargeted metabolomic analyses were carried out on the olives harvested from treated plants. There was a tendency for the number of down-regulated compounds to increase (Table 3). In general, a more significant number of compounds were observed whose abundance was lower when compared to the control. The metabolite 6PP and *Trichoderma* sp. strains GV41 and KV906 influenced the metabolic response in drupes more than the control.

Replicate samples were grouped and subjected to variance analysis (one-way ANOVA, $p < 0.05$) and to fold change (FC > 2.0), comparing metabolite abundances in treatments vs. water-treated plants (CTRL). Hierarchical cluster analysis and statistical analysis revealed 88 metabolites differentially accumulated among treatments that are depicted as hierarchical cluster in Figure 1. The metabolic profiling revealed that drupes from olive plants treated with the *Trichoderma* sp. strain M10, KV906, T22, and metabolite HA were

grouped separately. TH1 determined no differences in terms of phenolic levels compared to the control.

Table 3. Number of metabolites whose production is higher (UP) or lower (DOWN) compared to the control (CTRL) in the drupes obtained from treated plants (*Trichoderma* spp. strains T22, M10, GV41, TH1, KV906 or its metabolites 6-pentyl- α -pyrone (6PP) and harzianic acid (HA)).

Treatments	UP vs. CTRL	DOWN vs. CTRL
GV41	21	30
M10	16	44
T22	10	38
TH1	16	35
KV906	21	44
HA	12	36
6PP	28	39

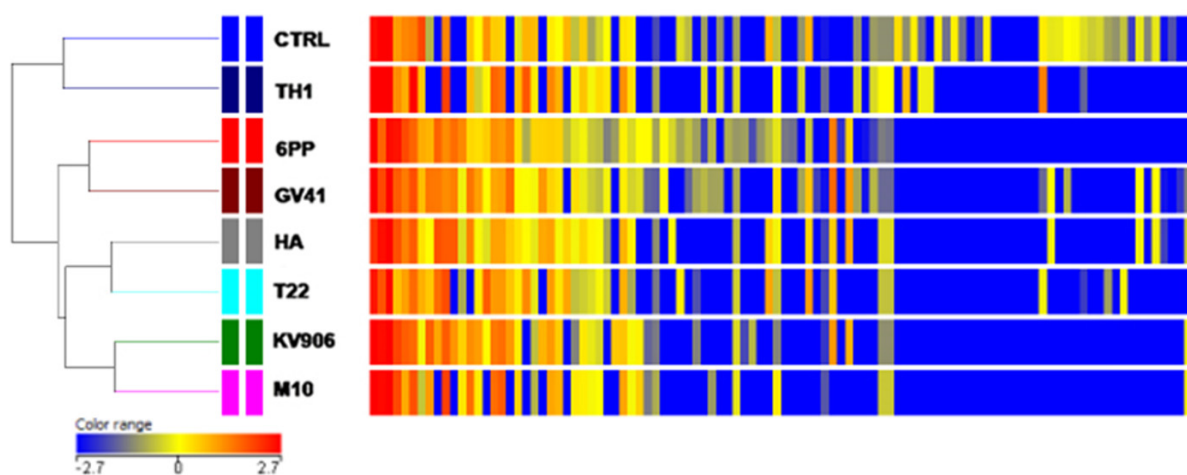


Figure 1. Hierarchical clustering heat map of differential metabolic profiles from olive drupe extracts. Samples are indicated by treatments with *Trichoderma* strains (TH1, GV41, T22, M10, and KV906) or secondary metabolites (HA and 6PP). Control was plants treated with water. Red color indicates higher phenolic abundance (>0), blue colors lower (<0), yellow a neutral change from the overall average abundance. Statistical significance was tested by one-way ANOVA ($p < 0.05$).

Successively, the variations in metabolite accumulation between increased (UP) or decreased (DOWN) compounds, as compared to the control, were analyzed for T22, M10 and HA.

A Venn diagram showed that 30 down-regulated compounds were in common among all treatments, while four metabolites were exclusively in the metabolome of T22-treated samples and four in those exposed to M10. No compound was found to be specific for HA treatment. Four metabolites were common to T22 and M10 treatments and six to M10 and HA. In contrast, no compounds were found to be common to T22 and HA treatments. Concerning the up-regulated compounds, five were common to all treatments, eight exclusive to M10, 1 to HA, and none for T22; one metabolite was common between treatments with T22 and M10, two between M10 and HA and four between HA and T22. (Figure 2).

Untargeted metabolomic analysis revealed the presence of several differentially accumulated metabolites in treated or non-treated drupes. Among these, 13 metabolites were putatively identified by comparison with an in-house database and standard compounds (Table 4).

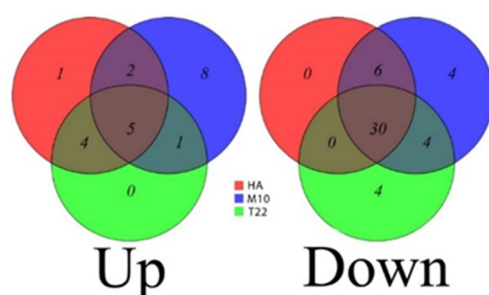


Figure 2. Venn diagrams of phenolic compounds whose abundance in the olive drupe metabolome is lower (**DOWN**) or higher (**UP**) compared to the control (CTRL). Samples treated with T22 are reported in green; with M10 in blue and with HA in red.

Table 4. Metabolites identified in the drupe extracts of olive trees subjected to field applications of biological treatments. Samples are indicated by treatments with *Trichoderma* strains (TH1, GV41, T22, M10, and KV906) or secondary metabolites (HA and 6PP). Identifications were confirmed by comparing results with known compounds in an in-house database/standards and selecting matching ($\geq 95\%$).

Compound	Regulation against Control Group (CTRL)						
	M10	KV906	GV41	TH1	T22	6PP	HA
Oleuropein aglycon	↑	↑	↑	↑	↑	↑	↑
Oleuropein isomer a	↓	↓	↓	↓	↓	↓	↓
Oleuropein isomer b	↓	↓	↓	↓	↓	↓	↓
2-Methoxyoleuropein	↓	↓	↑	↑	↓	↓	↓
Ligstroside	↓	↓	↓	↓	↓	↓	↓
Luteolin	↑	↑	↑	↑	↓	↑	↑
Luteolin rutinoside	↑	↑	↑	↑	↑	↑	↑
Luteolin di-glucoside	↑	↑	↑	↑	↑	↑	↑
Rutin	↓	↓	↓	↓	↓	↑	↓
Hydroxytyrosol glucoside	↓	↓	↑	↑	↑	↓	↑
Verbascoside	↑	↑	↑	↑	↓	↑	↓
Oleoside methyl ester	↓	↓	↑	↑	↑	↓	↑
Secologanoside	↓	↓	↓	↑	↓	↓	↓

↑ Increased production of the metabolite in treated vs. control. ↓ Decreased production of the metabolite in treated vs. control.

3.4. Targeted Metabolomics Analyzes of Phenolics in Olive Drupes

Oleuropein (secoiridoid) and luteolin (flavonoid) concentrations were considered to evaluate the phenolics' level trends. Oleuropein content increased in the drupes of plants treated with 6PP, GV41, and T22 (Figure 3).

The secondary metabolite 6PP and the *Trichoderma* strains GV41, KV906, HA, and M10, increased the luteolin's level in drupes (Figure 4).

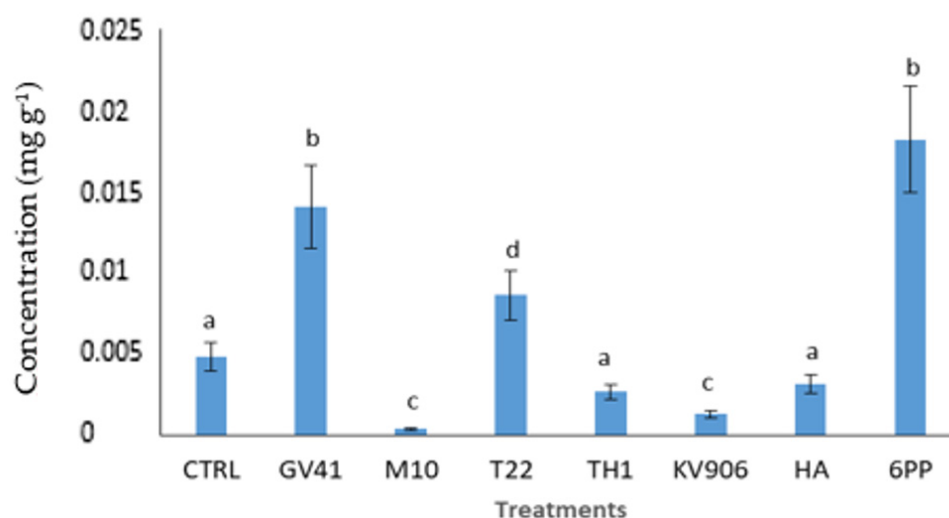


Figure 3. Oleuropein content in drupes. Samples are indicated by treatments with *Trichoderma* strains (TH1, GV41, T22, M10, and KV906) or secondary metabolites (HA and 6PP). Values are the means of three replicates \pm SE. Different letters indicate significant differences ($p < 0.05$).

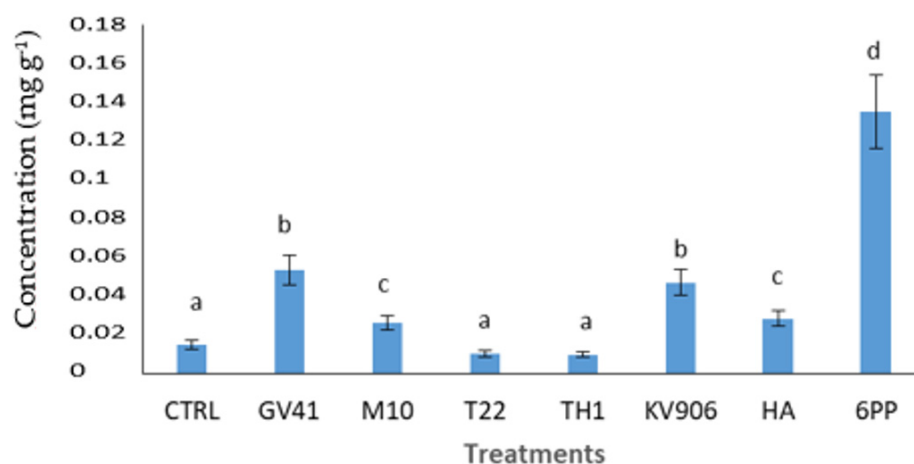


Figure 4. Luteolin content in drupes. Samples are indicated by treatments with *Trichoderma* strains (TH1, GV41, T22, M10, and KV906) or secondary metabolites (HA and 6PP). Values are the means of three replicates \pm SE. Different letters indicate significant differences ($p < 0.05$).

4. Discussion

Today, it is considered a priority to limit pesticides against phytopathogenic agents or replace them with products of biological origin. The use of mycoparasitic fungi, particularly those belonging to the genus *Trichoderma*, has met considerable success [11]. The world market of biopharmaceuticals proposes over 50 bioformulates containing *Trichoderma* strains as active ingredients [51]. The considerable achievement of *Trichoderma* fungi in agriculture is due to their suppression of pathogenic species, both terricolous and foliar, among the most harmful, such as those belonging to the genera *Fusarium*, *Sclerotinia*, *Botrytis*, and *Pythium* [52]. Microbial inocula to promote plant development and/or control phytopathogenic agents is still not very widespread in tree crops. Few studies evaluated the effect of beneficial microorganisms on the fitness of the olive tree and provided helpful information for the use of bioformulates in the open field. The present work aims to evaluate the effects of the field applications of *Trichoderma* spp. strains and some of their metabolites on young olive trees' drupes weight and nutraceutical content contained therein.

The olive is the most representative tree in Italy, with both historical and productive significance. There are three types of olives on the market: olive for oil production, olive for

the table, and dual-use. Fruit size, oil content flesh, and weight/pit weight ratio determine the inclusion in the product classes. Olives for oil production have an average weight below 3, table-use have an average weight higher than 5, dual-use between 3 and 5 [53]. Extensive research has been conducted on multiple species to understand the mechanisms that control fruit size [54]. Environmental and genetic factors affect the fruits' growth potential [55]. In particular, the water volume and the type of irrigation used for cultivation are noteworthy [56]. Water deficit results in high reactive oxygen species (ROS) (e.g., hydrogen peroxide, hydroxyl radical superoxide anion, and singlet oxygen) [57]. ROS can damage DNA, lipids, and protein, thereby affecting plants' metabolism [58]. Plants react to ROS, making enzymes with different biological activities (e.g., catalase, ascorbate peroxidase, superoxide dismutase, monodehydroascorbate reductase, dehydroascorbate reductase, guaiacol peroxidase, and glutathione reductase) [59] and primary or secondary metabolites (e.g., polyols, soluble sugars, alkaloids, free amino acids, and phenols) [60]. In this work, all treatments (excluding M10 and KV906) enhanced drupe weight, probably due to *Trichoderma* strains' ability to induce root growth, preserve nutritional uptake, and interfere with phenolics' productions [61]. The diverse response to bio-treatments was probably linked to the different abilities of each strain and metabolite to interact with the plant's secondary defense mechanisms [15]. The variation in phenolics (variety and levels) was tested using targeted and untargeted metabolomics to evaluate the interaction of *Trichoderma* spp. and metabolites with the plant defense mechanism. Targeted metabolomics uses analytical techniques and suitable multivariate statistical analysis (MSA) tools to evaluate some metabolites simultaneously [62]. The untargeted approach determines unknown and known metabolic changes based on data-independent acquisition (DIA). DIA methods make complex fragmentation spectra. In downstream data analysis steps, fragment ions are matched with precursor ions based on mass and retention time [63].

In this study, an untargeted metabolomic approach was used to evaluate phenolic profiles for olive drupes. The analysis of chromatograms led to the putative identification of 13 differentially accumulated compounds among all treatments. Oleuropein (the main secoiridoid) and luteolin (the main flavonoid) concentration increased in treated samples compared to control. Metabolomic profiles of olive drupes following the application of M10, KV906, T22, and metabolite HA were grouped separately. This result may indicate that the olive plants have a similar response when inoculated with *Trichoderma* fungi and treated with their metabolites [51]. However, the identified differential metabolites were not always common to all treatments (Figure 1). The targeted metabolomics method estimated the effects of each biological treatment on the phenolic' levels in the olive's drupes. Four more representative phenolic classes (flavonoids, secoiridoids, simple phenols, and oleosides) were considered. Following the treatments, an increase in the oleuropein's content and a decrease in the oleuropein's precursors (11-methyl ester and ligstroside) concentrations were determined, demonstrating the ability of *Trichoderma* and its metabolites to interact with the enzyme β -glucosidase [64] responsible for this biotransformation (Figure 5) [65].

About flavonoids, an improvement of the flavone luteolin's level (except in olive drupes obtained from plants treated by T22) and a decrease in the flavonol rutin's concentration (except in olive drupes obtained from plants treated by 6PP) were observed in all tested samples (Table 4), indicating the ability of the treatments (except T22 and 6PP) to affect principally the flavone synthase activity rather than flavonol synthase (Figure 6).

Finally, the levels' variation of oleuropein and luteolin in olive drupes after the bio-treatment was studied in detail. Oleuropein and luteolin were used as indicators of the secoiridoids and flavonoids variation, respectively. Oleuropein acts in *Olea europaea* as signaling molecule to protect the plant against UV-B radiation [67]. It has beneficial properties on human health, preventing cancer, cardiovascular diseases, inflammatory and oxidant damage [68]. Luteolin avoids oxidative and inflammatory processes with important implications for preventing neurodegenerative, cancer, and cardiovascular diseases and fortifying the immune system [69,70]. All *Trichoderma* spores and metabolites treatments affected the secoiridoid oleuropein and flavonoid luteolin levels (in some cases,

they were less abundant in treated plants) and generally influenced the flavonoids more than secoiridoids production. The biological treatment (6PP, GV41, and T22) improved the oleuropein content, while the luteolin levels were higher after 6PP, GV41, M10, HA, and KV906 treatments confirming a different interaction capacity of the biotreatments with the enzymes involved in the two biosynthetic pathways.

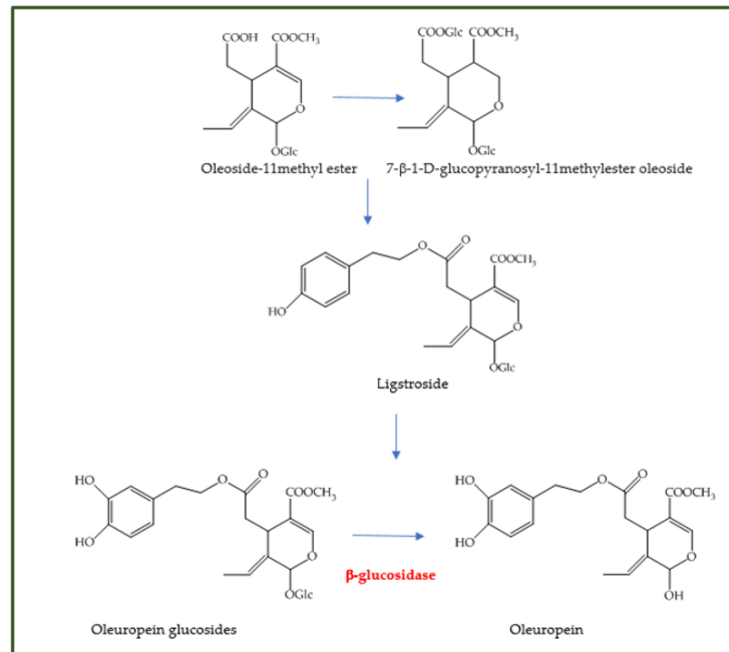


Figure 5. Oleuropein biosynthesis.

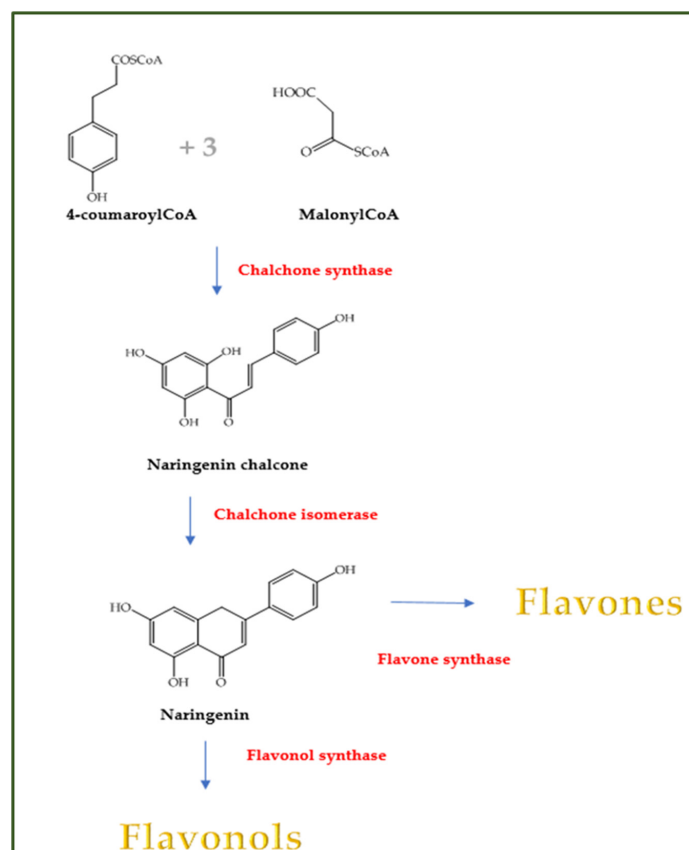


Figure 6. Flavonoid's biosynthesis [66].

5. Conclusions

Trichoderma strains and their bioactive metabolites used to cultivate the olive tree (*O. europaea* cv. Carolea) influence the weight of the drupes and the composition of phenolic compounds they contain, although in different ways depending on the strain or metabolite applied. All *Trichoderma* treatments influenced the production of flavonoids more than secoiridoids. The biological treatments' different abilities could depend on their selective aptitude to interact with the enzymes involved in flavonoid and secoiridoid production. Our results show that using the *Trichoderma* fungi and their metabolites represents a suitable alternative to synthetic fungicide since they are biocontrol agents and influence other desirable characteristics such as the size and nutraceutical properties of the olives. Furthermore, they suggest that in the future, the use of metabolites is preferable to that of living fungi as they give the same biological effects beneficial for cultivation and guarantee the nutraceutical properties of olives, avoiding some of the limitations related to the application of living microbes (difficulty in dosing concentrations to be applied on the plant and storage complications).

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11188710/s1>, Table S1. Analytical parameters used to perform the calibration curves.

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