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# Influence of Foliar Treatment with Suspensions Rich in *Trichoderma* Chlamydospores on *Momordica charantia* Physiology, Yield, and Quality

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**Abstract:** Several strategies promote phyllosphere colonization by soil-born *Trichoderma* plant-beneficial strains. One of these strategies is foliar spraying with suspensions containing large amounts of chlamydospores—spores with thick cell wall structures that make them highly resistant to harsh environmental conditions. *Trichoderma* biomass was produced by cultivation on a cornmeal medium and compared with the biomass produced on potato dextrose broth by microscopic and thermogravimetric analyses. The analyses revealed increased chlamydospore content and thermostability in the fungal biomass produced on the corn meal medium. The *Trichoderma* suspension rich in chlamydospores was sprayed on bitter gourd (*Momordica charantia*) leaves at two inoculant concentrations, $10^6$ and $10^8$ ufc/mL. The effect of these treatments on the plant physiological parameters, leaf photosynthetic pigments, polyphenol and flavonoid contents, antioxidant activities of the leaves and fruits, and yield was compared to the control (plants sprayed with water) and to the experimental treatment involving spraying with $10^8$ ufc/mL of propagules produced in potato dextrose broth. The effect of chlamydospore-rich suspensions on plant physiological parameters was more pronounced and long-lasting compared with the other treatments. The treatment with chlamydospore-rich suspension enhanced the accumulation of polyphenols and flavonoids in the leaves (by 17% and 50%, respectively) and fruits (by 18% and 31%, respectively) and increased the antioxidant activity. The *Trichoderma* treatment increased the yield by +25.33–53.07%. The application of the foliar treatment with *Trichoderma* suspensions did not modify the cytocompatibility of the extracts from the fruits determined on the L929 cells.

**Keywords:** maximum quantum yield of photosystem II; net photosynthetic rate; stomatal conductance; polyphenols; flavonoids; antioxidant activity; cytocompatibility

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## 1. Introduction

The fungal strains from *Trichoderma* (*Hypocrea*) genera are largely used as microbial inputs in agricultural technologies [1]. For decades, bioproducts based on selected *Trichoderma* strains were used to protect cultivated plants against fungal pathogens [2,3]. In the

21st century, *Trichoderma* strains were reported to be active against nematodes [4,5] and insects [6,7]. The plant-beneficial strains from *Trichoderma* genera were also recognized as plant biostimulants [8], increasing plants' nutrient uptake and nutrient use efficiency, enhancing plants' tolerance to abiotic stress, and improving crop quality traits.

The multifaceted benefits of *Trichoderma* strains on cultivated plants result after establishing and colonizing the microbiocenosis defined by belowground and above-ground plant organs, rhizospheres, and phyllospheres, respectively [9]. Colonizing strains produce various categories of bioactive compounds: lytic exo-enzymes [10], expansin-like proteins [11], other protein elicitors [12], and secondary metabolites [13] such as volatiles 6-pentyl-pyrones [14] or trichothecenes [15]. These bioactive compounds produced by *Trichoderma* populations established in the plant microbiocenosis (microbial ecological niche) control plant pathogens [16,17] and plant-feeding insects [18] and/or activate plant defense pathways [19]. Colonizing *Trichoderma* strains mediate plant interaction at multiple trophic levels [20]

Various *Trichoderma* strains were demonstrated to activate jasmonic and salicylic acid plant defense pathways [21], priming plant innate immunity in a balanced manner [22]. Plant defense elicitation by compounds produced by the *Trichoderma* microbial colonizer boosts primary metabolism, increasing nutrient uptake and nutrient use efficiency [23]. The activation of plant defense pathways results in higher plant tolerance to abiotic stress [24]. The secondary metabolism pathways associated with different plant defense pathways result in bioactive compounds being accumulated in leaves and fruits [14]. Applying the *Trichoderma* strain on plants mainly activates the phenylpropanoid pathway t [25,26], leading to a higher accumulation of polyphenols [27,28].

*Trichoderma* strains are versatile plant symbionts [29], specific to the rhizosphere and plant root surface [16,30,31]. A harsher phyllosphere environment with lower levels of nutrients and water, a highly variable temperature, and high light irradiation [32] is less permissive to non-native *Trichoderma* strains. Introducing beneficial *Trichoderma* strains into the phyllosphere is challenging. Several strategies were developed to promote the introduction of beneficial *Trichoderma* strains in the phyllosphere, with the phyllosphere microbiome being essential for plant health and productivity in a changing environment [33–36]. One strategy is to utilize protective formulations, such as dry flowable formulations that include antioxidants [37] or a talc formulation [38]. The application of a $10^{-8}$ cfu/mL suspension of *T. harzianum* TriH_JSB36 in a sterile talc mixture (1:10 v/w) to grape leaves promoted *Plasmopara viticola* biocontrol by priming grape defense responses [38]. Recently, it was suggested that oil-based formulations are important for foliar-applied products due to their ability to provide protection against UV radiation, enhance biocontrol activity, and promote better adhesion on the hydrophobic surface of the leaf cuticle [39].

Another strategy is to repeatedly apply highly active *Trichoderma* strains. *T. harzianum strain* T22 (ATCC® 20847™) was used to induce the accumulation of polyphenols in *Vitis vinifera* cv. Sangiovese grapes. Solutions with $10^8$ spores were drenched on grape leaves every 14 days, and 10 treatments were applied [28]. Such strategy determined the accumulation of polyphenols in grapes, with concomitant control of *Uncinula necator* [28]. However, the high number of treatments makes the large-scale adoption of such strategy difficult.

In a previous work, we used an alternative strategy. We applied higher concentrations of a plant biostimulant *Trichoderma* consortium with a high amount of chlamydospores to the leaves of *Passiflora cearulea* [40]. This strategy proved to be effective—the treatment induced increases in the polyphenol content and the antioxidant activities of the leaves and fruits of *P. caerulea*. We showed that the effect of the applied *Trichoderma* consortium involved chloroplast proliferation to compensate for the high light stress exerted on a shadow plant by cultivation in an open space. In this work, we intended to differentiate between the contribution of inoculation with two compatible strains, which have more chances to colonize different leaves' microhabitats, and the contribution of chlamydospores, which survive better in harsh conditions. Also, an additional goal of the present work was

to investigate the effects of the foliar application of *Trichoderma*-based plant biostimulants on another cultivated nutraceutical plant species.

The experiments were carried out by applying the same *Trichoderma* consortium, prepared from biomass obtained by cultivation on cornmeal medium (which promotes chlamydospore accumulation) and from biomass obtained from potato dextrose broth (PDB). The *Trichoderma* suspension produced on cornmeal was sprayed at two inoculant densities, $10^6$ and $10^8$ ufc/mL, and that produced on potato dextrose broth was sprayed at one inoculant density, $10^8$ ufc/mL. The plant physiological parameters; the photosynthetic pigment, polyphenol, and flavonoid contents; and the antioxidant activities of the leaves and fruits as well as the yield of the treated plants were determined and compared with those of the control group (water treatment).

The *Trichoderma* consortium treatments were applied on bitter gourd, *M. charantia*. Bitter gourd (*M. charantia*), acclimated in Romania in the past decade due its economic value [41], was chosen as a test plant due to its nutraceutical value, owing to a high accumulation of bioactive compounds in the leaves and fruits following secondary metabolism activation [42,43].

## 2. Materials and Methods

### *2.1. Biological Material*

*M. charantia* (bitter gourd, bitter cucumber) plants were cultivated under greenhouse conditions (Amia, Otopeni, Ilfov, Romania) in anthropogenic soil (hortic cerno-cambic) with 3.15% humus near Bucharest—44°33′02″ N, 26°04′12″ E, altitude 91 m. The pH of the soil horizons of 0–20 cm and 0–40 cm was slightly acidic, namely 6.12 and 6,37, respectively. The total nitrogen contents (Nt%) in the soil horizons of 0–20 cm and 0–40 cm were 0.228 and 0.182, respectively. The mobile phosphorus was 51 ppm in the 0–20 cm horizon and 43 ppm in the 0–40 cm horizon. The soil of the experimental plots was prepared by disking and raised bedding. Trellises (4.2 m long) were built for each replicate block with 3 m of space within the same raised beds and connected with ropes at a 2 m height. Irrigation was carried out using a drip irrigation system (provided by Netafim, Bucharest, Romania) with the emitters at 30 cm. Composted manure was applied to the soil in November 2022 at an equivalent dose of 5000 kg/ha, and granulated fertilizer was applied with an N:P:K ratio of 80:40:40 kg/ha. Bitter ground seedlings (cv. Avangard F1, Opal, Plovdiv, Bulgaria) of around 20 cm in height were transplanted at the beginning of May 2023 at a distance of 1.4 m × 0.75 m (equivalent to 9000 plants per ha). The cultivar was selected due to its suitability for the local conditions. The temperature was maintained between 25 ± 5 °C and 18 ± 3 °C during the day and during the night, respectively, and the relative humidity was maintained between 60 and 80% in the greenhouse.

The used *Trichoderma* consortium included two multi-functional strains from the INCDCP-ICECHIM collection, *T. harzianum* Td50b (NCAIM F001412) and *T. asperellum* T36 (NCAIM F 001434), which were proven to be effective for phyllosphere colonization in our previous experiment conducted on *P. caerulea* [40]. The used strains produce lytic exo-enzymes and volatiles, including 6-pentyl-pyrone [44,45].

The mouse fibroblast L929 cell line (ATCC, NCTC clone 929, 128 cells) supplied by the European Collection of Cell Cultures (ECACC), was used for the cytocompatibility test.

### *2.2. (Bio)Chemical Material*

Potato dextrose agar (PDA), potato dextrose broth (PDB), glycerol, and hydrochloric acid 1 M were purchased from Scharlab (Barcelona, Spain). The 96% ethanol was supplied by ChimReactiv (Bucharest, Romania). The reagent used for the determination of total polyphenols (Folin-Ciocâlteu reagent, sodium carbonate), total flavonoids (aluminum chloride, potassium acetate), and antioxidant activity (DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate, 7 mM 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, ABTS, potassium persulfate) and the standards used for calibration, i.e.,

trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, gallic acid, and quercetin, were acquired from Sigma-Aldrich (Merck Group, Darmstadt, Germany).

### *2.3. Fungal Cultivation*

The two *Trichoderma* strains T36 and Td50b were first replicated on PDA medium in Petri dishes using fungal mycelium discs of 5 mm, obtained with a flame-sterilized core borer from older cultures. The Petri dishes were incubated for 5 days at 28 °C in a static incubator (PHCbi MIR-254-PE, PHC, Tokyo, Japan). The PDB medium was prepared according to the producer's recommendations (24 g of powder in 1 L of $ddH_2O$). The cornmeal medium was prepared by adding 62.86 g of cornmeal (finely ground in an M20 universal mill, IKA, Staufen, Germany) and 7.54 mL of glycerol to 927 mL of $ddH_2O$, followed by adjusting the pH to 4.17 using 0.1 M of HCl. All media were sterilized by autoclaving at 121 °C for 15 min. After cooling to room temperature, the media were distributed in 500 mL Erlenmeyer flasks (200 mL medium/flask) and inoculated with a spore suspension of $10^6$ conidia/mL of sterile physiological saline water (10% suspension in the final volume). The Erlenmeyer flasks were incubated at 28 °C and 130 rpm agitation, in a Unimax 1010 incubator (Heidolph, Schwabach, Germany) for 7 days.

### *2.4. Morphological Analysis of T36 and Td50b Biomass by Optical Microscopy*

The hyphae and spores from the biomass of the two *Trichoderma* strains, T36 and Td50b, were visualized with a DM 1000 LED optical microscope (Leica, Wetzlar, Germany) in the brightfield (BF) module at a 40× magnification. The microscope samples were mounted as fresh slide-coverslip preparations. The average diameter of chlamydospores (determined from 20 counts per variant from 10 images) and the average thickness of hyphae (determined from 20 counts per variant from 10 images) were measured with the microscope software LAS X 5.1.0.

### *2.5. Thermogravimetric Analysis*

A thermogravimetric analysis (TGA) was performed on dried fungal biomass using Q5000IR equipment (TA Instruments, New Castle, DE, USA) on a 16–25 mg sample in a 100 µL platinum pan. For TGA, the mycelium samples were dried in sterile Petri dishes at room temperature. The samples underwent heating from 20 °C to 700 °C with a rate of 10 °C per min under a nitrogen flow rate of 50 mL/min (99.999%). At 700 °C, the purge gas was changed to synthetic air (99.999%) in hi-res mode, and this configuration was maintained isothermally for 5 min to determine the ash content.

### *2.6. Experimental Treatments*

The experiment included the following: $V_1$—control (subjected to water treatment 200 L/ha spraying volume); $V_2$—foliar treatment with *Trichoderma* spores obtained in the cornmeal medium ($T_{CM}$), a suspension normalized to $10^6$ cfu/mL, a spraying volume of 200 L/ha, and a spore dose of $2 \times 10^{11}$ spores/ha; $V_3$—foliar treatment with *Trichoderma* spores obtained in PDB medium ($T_{PD}$), a suspension normalized to $10^8$ cfu/mL, a spraying volume of 200 L/ha, and a spore dose of $2 \times 10^{13}$ spores/ha; and $V_4$—foliar treatment with *Trichoderma* spores obtained in the cornmeal medium ($T_{CM}$), a suspension normalized to $10^8$ cfu/mL, a spraying volume of 200 L/ha, and a spore dose of $2 \times 10^{13}$ spores/ha. Each experimental repetition included 30 *M. charantia* plants. There were 3 repetitions in a randomized block design. Each repetition was separated by a 3 m space. An SG20 backpack sprayer (Stihl AG, Waiblingen, Germany) with an applied pressure of 275 kPa was used for the application of the treatment. The experimental treatment involved the application of two separate foliar spraying events at the beginning of June (beginning of male flowering) and, after 4 weeks, at the beginning of July (female flower development). The spraying on leaves was carried out with a flat jet and low drift nozzle (TeeJett® flat-fan TT11002 model, Spraying Systems, Wheaton, IL, US). Yield was determined for all harvesting waves from all plants in a plot and normalized as kg/ha.

### *2.7. Determination of Plant Physiological Characteristics*

The following physiological characteristics were determined one week after the 1st treatment and 4 weeks after the 2nd treatment (8 weeks after the 1st treatment): maximum quantum yield of photosystem II—PSII, *Fv*/*Fm* (by chlorophyll fluorescence—saturation of the photosynthetic centers from PSII); net photosynthetic rate (Pn) ($CO_2$ fixed in the photosynthesis); and stomatal conductance (rate of exchange of gases through stomata, including water vapors). Ten randomly selected leaves from each repetition were selected for chlorophyll fluorescence determination. The measurements were carried out with a pulse-amplitude-modulated (PAM) fluorometer (Walz PAM 2500, Effertlich, Germany). According to the protocol for correctly determining the chlorophyll fluorescence parameters, the leaves were darkened for 30 min. After the darkening period, saturation light pulses were applied. The maximum photosystem II (PSII) quantum efficiency was determined as the ratio between the *Fv*, the variable fluorescence, and the *Fm*, the maximum fluorescent yield in the dark-adapted state [46]. The net photosynthetic rate was assessed using a broadleaf chamber integrated in a leaf gas exchange unit (model LCpro T, ADC Bioscientific Ltd., Herts, England). A porometer (AP4, Delta-T Devices, Burwell, UK) was used to determine the stomatal conductance according to the manufacturer's instructions. The determination of each plant's physiological parameters was repeated three times; middle and upper leaves were randomly selected from 10 plants for each repetition.

### *2.8. Determination of Total Polyphenols and Total Flavonoids*

The plant materials, leaves, and fruits were sampled at the end of August, 12 weeks after the first treatment. The leaves' material was sampled from the middle and upper leaves from 10 plants from each repetition, randomly selected. The immature, pre-climacteric fruits were randomly sampled from 10 plants from each repetition. The plant material was dried under a vacuum in a VD56 oven (Binder, Tuttlingen, Germany) at 50 °C for 24 h. The dried bitter gourd biomass was ground using a laboratory mill (SM2000, Retsch, Haan, Germany) and extracted by maceration in a 70% ethanol/water (*v*/*v*) solution for 10 days. After 10 days of maceration, the non-extracted plant material was separated by filtration. The Folin–Ciocâlteu method [47] with a slight adaptation [48] was used to determine the total polyphenol content from the ethanolic extract. Briefly, 150 µL of each sample was mixed with 750 µL of Folin–Ciocâlteu reagent, 4 mL of 15% $Na_2CO_3$, and 10.1 mL of $ddH_2O$, resulting in a final reaction volume of 15 mL. The reaction was incubated for 2 h at room temperature in the dark. The optical density (OD) was measured at λ = 756 nm and converted to polyphenol concentration using a calibration curve constructed with gallic acid (GA). The colorimetric method with aluminum chloride was used to determine the total flavonoids using a calibration curve with quercetin (Q) [49]. Briefly, 0.5 mL of bitter gourd macerate sample was mixed with 1.5 mL of ethanol, 0.1 mL of 1 M potassium acetate, 0.1 mL of 10% aluminum chloride, and 2.8 mL of $ddH_2O$. The mixture was incubated for 30 min at room temperature, and the OD was measured at λ = 415 nm. All analysis were performed in triplicate.

### *2.9. Antioxidant Activity Assay*

The antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method and the Trolox equivalent antioxidant capacity (TEAC), as described in our previous work. Known quantities of dried extracts were resolubilized using known volumes of absolute alcohols.

#### 2.9.1. DPPH Scavenging Activity Assay

The method adapted in our previous work from Braca et al. [50] was used. Briefly, the mixture consisted of 150 µL 0.25 mM methanolic solution of DPPH, 90 µL 0.1 M of Tris-HCl buffer, and 15 µL of the sample (resolubilized in methanol). After incubating in the dark at

37 °C for 30 min., the absorbance was read at λ = 520 nm against methanol as a blank and Equation 1 was used to estimate the DPPH inhibition (%):

$$\% \, Inhibition = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100 \quad (1)$$

where $A_{sample}$ is the absorbance of the sample and $A_{blank}$ is the absorbance of the blank (methanol).

#### 2.9.2. Antioxidant Capacity (TEAC) Assay

The method adapted after Re et al. [51] is described in depth in our previous work. Briefly, after generating the ABTS radical and bringing the ABTS solution to an absorbance of 0.7 ± 0.02 at λ = 734 nm, it was mixed with the sample in a 10:1 ratio, and the mixture was incubated for 6 min. The absorbance at 734 nm was expressed as μg Trolox/mL.

All assays of DPPH and TEAC were performed in triplicate.

### *2.10. Cytocompatibility Assay*

The dried macerate sample from bitter gourd fruits were resuspended into phosphate saline buffer (PBS) and sterilized through 0.22 μm syringe filters (Merck Millipore, Darmstadt, Germany). The stabilized line of mouse fibroblast L929 cells (ATCC NCTC clone 929 cell line) provided by the European Collection of Cell Cultures (ECACC) was used for the cytocompatibility assay. Three concentrations (50, 100, and 200 μg/mL) of bitter gourd extracts were tested. The NCTC L-929 cells were cultivated as already described [40]. The cell viability was determined using a colorimetric method based on the ability of viable cells to incorporate a supravital dye, Neutral Red (NR) [52], as described in our previous work [40]. The results represent the viability percentage from the control sample V1, which is considered to be 100%.

### *2.11. Statistical Analysis*

A statistical analysis was conducted (One-Way ANOVA) using the SPSS 21 software package (IBM, Armonk, NY, USA) with Duncan's multiple range test. The data were analyzed in evolution. A least significant difference (LSD) test was employed at a significance level of $p < 0.05$.

## 3. Results and Discussion

The morphological features of the *Trichoderma* strains used, T36 and Td50b, in both PDB and the cornmeal medium were long cylindrical hyphae with a straight or sinuous aspect, segmented in several compartments with one or more nuclei; green micro- and macroconidia with a globular aspect; and spherical chlamydospores with thick walls, either intercalated or at the end of the hyphae (Figures 1, S1 and S2).

Chlamydospores were more abundant in the culture with cornmeal than with PDB, which is in agreement with previous studies on corn flour and *T. harzianum* [53]. Another observation worth mentioning is the presence of numerous vacuoles in the hyphae grown in the cornmeal medium (see blue arrows in Figures 1c,d, S1 and S2) that were not observed in PDB. Overall, the mycelia from the cornmeal samples had a glassy, bright aspect.

The average diameter of the chlamydospores was slightly higher in the cornmeal medium (15.19 ± 2.91 μm and 16.32 ± 3.59 μm for T36 and Td50b, respectively) than in PDB (10.55 ± 2.48 μm and 10.49 ± 2.11 μm for T36 and Td50b, respectively), as shown in Figure Figure 2a. The hyphae developed in the cornmeal medium had a higher average thickness (7.49 ± 1.45 and 7.59 ± 1.79 μm for T36 and Td50b, respectively) than in PDB (5.20 ± 1.75 μm and 5.26 ± 0.86 μm for T36 and Td50b, respectively), as shown in Figure 2b.

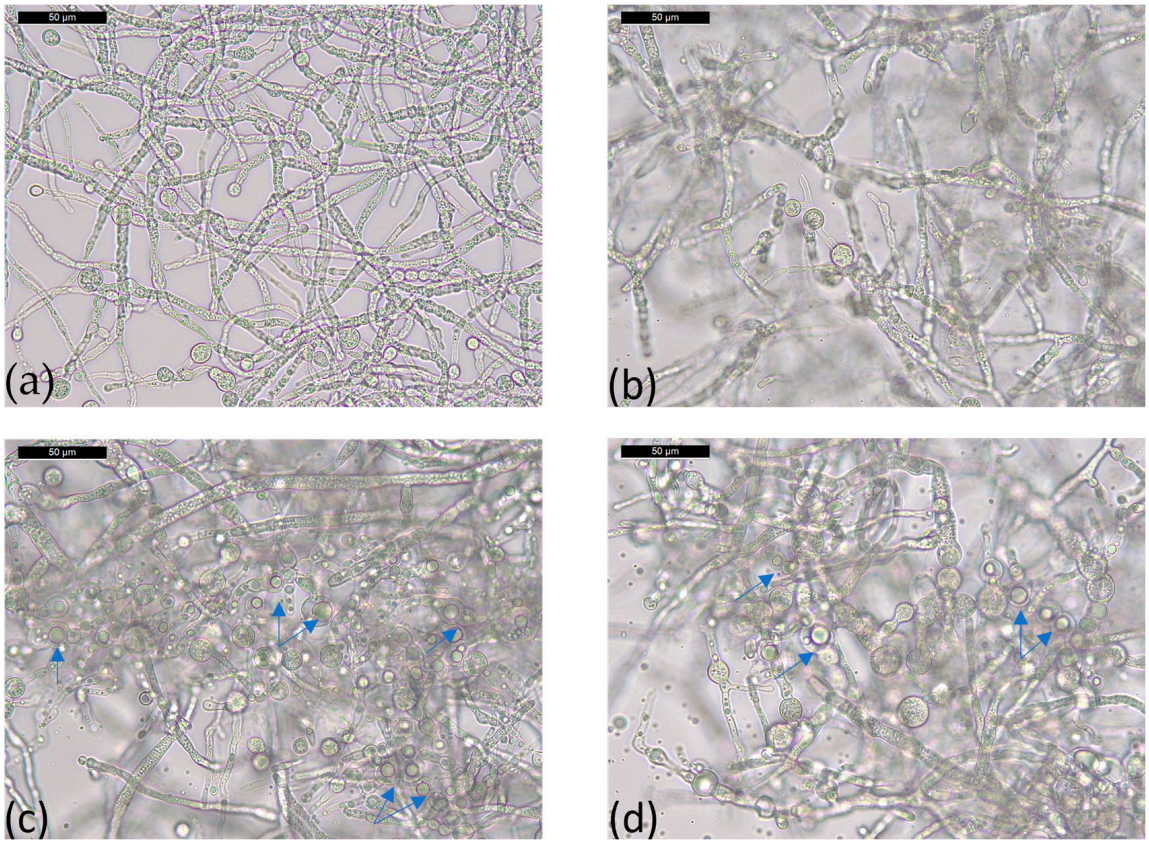


**Figure 1.** Optical microscopical images of *T. asperellum* T36 NCAIM F 001434 (**a**,**c**) and *T. harzianum*, Td50b, NCAIM F001412 (**b**,**d**) grown in PDB (**a**,**b**) and cornmeal medium (**c**,**d**). The blue arrows indicate the presence of vacuoles/droplets inside the hyphae in the case of the cornmeal medium.

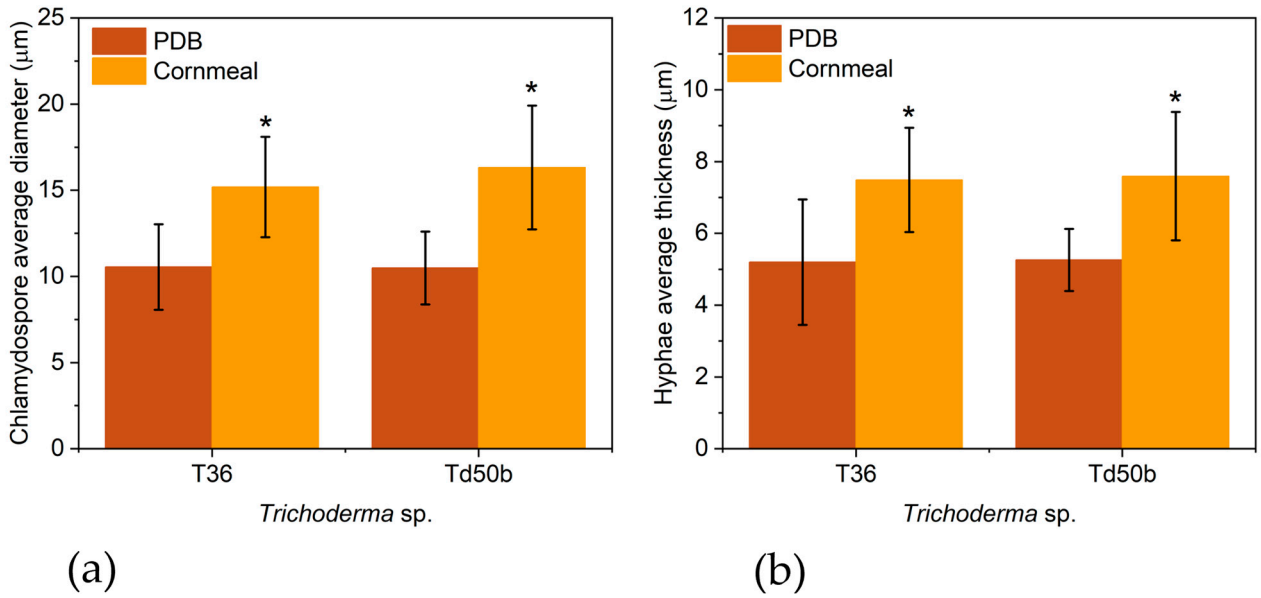


**Figure 2.** The average diameter of chlamydospores of *T. asperellum* T36 NCAIM F 001434 and *T. harzianum*, Td50b, NCAIM F001412 (**a**) grown in PDB and cornmeal medium, respectively, and the average thickness of the hyphae of *T. asperellum* T36 NCAIM F 001434 and *T. harzianum*, Td50b, NCAIM F001412 (**b**) grown in PDB and cornmeal medium, respectively (±error bars, α extless 0.05, $n = 20$). The asterisks represent statistically significant differences between cornmeal and PDB for T36 and Td50b: * ($0.01 < \alpha < 0.05$).

The thermogravimetric analysis revealed three main transitions in the thermograms of the *Trichoderma* biomass (Table 1, Figure 3 and Figure S3).

**Table 1.** Quantitative parameters from TGA analysis of T36 and Td50b.

| Parameter | T36 PDB | T36 Cornmeal | Td50b PDB | Td50b Cornmeal | FC |
|---|---|---|---|---|---|
| T1 (°C) | 116.5 ± 9.5 | 142.0 ± 10.5 * | 101.6 ± 3.0 | 133.0 ± 4.6 ** | 43 |
| WL1 (%) | 4.41 ± 0.12 | 4.73 ± 0.10 | 4.48 ± 0.11 | 4.65 ± 0.14 | 5.36 |
| T2 (°C) | 299.1 ± 1.1 | 305.2 ± 1.7 * | 302.1 ± 0.4 | 307.2 ± 0.7 ** | 380.0 |
| WL2 (%) | 27.89 ± 0.47 | 34.39 ± 1.74 * | 31.58 ± 0.34 | 36.44 ± 0.66 ** | 76.14 |
| T3 (°C) | 411.0 ± 1.8 | 408.8 ± 1.5 | 409.3 ± 0.6 | 406.6 ± 0.7 * | 617.1 |
| WL3 (%) | 48.88 ± 0.72 | 49.45 ± 1.20 | 45.74 ± 0.43 | 47.58 ± 0.69 | 12.36 |
| Residue (%) # | 18.82 ± 0.13 | 11.43 ± 0.64 *** | 18.20 ± 0.51 | 11.33 ± 0.39 *** | 6.14 |
| Ash (%) ## | 7.19 ± 0.51 | 0.32 ± 0.05 ** | 4.78 ± 0.60 | 0.31 ± 0.03 ** | 0.67 |
| Residue-Ash | 11.63 ± 0.30 | 11.11 ± 0.37 | 13.42 ± 0.45 | 11.02 ± 0.23 | 5.47 |

PDB—potato dextrose broth medium; WL—weight loss; FC—fungal chitin; # (700 °C/N2); ## (700 °C/Air). The asterisks represent statistically significant differences between cornmeal and PDB for T36 and Td50b: * ($0.01 < \alpha < 0.05$), ** ($0.001 < \alpha < 0.01$), *** ($\alpha < 0.001$).

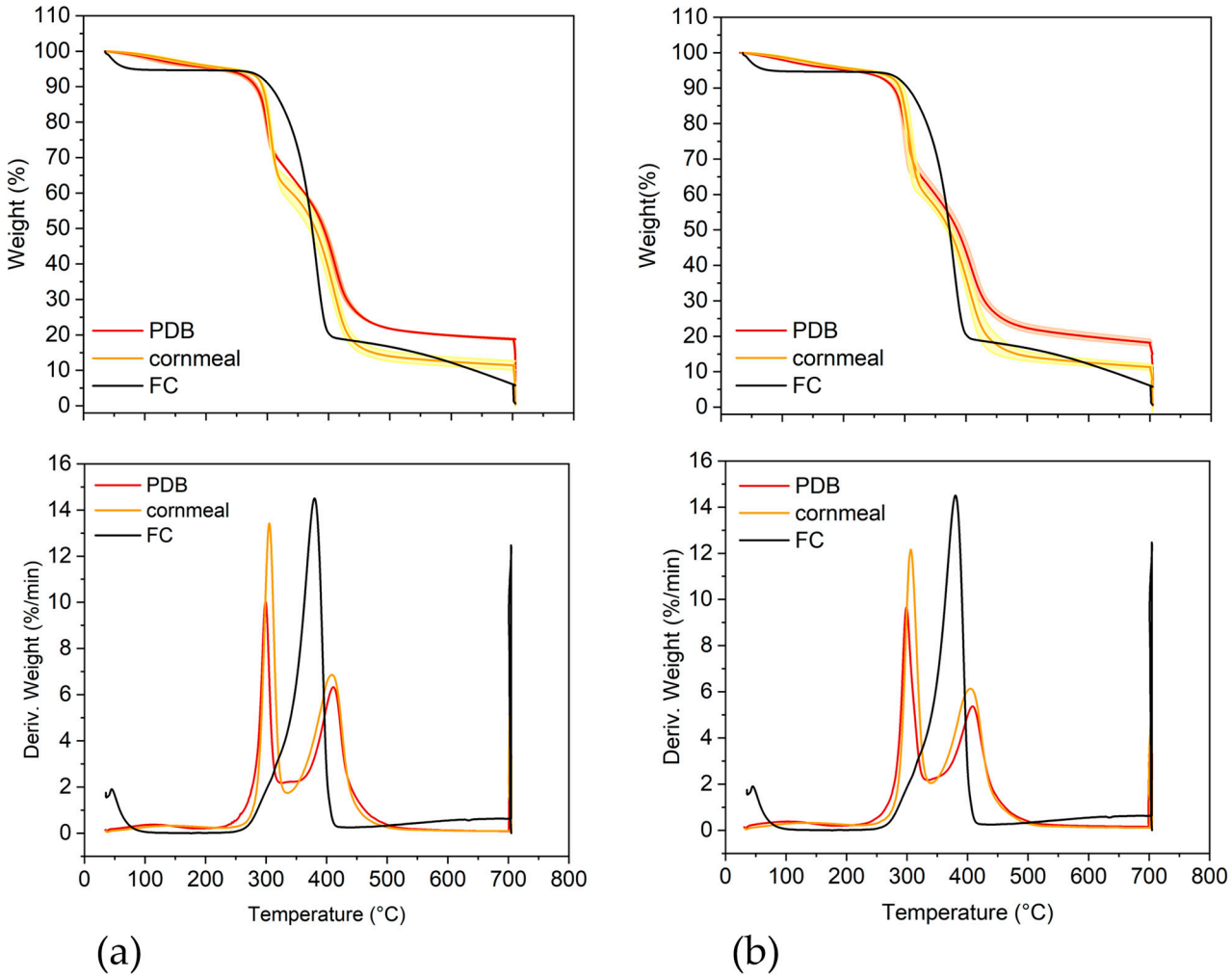


**Figure 3.** The thermogravimetric analysis of *T. asperellum* T36 NCAIM F 001434 (**a**) and *T. harzianum*, Td50b NCAIM F001412 (**b**) grown in potato dextrose broth (PDB) or cornmeal medium. The weights (%) of T36 and Td50b are shown with the error bar limits (light contour, 95% interval confidence). FC—commercial fungal chitin.

The first transition was between 30 °C and approximately 190 °C for PDB and 220 °C for cornmeal. The peak temperature of this transition (T1) was higher for the fungal biomass grown in the cornmeal medium (142.0 ± 10.5 °C for T36 and 133.0 ± 4.6 °C for Td50b) than in PDB (116.5 ± 9.5 °C for T36 and 101.6 ± 3.0 °C for Td50b). These differences were statistically significant. The weight loss (WL) was slightly higher for T36 and Td50b in cornmeal than in PDB. This transition was very broad and can be assigned to bound water, volatiles, and thermolabile compounds. The increase in the transition temperature, without significant changes in WL, suggests stronger bound water molecules, stronger interactions, and/or the presence of more thermostable compounds in the mycelium grown in the cornmeal than in PDB.

The second transition was up to 330 °C and 340 °C for mycelium from the cornmeal medium and from PDB, respectively. The peak temperature of this transition (T2) was higher for the fungal biomass grown in the cornmeal medium (305.2 ± 1.7 °C for T36 and 307.2 ± 0.7 °C for Td50b) than in PDB (299.1 ± 1.1 °C for T36 and 302.1 ± 0.4 °C for Td50b). The WL was higher as well by approximately 5–7% for the cornmeal medium compared to the PDB medium. This transition has previously been shown to have a significant contribution from β-glucan in macromycetes [53,54], and it probably includes other polysaccharides, lipids, and proteins as well. Our results indicate an increase in the biomasses of T36 and Td50b mycelium represented by these compounds when the strains were grown in the cornmeal medium compared to the control in PDB.

The third transition was from 330/340 °C to 700 °C. This transition was characterized by a peak temperature (T3) and WL that were slightly higher than the mycelium from the cornmeal medium compared to that of PDB. This transition has been shown to have a contribution from chitin in macromycetes [54,55]. In our case, the standard chitin had the main peak at an intermediate temperature (380 °C) between T2 and T3, closer to T3. These data suggest that the chitin in these strains is more thermostable than the isolated chitin and the chitin from the macromycetes.

A significant effect was observed for the residue and ash contents that were significantly decreased in the cornmeal compared with PDB (Table 1). This indicates a lower accumulation of minerals. It is worth mentioning that the ash content of T36 was higher than that of Td50b in PDB but became similar in the cornmeal. This indicates that the effect of cornmeal on ash accumulation was higher for T36 than for Td50b. The difference between the residue and the ash represents carbonaceous structures that formed upon thermal degradation. This was similar for T36 grown in PDB and cornmeal (11.63 ± 0.30 versus 11.11 ± 0.37%, respectively) but slightly lower for Td50b grown in cornmeal medium than in PDB (11.02 ± 0.23 versus 13.42 ± 0.45%, respectively). Td50b seemed to accumulate less carbon in cornmeal than in PDB, and the values in cornmeal were similar between T36 and Td50b.

The optical microscopy and the thermogravimetric analysis demonstrated a more significant proportion of chlamydospores in the biomass produced in the cornmeal medium than in PDB. The larger amount of chlamydospore in the biomass produced in cornmeal than in the PDB medium is also demonstrated by the thermogravimetric analysis. To the best of our knowledge, a thermogravimetric analysis has not been previously used for the characterization of the *Trichoderma* biomass containing spores—it was only used for the characterization of different types of *Trichoderma* formulations, such as *T. harzianum* LQC-99 encapsulated in cellulose nanocrystals (CNC) or in a nanocomposite of CNC with carboxymethyl cellulose (CMC) [56] or *T. harzianum* UPM40 encapsulated in Ca-alginate–montmorillonite clay beads [57].

In the case of bacterial spores, a thermogravimetric analysis was used to demonstrate that water distribution is the key factor in spore resistance to adverse conditions such as high temperatures [58]. Our TGA data reveal a higher evaporation temperature for the bound water in the biomass obtained from cornmeal than the PDB medium—this demonstrates that a higher proportion of spores with higher resistance (chlamydospores) in the biomass was produced in the cornmeal medium than in PDB.

Figure 4 illustrates the influence of different treatments with the *Trichoderma* consortium on *M. charantia*'s physiological parameters. The foliar treatment with the *Trichoderma* consortium improved the photosynthesis in *M. charantia.* The yield of PS II was slightly enhanced (8% increase compared with the control V1). A similar low increase (3%) compared to the control (V1) at the limit of the statistical significance was demonstrated for the net photosynthetic rate. The most significant effect was seen for the stomatal conductance, which was very significantly improved by the treatment with the *Trichoderma* consortium.

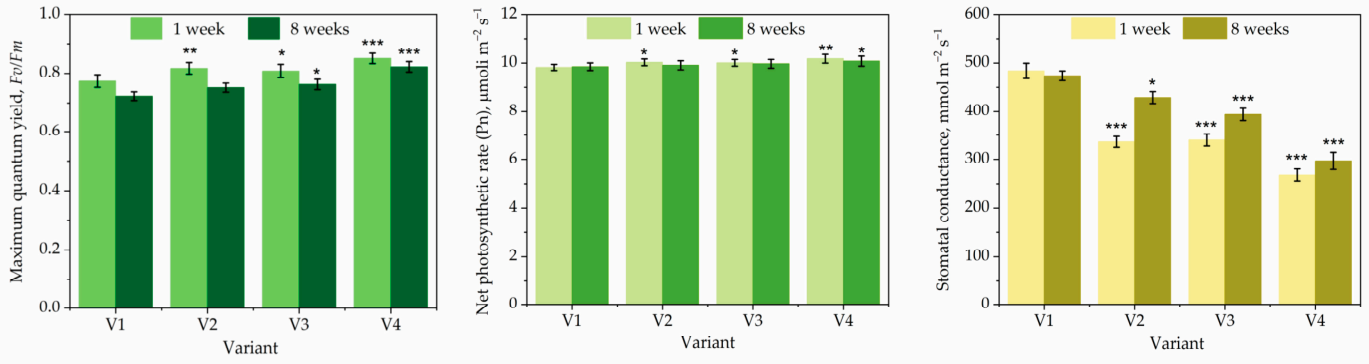


**Figure 4.** The influence of different treatments with the *Trichoderma* consortium on *M. charantia*'s physiological parameters. V1—control (foliar treatment with water, 200 L/ha spraying volume); V2—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^6$ cfu/mL, 200 L/ha spraying volume; V3—foliar treatment with *Trichoderma* spores obtained in potato-dextose broth medium ($T_{PD}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; V4—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume. The bars represent the means ± standard errors ($n$ = 30 plants). The asterisks represent statistically significant differences between V2–V4 and V1: * ($0.01 < \alpha < 0.05$), ** ($0.001 < \alpha < 0.01$), *** ($\alpha < 0.001$).

In our previous work, we demonstrated a similar effect on blue passion flower, *P. caerulea* [40]. In this work, we differentiate the effect of a compatible consortium, with strains showing different plasticity and adaptability to the highly variable conditions of the phyllosphere and the abundance of chlamydospores. The treatment with chlamydospore formulation lasts for a longer period. The application of a lower dose of from propagules, namely $10^6$ ufc/mL, which resulted from the biomass rich in chlamydospores produced in cornmeal, is almost similar with the higher dose of $10^8$ ufc/mL, produced in PDB. *Trichoderma* rich in chlamydospores has a long-lasting plant biostimulant effect. Most probably, the higher survival rate of the chlamydospore in the harsh environment determines such effect. Understanding the exact mechanism of such biostimulant action needs further investigations.

*Trichoderma* application in the rhizosphere was demonstrated to cause metabolic reprogramming. The application of *T. harzianum* T22 on zucchini squash (*Cucurbita pepo* L.) cv. 'San Pasquale' triggered ROS-mediated signaling processes and improved the photosynthetic process by 4% after 30 days in one study [59] compared with the approximately 8% improvement seen after just 1 week in our case, which was maintained even after 8 weeks. *T. harzianum* T6776 inoculated to the root system of Micro-Tom tomatoes (*Solanum lycopersicum*) improved the photosynthetic activity under lab conditions at 200–300 μmol $m^{-2}$ $s^{-1}$ light intensity; the quantum yield of the PSII system increased slightly (1%) but with a statistically significant difference compared to the control, and the net photosynthetic rate increased by a significant 30% compared with the control [60].

The yields of the different experimental treatments are presented in Table 2.

**Table 2.** The effects of different experimental treatments on the yield of bitter gourd in a Bucharest greenhouse.

| Nr. crt. | Treatment * | Dose | No. of Applications | Production (kg/ha) | Additional Yield | |
|---|---|---|---|---|---|---|
| | | | | | kg/ha | % |
| V1 | Control | - | - | 12,019 | | 100 |
| V2 | *Trichoderma* $T_{CM}$ | $2 \times 10^{11}$ ufc/ha | 2 | 16,962 | 4943 *** | 141.13 |
| V3 | *Trichoderma* $T_{PD}$ | $2 \times 10^{13}$ kg/ha | 2 | 15,064 | 3045 ** | 125.33 |
| V4 | *Trichoderma* $T_{CM}$ | $2 \times 10^{13}$ ufc/ha | 2 | 18,398 | 6379 *** | 153.07 |
| | | | | | LD 5%—1465 kg/ha<br>LD 1%—2013 kg/ha<br>LD 0.1%—3059 kg/ha | |

* V1, control, water treatment, 200 L/ha spraying volume; V2, foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^6$ cfu/mL, 200 L/ha spraying volume; V3—foliar treatment with *Trichoderma* spores obtained in potato-dextrose broth medium ($T_{PD}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; V4—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; LD: limit differences. The asterisks represent statistically significant differences between V2–V4 and V1 (control): * ($0.01 < p < 0.05$), ** ($0.001 < p < 0.01$), *** ($p < 0.001$).

The treatment with the *Trichoderma* consortium grown on cornmeal induced higher yields (141% and 153% of the control at the doses of $2 \times 10^{11}$ ufc/ha and $2 \times 10^{13}$ ufc/ha, respectively) than the treatment with the *Trichoderma* consortium grown on PDB (125% of control at the dose of $2 \times 10^{11}$ ufc/ha). The yield values demonstrated that the highest influence was exerted by the *Trichoderma* consortium grown in the cornmeal medium ($T_{CM}$), which contains a higher proportion of chlamydospores, which are more resistant to harsh environmental conditions than conidia. Such chlamydospores sustain the colonization of the phyllosphere by *Trichoderma* strains originating from soil and enhance the biostimulant effects. Very few studies are related to the use of *Trichoderma* as a plant biostimulant applied through foliar treatment. *Trichoderma* applied as a foliar treatment increased the quality of the tomatoes grown in different hydroponic substrates [61]. The accumulation of the total soluble solid (TSS, Brix) was enhanced by 14% at the plant from the first level after treatment with a mixture of *T. asperelloides* VSL4 (MH370294) and *T. koningiopsis* VSL185 (KU215377).

*Trichoderma* was tested for foliar application mainly for plant pathogen control [17,62]. One of the mechanisms involved in plant pathogen control is the activation of the plant's defense system [17]. The activation of plant defense is also involved in the biostimulant effect [40]. In several situations, the control of plant pathogens following the foliar application of *Trichoderma* was associated with an increase in the crop quality, e.g., an accumulation of 35% more polyphenols in grape and a 48.7% increase in the antioxidant activity compared to the control [28]. *T. harzianum* T39 controls *Plasmopara viticola* on susceptible grapevine, cv Pinot Noir, grown in pots, through the activation of phenylalanine ammonia lyase (PAL) and stilbene synthase (STS) [63]. The activation of these stress-related enzymes enhance the biosynthesis and accumulation of resveratrol in grape [64,65]. However, to date, there are no reports on the elicitation of the resveratrol biosynthesis and accumulation in grape following *Trichoderma* foliar treatment to the best of our knowledge.

The foliar application of *Trichoderma* enhanced the accumulation of polyphenols and flavonoids in the leaves of bitter gourd (Figure 5a) and the antioxidant activity (Figure 5b).

The chlamydospore formulations are more effective than the control formulation from the PDB medium in inducing the biosynthesis and accumulation of antioxidant polyphenols/flavonoids. The low dose of $10^6$ ufc/mL produced on cornmeal ($T_{CM}$) has a similar effect with the high dose produced in PBD ($T_{PD}$). A similar effect of a higher accumulation of polyphenols (35% more TPC and 24% more TFC) in the leaves compared to the control was demonstrated for the application of the same consortia, T36 and Td50b, on blue passion flower [40]. The application of *Trichoderma* strains on the rhizosphere was demonstrated to induce the accumulation of polyphenols [59]. The effect on inducing the biosynthesis and accumulation of antioxidant polyphenols/flavonoids is similar for fruits

(18%/31% higher than the control) and leaves (17%/50% higher than the control), as shown in Figures 5a and 6a.

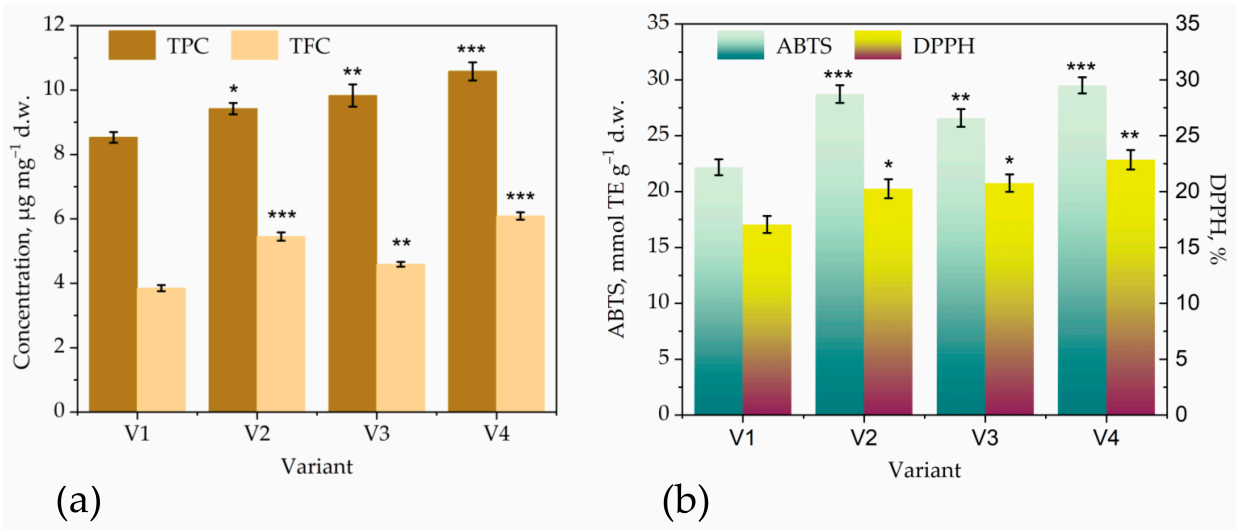


**Figure 5.** The total polyphenol and total flavonoid contents (**a**) and antioxidant activity (**b**) of *M. carnantia* leaves, as a function of the treatments with the *Trichoderma* consortium. V1—control (foliar treatment with water, 200 L/ha spraying volume); V2—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^6$ cfu/mL; V3—foliar treatment with *Trichoderma* spores obtained in potato-dextose broth medium ($T_{PD}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; V4—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume. The values represent the means ± standard errors ($n$ = 30 plants). The asterisks represent statistically significant differences between V2–V4 and V1: * ($0.01 < \alpha < 0.05$), ** ($0.001 < \alpha < 0.01$), *** ($\alpha < 0.001$).

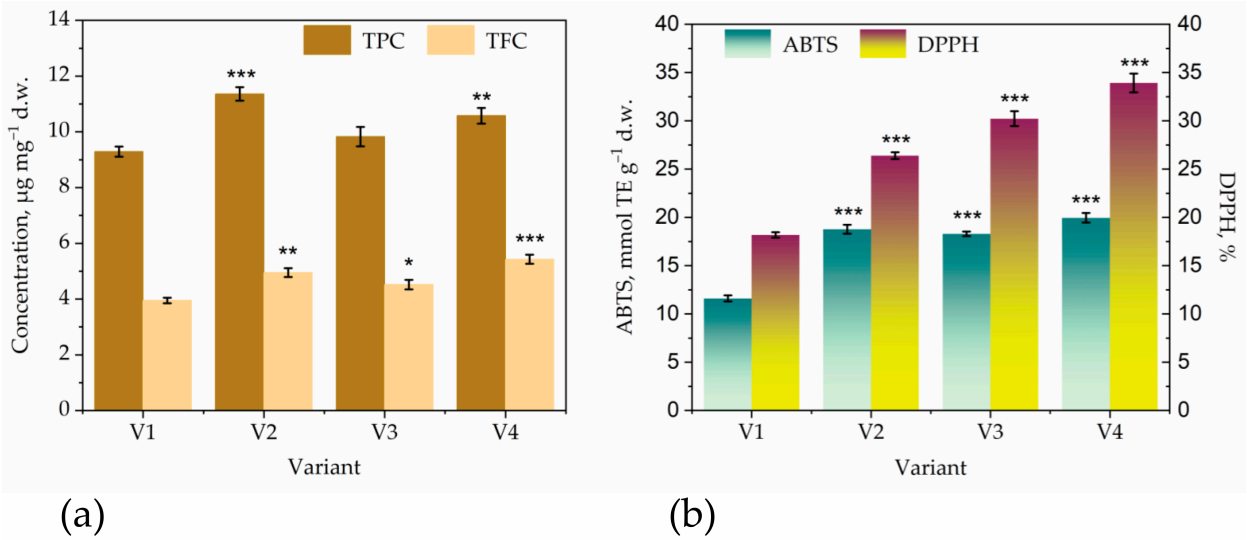


**Figure 6.** Total polyphenol and total flavonoid contents (**a**) and antioxidant activity (**b**) of bitter gourd (*M. charantia*) fruits. V1—control (foliar treatment with water, 200 L/ha spraying volume); V2—foliar treatment with *Trichoderma* spores obtained in the cornmeal medium ($T_{CM}$), suspension normalized to $10^6$ cfu/mL, 200 L/ha spraying volume; V3—foliar treatment with *Trichoderma* spores obtained in potato-dextose broth medium ($T_{PD}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; V4—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume. Values represent means ± standard errors ($n$ = 30 plants). Asterisks represent statistically significant differences between V2–V4 and V1: * ($0.01 < \alpha < 0.05$), ** ($0.001 < \alpha < 0.01$), *** ($\alpha < 0.001$).

The increase in the polyphenol content in the fruits was not associated with an enhancement in the cytotoxic effect of fruit extracts, as shown in Figure 7. The change in cell viability was similar between V1, V2, V3, and V4 compared with the negative control C, and the values were not statistically reduced below 80% viability even at the highest dose tested, i.e., 200 μg $mL^{-1}$ of fruit extract.

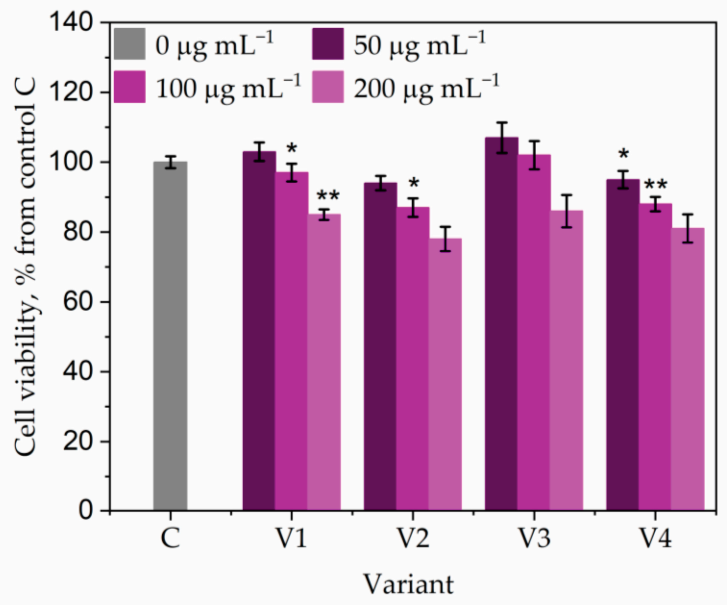


**Figure 7.** The influence of different treatments with the *Trichoderma* consortium on the cytocompatibility of *M. charantia* fruit extracts. C—negative control (no cell treatment); V1—control (foliar treatment with water, 200 L/ha spraying volume); V2—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^6$ cfu/mL, 200 L/ha spraying volume; V3—foliar treatment with *Trichoderma* spores obtained in potato-dextose broth medium ($T_{PD}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume; V4—foliar treatment with *Trichoderma* spores obtained in cornmeal medium ($T_{CM}$), suspension normalized to $10^8$ cfu/mL, 200 L/ha spraying volume. The values represent means ± standard errors ($n$ = 30 plants). The asterisks represent statistically significant differences between V1-V4 and C: * ($0.01 < \alpha < 0.05$), ** ($0.001 < \alpha < 0.01$).

## 4. Conclusions

The foliar application of a *Trichoderma* consortium chlamydospore formulation to *M. charantia* promotes phyllosphere colonization and determines a long-lasting biostimulant effect, which is more pronounced when applying the *Trichoderma* biomass with a higher abundance of chlamydospores. The photosynthetic activity was increased by 8%, the yield was increased by 53%, the polyphenol and flavonoid contents of the fruits were increased by 18% and 31%, respectively, and the antioxidant activity was increased by 80% compared with the control. In conclusion, the treatment had the highest impact on the yield and crop quality (antioxidant compounds and antioxidant activity). More investigations related to the mechanism involved in phyllosphere colonization will offer new possibilities for the further improvement of the foliar treatment with plant biostimulant *Trichoderma*, including through "smart" controlled-release formulations.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10040371/s1, Figure S1: Optical microscopical images of *T. asperellum* T36 NCAIM F 001434 grown in PDB (a,b) and cornmeal medium (c,d); Figure S2: Optical microscopical images of *T. harzianum*, Td50b, NCAIM F001412 grown in PDB (a,b) and cornmeal medium (c,d); Figure S3: Thermogravimetric analysis of *T. asperellum* T36 NCAIM F 001434 (a,c) and *T. harzianum*, Td50b, NCAIM F001412 (b,d) grown in PDB (a,b) and cornmeal medium (c,d).

**Author Contributions:** Conceptualization, D.C.-A. and F.O.; methodology, I.-A.B., A.O., O.C., M.G., C.-A.N. and D.C.-A.; validation, D.C.-A., T.E.Ș. and F.O.; formal analysis, D.C-A. and B.T.; investigation, I.-A.B., A.O., O.C., M.G., I.R. and C.-A.N.; resources, F.O. and T.E.Ș.; data curation, D.C.-A.; writing—original draft preparation, I.-A.B. and T.E.Ș.; writing—review and editing, D.C.-A. and F.O.; visualization, D.C.-A., I.-A.B. and B.T.; supervision, F.O.; project administration, D.C.-A.; funding acquisition, D.C.-A. All authors have read and agreed to the published version of the manuscript.

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## References

1. Abdullah, N.S.; Doni, F.; Mispan, M.S.; Saiman, M.Z.; Yusuf, Y.M.; Oke, M.A.; Suhaimi, N.S.M. Harnessing *Trichoderma* in Agriculture for Productivity and Sustainability. *Agronomy* **2021**, *11*, 2559. [CrossRef]
2. Guzmán-Guzmán, P.; Kumar, A.; de los Santos-Villalobos, S.; Parra-Cota, F.I.; Orozco-Mosqueda, M.D.; Fadiji, A.E.; Hyder, S.; Babalola, O.O.; Santoyo, G. *Trichoderma* Species: Our Best Fungal Allies in the Biocontrol of Plant Diseases—A Review. *Plants* **2023**, *12*, 432. [CrossRef] [PubMed]
3. Rodrigues, A.O.; De Mio, L.L.M.; Soccol, C.R. *Trichoderma* as a powerful fungal disease control agent for a more sustainable and healthy agriculture: Recent studies and molecular insights. *Planta* **2023**, *257*, 31. [CrossRef] [PubMed]
4. Sharon, E.; Bar-Eyal, M.; Chet, I.; Herrera-Estrella, A.; Kleifeld, O.; Spiegel, Y. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma Harzianum*. *Phytopathology* **2001**, *91*, 687–693. [CrossRef]
5. Sahebani, N.; Hadavi, N. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Soil Biol. Biochem.* **2008**, *40*, 2016–2020. [CrossRef]
6. Rodríguez-González, A.; Casquero, P.A.; Suárez-Villanueva, V.; Carro-Huerga, G.; Alvarez-García, S.; Mayo-Prieto, S.; Lorenzana, A.; Cardoza, R.E.; Gutiérrez, S. Effect of trichodiene production by *Trichoderma harzianum* on *Acanthoscelides obtectus*. *J. Stored Prod. Res.* **2018**, *77*, 231–239. [CrossRef]
7. Rodriguez-Gonzalez, A.; Guerra, M.; Ramirez-Lozano, D.; Casquero, P.A.; Gutierrez, S. Germination and Agronomic Traits of *Phaseolus vulgaris* L. Beans Sprayed with *Trichoderma* Strains and Attacked by *Acanthoscelides obtectus*. *Agronomy* **2021**, *11*, 2130. [CrossRef]
8. López-Bucio, J.; Pelagio-Flores, R.; Herrera-Estrella, A. *Trichoderma* as biostimulant: Exploiting the multilevel properties of a plant beneficial fungus. *Sci. Hortic.* **2015**, *196*, 109–123. [CrossRef]
9. Woo, S.L.; Hermosa, R.; Lorito, M.; Monte, E. *Trichoderma*: A multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. *Nat. Rev. Microbiol.* **2023**, *21*, 312–326. [CrossRef]
10. Viterbo, A.; Ramot, O.; Chernin, L.; Chet, I. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek* **2002**, *81*, 549–556. [CrossRef]
11. Brotman, Y.; Briff, E.; Viterbo, A.; Chet, I. Role of swollenin, an expansin-like protein from Trichoderma, in plant root colonization. *Plant Physiol.* **2008**, *147*, 779–789. [CrossRef] [PubMed]
12. Sharma, A.; Salwan, R.; Sharma, V. Extracellular proteins of *Trichoderma* and their role in plant health. *S. Afr. J. Bot.* **2022**, *147*, 359–369. [CrossRef]
13. Vinale, F.; Sivasithamparam, K. Beneficial effects of *Trichoderma* secondary metabolites on crops. *Phytother. Res.* **2020**, *34*, 2835–2842. [CrossRef] [PubMed]
14. Carillo, P.; Woo, S.L.; Comite, E.; El-Nakhel, C.; Rouphael, Y.; Fusco, G.M.; Borzacchiello, A.; Lanzuise, S.; Vinale, F. Application of *Trichoderma harzianum*, 6-pentyl-α-pyrone and plant biopolymer formulations modulate plant metabolism and fruit quality of plum tomatoes. *Plants* **2020**, *9*, 771. [CrossRef]
15. Malmierca, M.; Cardoza, R.; Alexander, N.; McCormick, S.; Hermosa, R.; Monte, E.; Gutiérrez, S. Involvement of *Trichoderma* trichothecenes in the biocontrol activity and induction of plant defense-related genes. *Appl. Environ. Microbiol.* **2012**, *78*, 4856–4868. [CrossRef]
16. Shahriar, S.A.; Islam, M.N.; Chun, C.N.W.; Kaur, P.; Rahim, M.A.; Islam, M.M.; Uddain, J.; Siddiquee, S. Microbial metabolomics interaction and ecological challenges of *Trichoderma* species as biocontrol inoculant in crop rhizosphere. *Agronomy* **2022**, *12*, 900. [CrossRef]

17. Elad, Y. Biological control of foliar pathogens by means of Trichoderma harzianum and potential modes of action. *Crop Prot.* **2000**, *19*, 709–714. [CrossRef]
18. Coppola, M.; Cascone, P.; Lelio, I.D.; Woo, S.L.; Lorito, M.; Rao, R.; Pennacchio, F.; Guerrieri, E.; Digilio, M.C. Trichoderma atroviride P1 Colonization of Tomato Plants Enhances Both Direct and Indirect Defense Barriers Against Insects. *Front. Physiol.* **2019**, *10*, 464382. [CrossRef] [PubMed]
19. Salwan, R.; Sharma, A.; Kaur, R.; Sharma, R.; Sharma, V. The riddles of *Trichoderma* induced plant immunity. *Biol. Control* **2022**, *174*, 105037. [CrossRef]
20. Macías-Rodríguez, L.; Contreras-Cornejo, H.A.; Adame-Garnica, S.G.; del-Val, E.; Larsen, J. The interactions of *Trichoderma* at multiple trophic levels: Inter-kingdom communication. *Microbiol. Res.* **2020**, *240*, 126552. [CrossRef]
21. Nawrocka, J.; Małolepsza, U. Diversity in plant systemic resistance induced by *Trichoderma*. *Biol. Control* **2013**, *67*, 149–156. [CrossRef]
22. Morán-Diez, M.E.; Martínez de Alba, Á.E.; Rubio, M.B.; Hermosa, R.; Monte, E. *Trichoderma* and the Plant Heritable Priming Responses. *J. Fungi* **2021**, *7*, 318. [CrossRef]
23. Tyśkiewicz, R.; Nowak, A.; Ozimek, E.; Jaroszuk-Ściseł, J. Trichoderma: The Current Status of Its Application in Agriculture for the Biocontrol of Fungal Phytopathogens and Stimulation of Plant Growth. *Int. J. Mol. Sci.* **2022**, *23*, 2329. [CrossRef] [PubMed]
24. Pacheco-Trejo, J.; Aquino-Torres, E.; Reyes-Santamaría, M.I.; Islas-Pelcastre, M.; Pérez-Ríos, S.R.; Madariaga-Navarrete, A.; Saucedo-García, M. Plant Defensive Responses Triggered by *Trichoderma* spp. as Tools to Face Stressful Conditions. *Horticulturae* **2022**, *8*, 1181. [CrossRef]
25. Shang, J.; Liu, B.; Xu, Z. Efficacy of *Trichoderma asperellum* TC01 against anthracnose and growth promotion of *Camellia sinensis* seedlings. *Biol. Control* **2020**, *143*, 104205. [CrossRef]
26. Coppola, M.; Diretto, G.; Digilio, M.C.; Woo, S.L.; Giuliano, G.; Molisso, D.; Pennacchio, F.; Lorito, M.; Rao, R. Transcriptome and Metabolome Reprogramming in Tomato Plants by *Trichoderma harzianum* strain T22 Primes and Enhances Defense Responses Against Aphids. *Front. Physiol.* **2019**, *10*, 745. [CrossRef]
27. Dini, I.; Pascale, M.; Staropoli, A.; Marra, R.; Vinale, F. Effect of Selected *Trichoderma* Strains and Metabolites on Olive Drupes. *Appl. Sci.* **2021**, *11*, 8710. [CrossRef]
28. Pascale, A.; Vinale, F.; Manganiello, G.; Nigro, M.; Lanzuise, S.; Ruocco, M.; Marra, R.; Lombardi, N.; Woo, S.L.; Lorito, M. *Trichoderma* and its secondary metabolites improve yield and quality of grapes. *Crop Prot.* **2017**, *92*, 176–181. [CrossRef]
29. Guzmán-Guzmán, P.; Porras-Troncoso, M.D.; Olmedo-Monfil, V.; Herrera-Estrella, A. *Trichoderma* species: Versatile plant symbionts. *Phytopathology* **2019**, *109*, 6–16. [CrossRef] [PubMed]
30. Pacheco-Ordaz, R.; Wall-Medrano, A.; Goñi, M.G.; Ramos-Clamont-Montfort, G.; Ayala-Zavala, J.F.; González-Aguilar, G. Effect of phenolic compounds on the growth of selected probiotic and pathogenic bacteria. *Lett. Appl. Microbiol.* **2018**, *66*, 25–31. [CrossRef]
31. Taylor, J.T.; Harting, R.; Shalaby, S.; Kenerley, C.M.; Braus, G.H.; Horwitz, B.A. Adhesion as a Focus in Trichoderma-Root Interactions. *J. Fungi* **2022**, *8*, 372. [CrossRef] [PubMed]
32. Vacher, C.; Hampe, A.; Porté, A.J.; Sauer, U.; Compant, S.; Morris, C.E. The Phyllosphere: Microbial Jungle at the Plant-Climate Interface. *Annu. Rev. Ecol. Evol. Syst.* **2016**, *47*, 1–24. [CrossRef]
33. Xu, N.H.; Zhao, Q.Q.; Zhang, Z.Y.; Zhang, Q.; Wang, Y.; Qin, G.Y.; Ke, M.J.; Qiu, D.Y.; Peijnenburg, W.; Lu, T.; et al. Phyllosphere Microorganisms: Sources, Drivers, and Their Interactions with Plant Hosts. *J. Agric. Food Chem.* **2022**, *70*, 4860–4870. [CrossRef]
34. Zhu, Y.G.; Xiong, C.; Wei, Z.; Chen, Q.L.; Ma, B.; Zhou, S.Y.D.; Tan, J.Q.; Zhang, L.M.; Cui, H.L.; Duan, G.L. Impacts of global change on the phyllosphere microbiome. *New Phytol.* **2022**, *234*, 1977–1986. [CrossRef] [PubMed]
35. Sohrabi, R.; Paasch, B.C.; Liber, J.A.; He, S.Y. Phyllosphere Microbiome. *Annu. Rev. Plant Biol.* **2023**, *74*, 539–568. [CrossRef]
36. Mandal, S.D.; Jeon, J. Phyllosphere Microbiome in Plant Health and Disease. *Plants* **2023**, *12*, 3481. [CrossRef] [PubMed]
37. Oancea, F.; Raut, I.; Sesan, T.E.; Cornea, P.C. Dry Flowable Formulation of Biostimulants *Trichoderma* strains. *Agric. Agric. Sci. Procedia* **2016**, *10*, 494–502. [CrossRef]
38. Kamble, M.V.; Joshi, S.M.; Hadimani, S.; Jogaiah, S. Biopriming with rhizosphere *Trichoderma harzianum* elicit protection against grapevine downy mildew disease by triggering histopathological and biochemical defense responses. *Rhizosphere* **2021**, *19*, 100398. [CrossRef]
39. Martinez, Y.; Ribera, J.; Schwarze, F.W.; De France, K. Biotechnological development of *Trichoderma*-based formulations for biological control. *Appl. Microbiol. Biotechnol.* **2023**, *107*, 5595–5612. [CrossRef]
40. Şesan, T.E.; Oancea, A.O.; Ştefan, L.M.; Mănoiu, V.S.; Ghiurea, M.; Răut, I.; Constantinescu-Aruxandei, D.; Toma, A.; Savin, S.; Bira, A.F. Effects of foliar treatment with a *Trichoderma* plant biostimulant consortium on *Passiflora caerulea* L. yield and quality. *Microorganisms* **2020**, *8*, 123. [CrossRef]
41. Sârbu, A.; Şesan, T.E.; Tănase, T.; Paraschiv, A.M.; Cîşlariu, A.G.; Oancea, F. Anatomical investigations on *Momordica charantia* L. plants, newly acclimated in Romania. *J. Plant Dev.* **2022**, *29*, 25–44. [CrossRef]
42. Tan, S.P.; Kha, T.C.; Parks, S.E.; Roach, P.D. Bitter melon (*Momordica charantia* L.) bioactive composition and health benefits: A review. *Food Rev. Int.* **2016**, *32*, 181–202. [CrossRef]
43. Richter, E.; Geetha, T.; Burnett, D.; Broderick, T.L.; Babu, J.R. The Effects of Momordica charantia on Type 2 Diabetes Mellitus and Alzheimer Disease. *Int. J. Mol. Sci.* **2023**, *24*, 4643. [CrossRef] [PubMed]

44. Oancea, F.; Mara, G.; Sesan, T.E.; Máthé, I.; Răut, I.; Ábrahám, B.; Lányi, S. Strain of Trichoderma harzianum and Controlled Release Composition which Contains Said Strain. EU Patent 2735607A1, 28 May 2014.
45. Raut, I.; Badea-Doni, M.; Calin, M.; Oancea, F.; Vasilescu, G.; Sesan, T.E.; Jecu, L. Effect of volatile and non-volatile metabolites from *Trichoderma* spp. against important phytopathogens. *Rev. Chim.* **2014**, *65*, 1285–1288.
46. Lichtenthaler, H.; Buschmann, C.; Knapp, M. How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio R Fd of leaves with the PAM fluorometer. *Photosynthetica* **2005**, *43*, 379–393. [CrossRef]
47. Huang, D.; Ou, B.; Prior, R.L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856. [CrossRef] [PubMed]
48. Craciunescu, O.; Constantin, D.; Gaspar, A.; Toma, L.; Utoiu, E.; Moldovan, L. Evaluation of antioxidant and cytoprotective activities of *Arnica montana* L. and *Artemisia absinthium* L. ethanolic extracts. *Chem. Cent. J.* **2012**, *6*, 97. [CrossRef]
49. Chang, C.-C.; Yang, M.-H.; Wen, H.-M.; Chern, J.-C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178–182.
50. Braca, A.; De Tommasi, N.; Di Bari, L.; Pizza, C.; Politi, M.; Morelli, I. Antioxidant principles from *Bauhinia tarapotensis*. *J. Nat. Prod.* **2001**, *64*, 892–895. [CrossRef]
51. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
52. Repetto, G.; Del Peso, A.; Zurita, J.L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* **2008**, *3*, 1125. [CrossRef] [PubMed]
53. Li, Y.-Q.; Song, K.; Li, Y.-C.; Chen, J. Statistical culture-based strategies to enhance chlamydospore production by Trichoderma harzianum SH2303 in liquid fermentation. *J. Zhejiang Univ. Sci. B* **2016**, *17*, 619–627. [CrossRef] [PubMed]
54. Girometta, C.; Dondi, D.; Baiguera, R.M.; Bracco, F.; Branciforti, D.S.; Buratti, S.; Lazzaroni, S.; Savino, E. Characterization of mycelia from wood-decay species by TGA and IR spectroscopy. *Cellulose* **2020**, *27*, 6133–6148. [CrossRef]
55. Cartabia, M.; Girometta, C.E.; Milanese, C.; Baiguera, R.M.; Buratti, S.; Branciforti, D.S.; Vadivel, D.; Girella, A.; Babbini, S.; Savino, E.; et al. Collection and Characterization of Wood Decay Fungal Strains for Developing Pure Mycelium Mats. *J. Fungi* **2021**, *7*, 1008. [CrossRef] [PubMed]
56. Brondi, M.; Florencio, C.; Mattoso, L.; Ribeiro, C.; Farinas, C. Encapsulation of *Trichoderma harzianum* with nanocellulose/carboxymethyl cellulose nanocomposite. *Carbohydr. Polym.* **2022**, *295*, 119876. [CrossRef] [PubMed]
57. Adzmi, F.; Meon, S.; Musa, M.H.; Yusuf, N.A. Preparation, characterisation and viability of encapsulated *Trichoderma harzianum* UPM40 in alginate-montmorillonite clay. *J. Microencapsul.* **2012**, *29*, 205–210. [CrossRef] [PubMed]
58. Tiburski, J.H.; Rosenthal, A.; Guyot, S.; Perrier-Cornet, J.-M.; Gervais, P. Water distribution in bacterial spores: A key factor in heat resistance. *Food Biophys.* **2014**, *9*, 10–19. [CrossRef]
59. Formisano, L.; Miras-Moreno, B.; Ciriello, M.; El-Nakhel, C.; Corrado, G.; Lucini, L.; Colla, G.; Rouphael, Y. Trichoderma and Phosphite Elicited Distinctive Secondary Metabolite Signatures in Zucchini Squash Plants. *Agronomy* **2021**, *11*, 1205. [CrossRef]
60. Fiorini, L.; Guglielminetti, L.; Mariotti, L.; Curadi, M.; Picciarelli, P.; Scartazza, A.; Sarrocco, S.; Vannacci, G. Trichoderma harzianum T6776 modulates a complex metabolic network to stimulate tomato cv. Micro-Tom growth. *Plant Soil* **2016**, *400*, 351–366. [CrossRef]
61. Palacios-Torres, R.E.; Bustamante-Ortiz, A.G.; Prieto-Baeza, L.A.; Hernández-Hernández, H.; Ramírez-Seañez, A.R.; Yam-Tzec, J.A.; Díaz-Félix, G. Effect of foliar application of *Trichoderma* on the quality of tomato fruits grown in different hydroponic substrates. *Folia Hortic.* **2019**, *31*, 355–364. [CrossRef]
62. Lo, C.-T.; Nelson, E.; Harman, G. Improved biocontrol efficacy of *Trichoderma harzianum* 1295-22 for foliar phases of turf diseases by use of spray applications. *Plant Dis.* **1997**, *81*, 1132–1138. [CrossRef] [PubMed]
63. Perazzolli, M.; Moretto, M.; Fontana, P.; Ferrarini, A.; Velasco, R.; Moser, C.; Delledonne, M.; Pertot, I. Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genom.* **2012**, *13*, 660. [CrossRef] [PubMed]
64. Hasan, M.M.; Bae, H. An overview of stress-induced resveratrol synthesis in grapes: Perspectives for resveratrol-enriched grape products. *Molecules* **2017**, *22*, 294. [CrossRef] [PubMed]
65. Sun, Y.; Xi, B.; Dai, H. Effects of Water Stress on Resveratrol Accumulation and Synthesis in 'Cabernet Sauvignon' Grape Berries. *Agronomy* **2023**, *13*, 633. [CrossRef]