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# Multispecies *Trichoderma* in Combination with Hydrolyzed Lignin Improve Tomato Growth, Yield, and Nutritional Quality of Fruits

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**Abstract:** The application of biological pesticides as alternatives to chemical phytosanitary products is a natural and innovative method to improve environmental protection and sustainable agricultural production. In this work, the compatibility between *Trichoderma* spp. and a commercial lignin extract was assessed in vitro and in vivo. The beneficial effects of lignin in combination with different *Trichoderma* consortia were evaluated in terms of improved growth and quantitative and qualitative tomato productivity. *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin formulation was the most effective in growth promotion and increased root and stem dry weight compared to control (45.4 and 43.9%, respectively). This combination determined a 63% increase in tomato yield compared to the control, resulting in the best-performing treatment compared to each individual constituent. Consistent differences in terms of lycopene, GABA, ornithine, total, essential, and branched-chain amino acids were revealed in fruits from tomato plants treated with *Trichoderma*–lignin formulations (*T. asperellum* + *T. virens* GV41 + lignin) or with the microbial consortia (*T. asperellum* + *T. virens* GV41, *T. atroviride* + *T. virens* GV41). The developed bioformulations represent a sustainable biological strategy to increase yield and produce nutritional compound-enriched vegetables.

**Keywords:** biostimulants; microbial consortia; sustainable agriculture; GABA; essential amino acids; lycopene

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## 1. Introduction

Chemical inputs have played a crucial role in the success of agricultural production systems. The widespread use of fertilizers has greatly contributed to the substantial increase in the global harvest of grains, rice, corn, and wheat varieties, which almost doubled yields in the last six decades [1]. However, this success has resulted in many drawbacks due to the intensive application of nitrogen (N)- and phosphate (P)-based fertilizers that accumulate in the ecosystem and depletion of natural resources, including soil and water [2,3]. It is estimated that food production will need to increase by 60% by 2050 in order to cover the needs to feed the expected 10 billion people on Earth [4,5]. To achieve this goal, it is necessary to meet all the compliances associated with globalization, climate change, and emerging diseases, following the last guidelines of the European Community in terms of sustainable agriculture practices, to drastically reduce all the negative impacts on the environment [6]. The application of biological pesticides as alternatives to chemical

phytosanitary products can be considered a natural, safe, and innovative method to improve agricultural production in a sustainable manner, providing environmental protection [7,8].

Despite the enormous potential, the use of bioformulations in agriculture is still limited due to their short shelf life and the variability of their effectiveness in different environmental conditions [9–11]. To make these formulations economically competitive and equivalent or best performing compared to chemical products, it is necessary to overcome these limitations by looking for solutions that can expand their beneficial effects on crops both in terms of biocontrol and growth promotion [12–14].

The development and application of multispecies microbial consortia with functional complementation and high microbial and metabolic biodiversity can provide a wide spectrum of activity, higher reliability, and effectiveness in variable environmental conditions [15,16]. Research is strongly committed to the development of new formulations based on microbial communities, which are able to positively modulate the rhizosphere microbiome. Recent studies have been aimed at using *Trichoderma* as a component to enhance multispecies microbial consortia rather than applying a single individual but highly selected strain [17].

Some plant biostimulants contain *Trichoderma* spp., common beneficial fungi in the rhizosphere and soil [18]. Different species of *Trichoderma* are used as active ingredients in more than 200 products worldwide, e.g., biopesticides, biofertilizers, and bio-growth enhancers marketed for conventional and organic agriculture [19,20]. Many *Trichoderma*-based products are registered as biological control agents or biopesticides (microbial plant protection products) but are also recognized for their ability to improve plant development, productivity, and nutritional quality as well as to induce plant defense mechanisms to pathogen/pest attack and in response to numerous abiotic stresses [2,21]. Fungi belonging to the *Trichoderma* genus can also improve plant nutritional status through the production of chemicals, allowing them to solubilize or chelate many minerals, thus making them available for root absorption [22,23]. Growth promotion and tolerance to abiotic stresses have been correlated with the ability of *Trichoderma* spp. to stimulate the production of 3-indolacetic acid (IAA), which promotes plant growth and lateral root development, and gibberellins [24,25]. Although *Trichoderma* plant biostimulant activity is well known, only formulations containing microorganisms belonging to *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp., and *Azospirillum* spp. can be registered as biostimulants, as reported in the Component Material Categories, number 7 (CMC-7) of the EU Fertilizer Products Regulation (FPR) [26]. However, many microbial isolates not included in the cited genera can be considered promising candidates for the development of new biofertilizers or biostimulants, suggesting the need to expand the CMC-7 list or revise the definition of biostimulants [8].

In recent years, many studies have aimed at evaluating the potential association of microbial consortia and their bioactive molecules, but also with consideration to compounds originating from "botanicals" or plant extracts, capable of inhibiting pathogen development, inducing systemic resistance, and promoting plant growth [27,28]. Among the vegetal bioactive compounds, lignocellulosic biomass-based residues are attractive as a bio-based feedstock and potential source of other secondary products [29,30]. Lignin is the second most abundant polymer in nature, representing 15–30% of the total lignocellulosic biomass [31]. It is one of the main under-valued by-products from different industrial processes, including the agro–industrial sector, wood–paper industrial processes for cellulose extraction, and biorefinery for the production of biofuel, that have various potential applications in perspective to the circular economy [32]. In fact, due to its high availability and renewability, lignin is an excellent source for producing valuable functional molecules such as polyphenols with antimicrobial activity [33,34]. Different studies have also reported that lignin-based polyphenol biostimulants have IAA-like and GA-like activities [35,36]. This work was aimed at developing a new formulation based on a multispecies consortium of *Trichoderma* spp. in association with a lignin-derived polyphenolic mixture for improving tomato plant health and productivity, both in terms of quantitative and qualitative aspects.

The compatibility between lignin and three different species of *Trichoderma* (*T. asperellum*, *T. virens*, *T. atroviride*) was analyzed both in vitro and in vivo. The lignin mixture was applied at different concentrations in in vitro assays to evaluate the effects on tomato seed germination. Tests in vivo on tomato plants with the lignin–*Trichoderma* formulations were conducted in the greenhouse to evaluate the main biometric parameters (root and shoot fresh and dry weight), then subsequently, an experiment was performed in the field to assess the yield and nutritional quality of tomato production.

## 2. Materials and Methods

### *2.1. Tests of Lignin Effect on Tomato Germination*

All the experiments performed in this work applied a liquid solution of lignin-derived polyphenolic mixture that is commercially available (Solargo™, UPM-Kymmene Oyj, Kouvola, Finland), as of now referred to as lignin. Soil (Ptzer erden GmbH-Orange substrate) was double-sterilized at 121 °C for 40 min in plastic bags and then cooled down for 48 h. Then, the soil was distributed in plastic pots (12 cm Ø), and tomato seeds (*Solanum lycopersicum* cv. San Marzano nano) were sown. After seed planting, a spray application of lignin was carried out on the soil surface using 4 mL of solution for each of the six treatments at concentrations ranging from 0.01% to 2% (*v*/*v*) (0.01, 0.1, 0.3, 0.5, 1, and 2%), with a control of sterile distilled water. For each condition, 3 pots containing 20 seeds were prepared. The pots were randomly arranged in the greenhouse under controlled conditions of temperature, photoperiod, and humidity (25 °C, 14/10 h, 80%, respectively), and seed germination was monitored every 24 h for 10 days. The final germination percentage, mean germination time, and synchronization index were estimated 10 days after sowing, and data were analyzed using the GerminaR statistical package in R 4.1.2 1 [37].

### *2.2. Trichoderma spp.–Lignin Compatibility Tests*

*Trichoderma*–lignin compatibility was evaluated in vivo by determining the presence of the fungus in soil samples collected near the tomato plant roots previously treated with the combined formulations (Table 1).

**Table 1.** List of treatments including *Trichoderma* species and lignin in the different formulations tested in in vitro and in vivo assays with their relative code numbers.

| Treatment | Concentration |
|---|---|
| Control (water) | |
| *T. virens* GV41 | $4 \times 10^6$ sp $mL^{-1}$ |
| *T. asperellum* | $4 \times 10^6$ sp $mL^{-1}$ |
| *T. atroviride* | $4 \times 10^6$ sp $mL^{-1}$ |
| Lignin | 1% |
| *T. virens* GV41. + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T. asperellum* + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T. atroviride* + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T asperellum* + *T. atroviride* + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T. asperellum* + *T. virens* GV41 + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T. atroviride* + *T. virens* GV41 + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |
| *T. asperellum* + *T. atroviride* | $4 \times 10^6$ sp $mL^{-1}$ (1:1) |
| *T. asperellum* + *T. virens* GV41 | $4 \times 10^6$ sp $mL^{-1}$ (1:1) |
| *T. atroviride* + *T. virens* GV41 | $4 \times 10^6$ sp $mL^{-1}$ (1:1) |
| *T. asperellum* + *T. atroviride* + *T. virens* GV41 | $4 \times 10^6$ sp $mL^{-1}$ (1:1:1) |
| *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin | $4 \times 10^6$ sp $mL^{-1}$ + lignin |

Treatments with lignin were applied at 1% (*v*/*v*) concentration in water according to the application doses recommended by the manufacturer (10–20 L/ha on horticultural crops). Three hundred tomato seeds (cv. San Marzano nano) were sown in a planting tray containing sterile soil and germinated at 20 °C, in the dark, 100% RH. After 15 days, seedlings were transferred to 12 cm Ø plastic pots containing 200 g of double-sterilized soil

and irrigated with 20 mL of each *Trichoderma*–lignin formulation. Treatments were repeated every two weeks for 2 months. The inoculation of the microbial strains, single and in combination with lignin, was carried out at the final concentration of $4 \times 10^6$ spores $mL^{-1}$. The combined *Trichoderma*–lignin formulations were obtained by adding spore suspensions of each fungal strain to the lignin aqueous solution (1%) to the final concentration of $4 \times 10^6$ spores $mL^{-1}$. For all formulations based on multiple microbial strains, the *Trichoderma* suspensions were mixed in 1:1 (combination of two strains) or 1:1:1 (combination of three strains) ratios. The negative controls were irrigated with sterile distilled water. The tests were carried out in greenhouses under controlled conditions (25 °C, 14/10 h photoperiod, 80% RH). Three replicates of 5 plants organized in randomized blocks were considered for each treatment. To verify *Trichoderma* spp. presence and persistence in soil treated with the different formulations, 2 g of soil was collected three times—at transplant, before each treatment, and 30 days after the last treatment. Each sample was placed in a 50 mL Falcon® tube, resuspended in 8 mL of sterile distilled water, and vigorously shaken for 10 min at room temperature on a vortex shaker (Fisherbrand™, Hampton, NH, USA). Subsequently, serial dilutions were prepared ($1{:}10^7$) in sterile water, and then 100 µL aliquots of dilutions $10^5$, $10^6$, and $10^7$ were spread with L-shaped microbiological cell spreaders on PDA with Igepal at 0.1% $v/v$ (Sigma-Aldrich, St. Louis, MO, USA) in 90 mm Petri plates, with 3 replicates for each dilution. The plates were incubated in the dark at 25 °C for 5 days, monitoring the fungal colony growth every 24 h. Total counts of fungal colonies on PDA plates were expressed in Colony-Forming Units (CFU g of $soil^{-1}$). Morphologically different colonies were isolated in monocultures and subjected to macroscopic and microscopic characterizations.

To facilitate the descriptions of the bioformulation comparisons, number codes were assigned to the diverse treatments as indicated in Table 1, and subsequently, these code numbers will be referred to in the text for the following explanations of the Results.

### *2.3. Effect of Trichoderma–Lignin Formulations on Tomato Growth*

Tomato seeds (cv. San Marzano nano) were germinated in the greenhouse in sterile soil and grown at 20 °C, 100% RH. After 15 days, seedlings were transferred to 14 cm Ø plastic pots containing sterile soil and irrigated with 20 mL of each *Trichoderma*–lignin formulation. The treatment consisting of 1% lignin and conidia suspensions of the *Trichoderma* multispecies consortium (*T. virens* GV41–*T. asperellum*–*T. atroviride*) was applied by root drench to tomato seedlings, repeated weekly for 1 month, then evaluated for the effect on plant growth and development. Three biological replicates of 10 plants were considered for each treatment. Pots were arranged randomly in the greenhouse. After 4 weeks, the plants were harvested, and the main biometric parameters were assessed (shoot and root fresh and dry weight). Data were reported as a percentage of increase compared to untreated control calculated by applying the following formula:

$$\text{Increase } (\%) = \frac{(\text{treated sample values} - \text{untreated sample values})}{\text{untreated sample values}} \times 100$$

### *2.4. Field Experiments: Effect of Trichoderma–Lignin on Tomato Yield and Fruit Quality*

Experiments were carried out in a tunnel greenhouse (8 m × 20 m, covered with a diffused light PVC thermal plastic) located at the University of Naples Federico II, Portici (NA), south Italy (40° 490 N, 14° 150 E; 72 m a.s.l.). The main chemical and physical properties of the soil were a sandy loam texture (85% sand, 12% silt, and 3.2% clay), with a pH of 7.8, electrical conductivity of 0.195 dS $m^{-1}$, organic matter (O.M.) of 2.8% ($w/w$), C:N of 9.7, total nitrogen (N) of 0.16%, $P_2O_5$ at 165 mg $kg^{-1}$, and $K_2O$ at 801 mg $kg^{-1}$. One-month-old tomato seedlings (*S. lycopersicum* cv. pixel) were purchased from the Bene nursery (Poggiomarino NA). The experimental field was divided into 7 rows of 12 plots, each consisting of 5 plants with a planting density of 3.1 plants/$m^2$; treatments, arranged in randomized blocks, consisted of 5 biological replicates. After planting, seedlings were

drenched with 50 mL of each formulation (Table 1). Lignin was applied at a concentration of 1% in aqueous solution ($v/v$). The formulations based only on microbial strains were applied at the final concentration of $4 \times 10^6$ spores $mL^{-1}$. The combined *Trichoderma*–lignin formulations were obtained by adding a spore suspension of each *Trichoderma* strain to the lignin solution (1%) to obtain a final concentration of $4 \times 10^6$ spores $mL^{-1}$. Treatments were repeated every 15 days for three months, using increasing volumes of the different formulations (from 50 to 200 mL) in relation to the phenological plant status. The negative controls were drenched with the same volume of sterile distilled water. For the yield evaluation, a gradual fruit harvest was carried out at three different times (July, August, and September), considering the optimal ripeness degree evaluated by skin color classification. Yield was estimated as total weight and fruit number per plant, considering fruits collected from the first to the sixth fruiting cluster formed on the emerging branches. Three tomato fruits were collected from the third fruiting cluster, selected based on characteristics of uniformity in size and shape and lack of disease symptoms, lesions, or defects for analysis of the qualitative features. A sub-sample of these fruits was used for the determination of the shape index, the total content of soluble solids, pH, and electrical conductivity (EC) of the juice, while a second sub-sample was frozen in liquid nitrogen, freeze-dried, and subjected to further chemical analysis for starch, soluble carbohydrate, anthocyanin, lycopene, soluble protein, and free amino acid contents. The parameters indicated above were determined as reported by Carillo et al., 2020 [38].

### *2.5. Statistical Analyses*

The normal distribution of all datasets was verified by applying Shapiro–Wilk and Barlett tests by R software version 4.1.2. Biometric plant data, expressed as a percentage of increase compared to untreated control, were analyzed by applying the non-parametric Kruskal–Wallis test followed by the Dunn test, considering a $p$-value $\leq 0.05$.

A one-way ANOVA of all qualitative and nutritional data (shape index, dry matter percentage, pH, juice electrical conductivity, total soluble solids, lycopene, soluble proteins, and total, essential, branched-chain, and free amino acids) was performed using the Agricolae package by R software version 4.1.2. Duncan's multiple-range test was used to analyze the standard deviations of the means at $p \leq 0.05$. The principal component analysis was performed using Factoextra Package by R software version 4.1.2.

## 3. Results

### *3.1. Effect of Lignin on Tomato Germination*

Treatments with different concentrations of lignin (0.01, 0.1, 0.3, 0.5, 1, and 2% $v/v$) did not inhibit tomato seed germination. The best effect on germination occurred when seeds were treated with 0.3% lignin, demonstrating the highest increase at 4 and 7 days after sowing (D4–D5) compared to the untreated control (Table 2).

**Table 2.** Germination percentages ± standard deviation of tomato seeds treated with different concentrations of lignin (0.01, 0.1, 0.3, 0.5, 1, and 2% $v/v$) measured at 1, 4, 5, 7, and 10 days after sowing (D1, D4, D5, D7, and D10, respectively). Different letters indicate statistical differences between the treatment means according to different letters indicate significant differences between treatments according to Kruskal–Wallis and Dunn tests for multiple comparisons ($p \leq 0.05$).

| Lignin Concentrations | D1 | | D4 | | D5 | | D7 | | D10 | |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 (water) | 0 ± 0 | a | 17.78 ± 16.78 | c | 46.67 ± 6.67 | a | 51.11 ± 7.7 | f | 91.11 ± 10.18 | a |
| 0.01% | 0 ± 0 | a | 51.11 ± 7.7 | ab | 60 ± 13.33 | a | 88.89 ± 10.18 | ab | 97.78 ± 3.85 | a |
| 0.1% | 0 ± 0 | a | 40 ± 6.67 | abc | 51.11 ± 10.18 | a | 86.67 ± 6.67 | bc | 97.78 ± 3.85 | a |
| 0.3% | 0 ± 0 | a | 66.67 ± 6.67 | a | 86.67 ± 13.33 | a | 100 ± 0 | a | 100 ± 0 | a |
| 0.5% | 0 ± 0 | a | 26.67 ± 11.55 | c | 55.56 ± 10.18 | a | 73.33 ± 6.67 | de | 88.89 ± 7.7 | a |
| 1% | 0 ± 0 | a | 24.44 ± 3.85 | c | 55.56 ± 15.4 | a | 75.56 ± 10.18 | cd | 93.33 ± 6.67 | a |
| 2% | 0 ± 0 | a | 31.11 ± 10.18 | bc | 53.33 ± 6.67 | a | 64.44 ± 3.85 | ef | 80 ± 6.67 | a |

In addition, this concentration produced a positive effect on the timing of germination. In fact, seeds treated with 0.3% lignin showed a mean germination time (MGT) of 4.6 days in comparison to 7.06 days for the untreated control (Figure 1A), and the greatest value of synchrony (Syn) of 0.5, indicating more uniform germination (Figure 1B). No significant differences were observed in terms of MGT and Syn for any of the other treatments when compared to the untreated control.

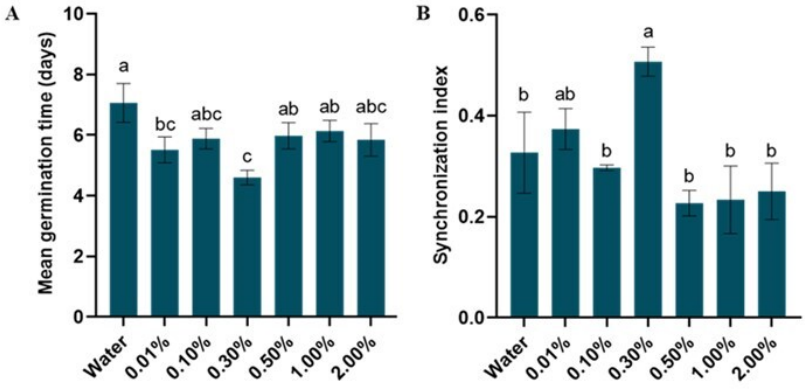


**Figure 1.** Effect of lignin treatments on the germination of tomato seeds: (**A**) Mean germination time (MGT) and (**B**) Synchrony Index at different concentrations (0.01, 0.1, 0.3, 0.5, 1, and 2% *v*/*v*) of lignin. Bars with different letters indicate statistical differences between treatments according to one-way ANOVA with Bonferroni correction ($p \leq 0.05$).

### *3.2. Trichoderma–Lignin Compatibility*

In order to develop a new formulation based on beneficial microorganisms and natural bioactive substances, it was necessary to test the effect of lignin on the growth of different *Trichoderma* species (*T. virens* GV41, *T. asperellum*, and *T. atroviride*). The lignin did not inhibit the growth of the beneficial fungal strains in in vitro assays (Table 3).

**Table 3.** *Trichoderma* abundance in soil samples obtained from the tomato rhizosphere, after the application of the diverse single and combined treatments, by determining the number of Colony-Forming Units (CFU) per gram of soil when samples were cultured on PDA + Igepal, then measured at (a) 15 days after the first treatment and (b) 30 days after the last treatment.

| Treatment | CFU/g (a) | CFU/g (b) |
|---|---|---|
| Water | 0 | 0 |
| *T. virens* | $3.24 \times 10^6 \pm 0.24$ | $5.24 \times 10^6 \pm 0.38$ |
| *T. asperellum* | $3.74 \times 10^6 \pm 0.21$ | $6.64 \times 10^6 \pm 0.17$ |
| *T. atroviride* | $4.21 \times 10^6 \pm 0.34$ | $8.01 \times 10^6 \pm 0.18$ |
| Solargo™ | 0 | 0 |
| *T. virens* + lignin | $4.88 \times 10^6 \pm 0.52$ | $3.74 \times 10^6 \pm 0.44$ |
| *T. asperellum* + lignin | $3.62 \times 10^6 \pm 0.31$ | $8.58 \times 10^6 \pm 0.31$ |
| *T. atroviride* + lignin | $4.26 \times 10^6 \pm 0.26$ | $9.63 \times 10^6 \pm 0.18$ |
| *T. asperellum* + *T. atroviride* + lignin | $5.02 \times 10^6 \pm 0.17$ | $1.03 \times 10^7 \pm 0.03$ |
| *T. asperellum* + *T. virens* + lignin | $6.04 \times 10^6 \pm 0.24$ | $1.12 \times 10^7 \pm 0.15$ |
| *T. atroviride* + *T. virens* + lignin | $3.42 \times 10^6 \pm 0.34$ | $7.24 \times 10^6 \pm 0.63$ |
| *T. asperellum* + *T. atroviride* | $4.73 \times 10^6 \pm 0.23$ | $7.42 \times 10^6 \pm 0.33$ |
| *T. asperellum* + *T. virens* | $2.1 \times 10^6 \pm 0.27$ | $1.69 \times 10^7 \pm 0.28$ |
| *T. atroviride* + *T. virens* | $4.8 \times 10^6 \pm 0.63$ | $8 \times 10^6 \pm 0.17$ |
| *T. asperellum* + *T. atroviride* + *T. virens* | $4.24 \times 10^6 \pm 0.19$ | $1.31 \times 10^7 \pm 0.18$ |
| *T. virens* + *T. asperellum* + *T. atroviride* + lignin | $4.32 \times 10^6 \pm 0.31$ | $7.68 \times 10^6 \pm 0.27$ |

The overall fungal abundance assessed in the soil samples collected 15 days after the first treatment ranged from 3 to $6 \times 10^6$ CFU $g^{-1}$, in line with the spore concentration of the *Trichoderma*–lignin formulations applied ($4 \times 10^6$ spores $mL^{-1}$). In soil samples collected

30 days after the last treatment, there was an increase of the *Trichoderma* spp. abundance observed in the growth and development of microorganisms in the soil. Colonies with different phenotypical traits were isolated from the plate cultures and purified in monoculture by single sporing and subsequently identified by morphological characterization of the macroscopic and microscopic structures typical of the three species of *Trichoderma* applied. No colonies afferent to the genus of *Trichoderma* were found in the control (water) and lignin-treated soil samples.

### *3.3. Effect of Trichoderma–Lignin Formulations on Tomato Growth*

Formulations of lignin and conidial suspensions of *T. virens* GV41, *T. asperellum*, and/or *T. atroviride* were applied to tomato seedlings to evaluate the effect on vegetative growth. Applications were conducted weekly, and after four weeks, the plants were harvested, and the main biometric indices (shoot and root dry weight) were assessed. From this point forward, treatments will be referred to following the codes reported (from 1 to 16) in Table 1 and as described in the Section 2.

As shown in Figure 2, formulation 16 was the most effective in terms of growth promotion, significantly increasing the dry weight of the root (Figure 2A) and stem (Figure 2B) compared to untreated control (45.4 and 43.9%, respectively).

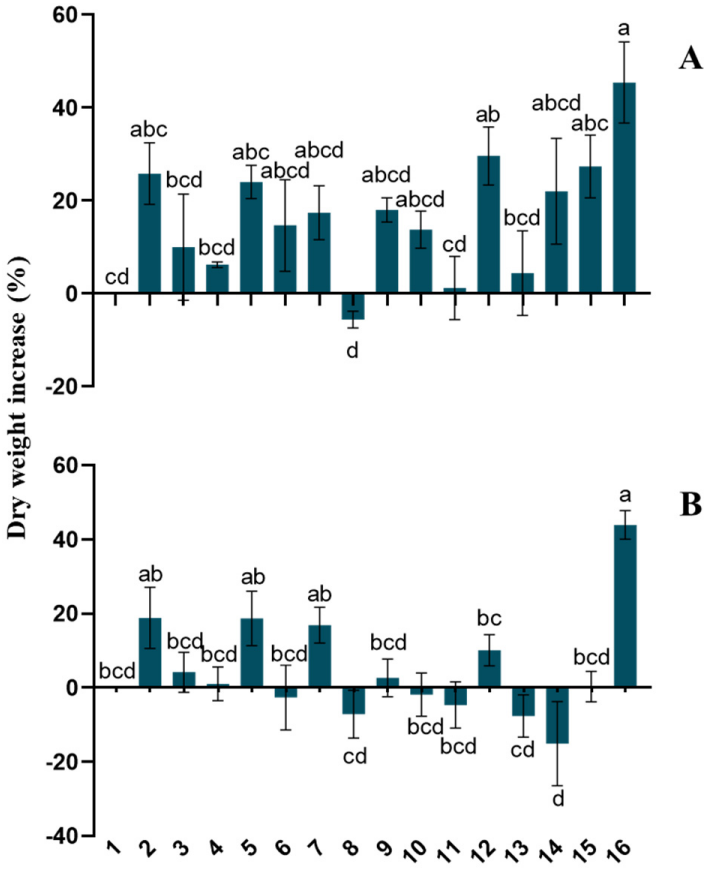


**Figure 2.** Percent increase in dry weight of (**A**) roots and (**B**) stems of tomato plants treated with the *Trichoderma*–lignin formulations. 1: Control (water); 2: *T. virens* GV41; 3: *T. asperellum*; 4: *T. atroviride*; 5: lignin (1%); 6: *T. virens* GV41 + lignin; 7: *T. asperellum* + lignin; 8: *T. atroviride* + lignin; 9: *T. asperellum* + *T. atroviride* + lignin; 10: *T. asperellum* + *T. virens* GV41 + lignin; 11: *T. atroviride* + *T. virens* + lignin; 12: *T. asperellum* + *T. atroviride*; 13: *T. asperellum* + *T. virens* GV41; 14: *T. atroviride* + *T. virens* GV41; 15: *T. asperellum* + *T. atroviride* + *T. virens* GV41; 16: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin. Bars with different letters indicate significant differences between treatments according to Kruskal–Wallis and Dunn tests for multiple comparisons ($p \leq 0.05$).

### *3.4. Field Experiments: Effect of Trichoderma–Lignin on Tomato Yield and Fruit Quality*

The effects of *Trichoderma*–lignin formulations applied to tomato plants were determined on the yield and quality of fruits collected from the field experiment. The plant growth promotion effects of the treatments were expressed as the percentage increase in fruit number (Figure 3A) and yield (Figure 3B) compared to the untreated control, considering the fruits collected from the first to the sixth fruiting cluster. The highest fruit number increase, around 27%, was determined when formulations 3 and 16 were applied to the plants. No statistical differences were detected among the other applications in comparison to the control (Figure 3A).

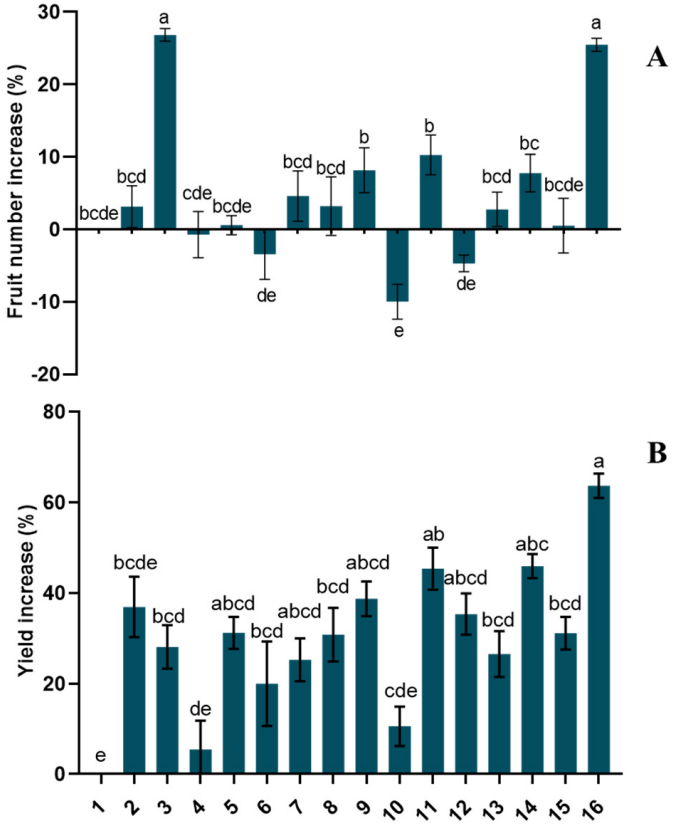


**Figure 3.** Effects of *Trichoderma*–lignin formulations on the increase of (**A**) fruit number increase and (**B**) yield recorded on treated tomato plants. 1: Control (water); 2: *T. virens* GV41; 3: *T. asperellum*; 4: *T. atroviride*; 5: lignin (1%); 6: *T. virens* GV41 + lignin; 7: *T. asperellum* + lignin; 8: *T. atroviride* + lignin; 9: *T. asperellum* + *T. atroviride* + lignin; 10: *T. asperellum* + *T. virens* GV41 + lignin; 11: *T. atroviride* + *T. virens* + lignin; 12: *T. asperellum* + *T. atroviride*; 13: *T. asperellum* + *T. virens* GV41; 14: *T. atroviride* + *T. virens* GV41; 15: *T. asperellum* + *T. atroviride* + *T. virens* GV41; 16: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin. Bars with different letters highlight statistical differences according to Kruskal–Wallis and Dunn tests for multiple comparisons ($p \leq 0.05$).

The best-performing treatment for increased yield was noted with the comprehensive combination of all three *Trichoderma* spp. and the lignin-comprising formulation 16. This treatment not only demonstrated a 63% greater productivity in comparison to the control, but it also showed a greater improvement in yield over each of the single microbial constituents used in the formulation (Figure 3B). The effect of the different formulations on the quality of tomato fruits was evaluated by analyzing multiple parameters of the qualitative aspects, including shape index, dry matter percentage, pH, juice electrical conductivity (EC), and total soluble solids (TSS). As reported in Table 4, no significant differences were recorded between the biotreated plants and the untreated control for the measured parameters.

**Table 4.** Effect of lignin and *Trichoderma* spp. treatments, alone and in combination, on tomato shape index (SI), fruit dry matter content (DM %), pH, electrical conductivity (EC), and total soluble solids (TSS) of tomato juice. Values are mean ± standard deviation. Treatment (TRT) **1**: Control (water); **2**: *T. virens* GV41; **3**: *T. asperellum*; **4**: *T. atroviride*; **5**: lignin (1%); **6**: *T. virens* GV41 + lignin; **7**: *T. asperellum* + lignin; **8**: *T. atroviride* + lignin; **9**: *T. asperellum* + *T. atroviride* + lignin; **10**: *T. asperellum* + *T. virens* GV41 + lignin; **11**: *T. atroviride* + *T. virens* + lignin; **12**: *T. asperellum* + *T. atroviride*; **13**: *T. asperellum* + *T. virens* GV41; **14**: *T. atroviride* + *T. virens* GV41; **15**: *T. asperellum* + *T. atroviride* + *T. virens* GV41; **16**: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin. Different letters highlight statistical differences according to one-way ANOVA followed by Duncan's multiple-range test ($p$-value $\leq 0.05$).

| Code | SI | | pH | | EC | | TSS | | DM (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1.28 ± 0.0761 | abc | 4.04 ± 0.0306 | ab | 4.51 ± 0.2442 | abc | 6.40 ± 0.4583 | a | 5.934 ± 0.0062 | a |
| **2** | 1.3 ± 0.0778 | ab | 4.07 ± 0.0702 | ab | 4.50 ± 0.1914 | abc | 6.40 ± 0.7 | a | 6.167 ± 0.0061 | a |
| **3** | 1.27 ± 0.0903 | abc | 3.99 ± 0.0361 | ab | 4.55 ± 0.1026 | abc | 6.26 ± 0.6506 | a | 6.091 ± 0.011 | a |
| **4** | 1.25 ± 0.0689 | bc | 4.04 ± 0.0608 | ab | 4.39 ± 0.581 | bc | 6.40 ± 0.9165 | a | 6.070 ± 0.0132 | a |
| **5** | 1.23 ± 0.0738 | c | 4.07 ± 0.0757 | ab | 4.45 ± 0.4341 | bc | 6.57 ± 1.0017 | a | 5.975 ± 0.0119 | a |
| **6** | 1.32 ± 0.0813 | ab | 4.11 ± 0.1266 | ab | 4.39 ± 0.2003 | bc | 6.20 ± 0.2646 | a | 5.779 ± 0.0028 | a |
| **7** | 1.3 ± 0.057 | abc | 4.09 ± 0.1 | ab | 4.55 ± 0.1908 | abc | 6.23 ± 0.7767 | a | 5.720 ± 0.0109 | a |
| **8** | 1.29 ± 0.0761 | abc | 4.07 ± 0.0961 | ab | 4.65 ± 0.3315 | ab | 6.17 ± 0.4041 | a | 5.699± 0.0073 | a |
| **9** | 1.32 ± 0.0761 | a | 4.28 ± 0.4508 | a | 4.42 ± 0.1823 | bc | 6.60 ± 0.4583 | a | 5.674 ± 0.0047 | a |
| **10** | 1.27 ± 0.1038 | abc | 4.35 ± 0.3889 | a | 5.01 ± 0.193 | a | 6.70 ± 0.6557 | a | 5.602 ± 0.0061 | a |
| **11** | 1.27 ± 0.0974 | abc | 4.03 ± 0.02 | ab | 4.70 ± 0.3672 | ab | 6.53 ± 0.3055 | a | 5.583 ± 0.0023 | a |
| **12** | 1.31 ± 0.1112 | ab | 3.87 ± 0.2804 | b | 4.35 ± 0.2227 | bc | 6.37 ± 0.7371 | a | 5.571 ± 0.003 | a |
| **13** | 1.29 ± 0.0574 | abc | 4.06 ± 0.0208 | ab | 4.29 ± 0.1361 | bc | 6.53 ± 0.5508 | a | 5.510 ± 0.0074 | a |
| **14** | 1.3 ± 0.05 | ab | 4.08 ± 0.0493 | ab | 4.43 ± 0.1007 | bc | 6.50 ± 0.5568 | a | 5.476 ± 0.0025 | a |
| **15** | 1.31 ± 0.07 | ab | 3.82 ± 0.3032 | b | 4.29 ± 0.0945 | bc | 6.13 ± 0.2082 | a | 5.378 ± 0.0023 | a |
| **16** | 1.31 ± 0.0686 | ab | 4.05 ± 0.0252 | ab | 4.08 ± 0.3331 | c | 6.20 ± 0.2646 | a | 5.873 ± 0.0058 | a |

The nutritional characteristics of fruits, such as starch, soluble carbohydrates (glucose, fructose, sucrose), lycopene, soluble proteins, and free amino acid contents, were also evaluated. Lycopene content was significantly affected by formulation 10, with a significant accumulation compared to treatments 1 (untreated control), the single components 2, 3, 5, and combinations 6, 7, and 13 (Table 5).

**Table 5.** Effect of *Trichoderma*–lignin formulations on Lycopene (Lyco), γ-amino butyric acid (GABA), monoethanolamine (MEA), essential amino acids (EAAs), total amino acids (TAAs), branched-chain amino acids (BCAAs) and ornitin (Orn). Values are expressed as mean ± standard deviation (micromole per gram of lyophilized tissue—μMol $g^{-1}$). Different letters highlight statistical differences according to Ducan's multiple-range test ($p$-value $\leq 0.05$). Treatment (TRT) **1**: Control; **2**: *T. virens* GV41; **3**: *T. asperellum*; **4**: *T. atroviride*; **5**: Lignin (1%); **6**: *T. virens* GV41 + lignin; **7**: *T. asperellum* + lignin; **8**: *T. atroviride* + lignin; **9**: *T. asperellum* + *T. atroviride* + lignin; **10**: *T. asperellum* + *T. virens* GV41 + lignin; **11**: *T. atroviride* + *T. virens* + lignin; **12**: *T. asperellum* + *T. atroviride*; **13**: *T. asperellum* + *T. virens* GV41; **14**: *T. atroviride* + *T. virens* GV41; **15**: *T. asperellum* + *T. atroviride* + *T. virens* GV41; **16**: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin.

| TRT | Lycopene | | TAAs | | GABA | | MEA | | Orn | | EAAs | | BCAAs | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1.03 ± 0.18 | cde | 425.45 ± 96.23 | def | 33.54 ± 6.72 | cde | 1.22 ± 0.36 | cdef | 1.74 ± 0.58 | d | 44.37 ± 10.92 | bcd | 6.99 ± 1.84 | cdefg |
| **2** | 0.79 ± 0.16 | ef | 423.07 ± 68.85 | def | 22.54 ± 3.46 | efg | 1.12 ± 0.26 | def | 1.74 ± 0.35 | d | 44.11 ± 8.33 | bcd | 7.64 ± 1.44 | bcdefg |
| **3** | 0.72 ± 0.084 | f | 459.74 ± 124.89 | def | 34.05 ± 4.30 | cde | 1.42 ± 0.38 | cdef | 1.59 ± 0.39 | d | 47.05 ± 10.41 | bcd | 9.25 ± 2.00 | abcd |
| **4** | 0.72 ± 0.20 | f | 300.73 ± 77.62 | f | 18.44 ± 3.10 | fg | 0.77 ± 0.19 | f | 1.17 ± 0.33 | d | 33.58 ± 5.47 | cd | 5.28 ± 1.15 | fg |
| **5** | 0.941 ± 0.075 | def | 370.23 ± 86.22 | ef | 36.98 ± 9.56 | bcd | 1.39 ± 0.27 | cdef | 1.36 ± 0.32 | d | 37.44 ± 10.71 | bcd | 5.74 ± 1.36 | efg |
| **6** | 1.04 ± 0.24 | cde | 285.51 ± 11.82 | f | 12.58 ± 0.81 | g | 1.02 ± 0.13 | ef | 1.32 ± 0.16 | d | 33.58 ± 1.63 | d | 4.94 ± 0.25 | g |
| **7** | 1.15 ± 0.27 | cde | 366.40 ± 8.74 | def | 18.30 ± 2.14 | fg | 1.42 ± 0.28 | cdef | 1.98 ± 0.36 | d | 45.81 ± 2.31 | bcd | 6.24 ± 0.67 | defg |
| **8** | 1.21 ± 0.09 | bcd | 494.05 ± 125.82 | bc | 25.71±3.67 | def | 2.50±0.53 | ab | 1.93±0.31 | d | 64.85±14.13 | b | 8.22±2.11 | bcdef |
| **9** | 1.45 ± 0.17 | ab | 405.69 ± 89.26 | cde | 33.54 ± 6.25 | cde | 1.33 ± 0.36 | cdef | 1.33 ± 0.20 | d | 53.60 ± 9.52 | bcd | 6.50 ± 1.73 | defg |
| **10** | 1.52 ± 0.10 | a | 726.72 ± 115.23 | ab | 43.39 ± 10.35 | abc | 2.46 ± 0.66 | ab | 6.90 ± 1.67 | a | 80.21 ± 13.84 | a | 10.46 ± 1.52 | ab |
| **11** | 0.93 ± 0.16 | def | 455.88 ± 53.85 | def | 30.32 ± 3.60 | cdef | 1.67 ± 0.44 | cde | 4.93 ± 0.01 | b | 42.48 ± 5.37 | bcd | 8.51 ± 1.54 | bcdef |
| **12** | 1.04 ± 0.06 | cde | 509.53 ± 105.93 | def | 37.79 ± 8.24 | bcd | 2.59 ± 0.58 | ab | 4.20 ± 0.60 | bc | 47.06 ± 7.05 | b | 10.11 ± 2.16 | abc |
| **13** | 1.02 ± 0.05 | cde | 726.22 ± 124.58 | a | 52.86 ± 16.25 | a | 3.10 ± 0.76 | a | 1.38 ± 0.30 | d | 93.41 ± 12.50 | a | 11.96 ± 2.32 | a |
| **14** | 1.16 ± 0.09 | cd | 755.55 ± 156.36 | a | 48.83 ± 6.67 | ab | 2.44 ± 0.31 | ab | 3.49 ± 0.81 | c | 92.95 ± 8.68 | a | 12.11 ± 1.97 | a |
| **15** | 1.26 ± 0.15 | abc | 488.44 ± 88.67 | bc | 28.46 ± 3.70 | def | 1.84 ± 0.33 | bcd | 3.77 ± 0.35 | c | 65.67 ± 11.70 | bc | 8.05 ± 0.87 | bcdefg |
| **16** | 1.01 ± 0.07 | cdef | 512.18 ± 110.75 | cd | 32.40 ± 5.99 | cde | 1.97 ± 0.19 | bc | 1.96 ± 0.68 | d | 58.53 ± 10.45 | b | 8.64 ± 2.26 | bcde |

GABA content resulted in a significant increase in formulation 13-treated plants, compared to control 1 and single constituents 2 and 3. A similar trend was observed for formulation 14.

Methanolamine (MEA) content was positively affected by the application of formulation 13, compared to treatments 1, 2, and 3 (Table 5). Plants treated with formulations 10, 13, and 14 showed the highest values of total (TAAs) and essential (EAAs) amino acids compared to the untreated control and single constituents (treatments from 1 to 5). Formulation 14 improved branched-chain amino acid (BCAA) content compared to 1 and the application of single components (2 and 4) (Table 5). Application of treatment 10 determined a significant increase in terms of ornithine content compared to 1, 2, 3, and 5.

The application of formulations 10, 13, and 14 strongly affected the tomato free amino acid content, positively influencing their abundance (Table 6, Figure 4). In particular, plants treated with formulation 10 produced fruits enriched in terms of glutamine (Gln) content compared to all other treatments. On the other hand, the application of formulations 13 and 14 increased the glutamic acid content (Glu) compared to control and single microbial constituents.

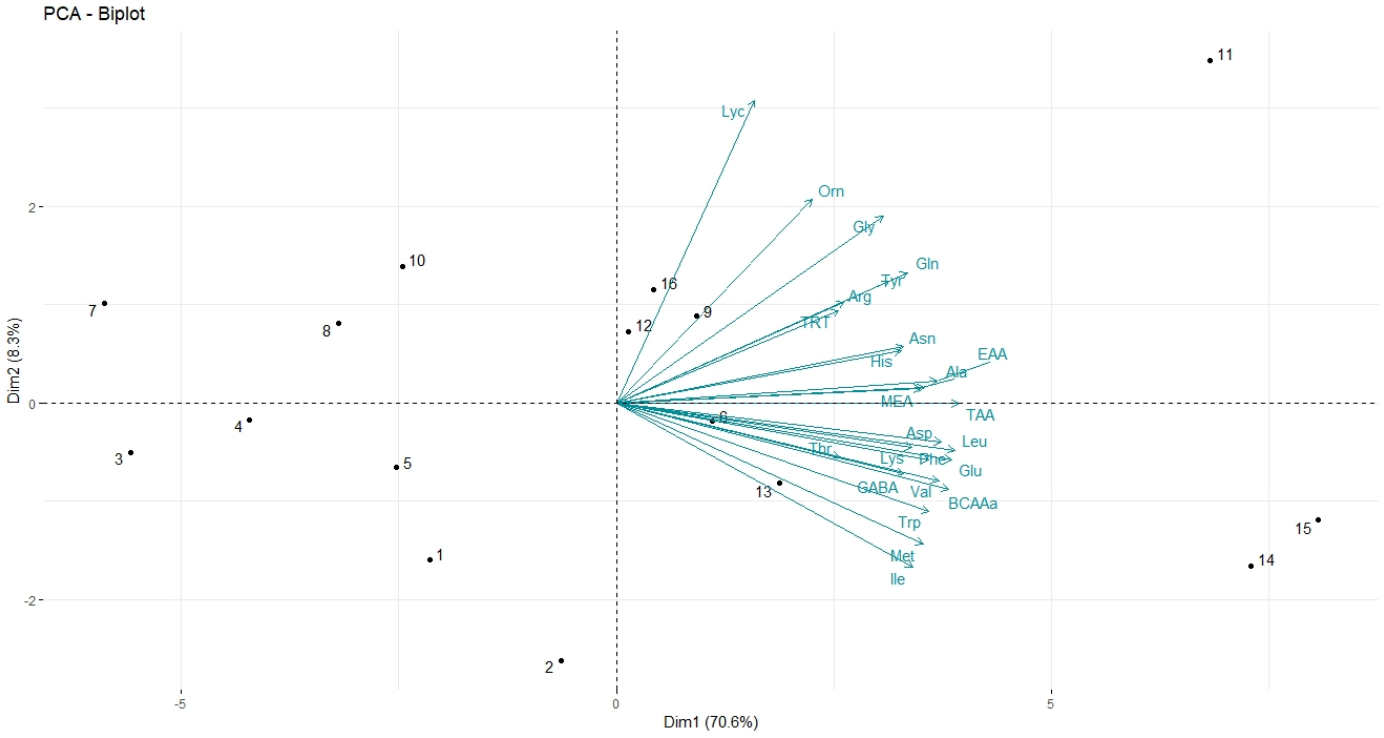


**Figure 4.** Principal component loading plot and scores of the Principal Component Analysis (PCA) of measured qualitative and nutritional parameters of tomato fruits as indicated in Tables 4–6 as affected by the different treatments. Treatment (TRT) **1**: Control; **2**: *T. virens* GV41; **3**: *T. asperellum*; **4**: *T. atroviride*; **5**: lignin (1%); **6**: *T. virens* GV41 + lignin; **7**: *T. asperellum* + lignin; **8**: *T. atroviride* + lignin; **9**: *T. asperellum* + *T. atroviride* + lignin; **10**: *T. asperellum* + *T. virens* GV41 + lignin; **11**: *T. atroviride* + *T. virens* + lignin; **12**: *T. asperellum* + *T. atroviride*; **13**: *T. asperellum* + *T. virens* GV41; **14**: *T. atroviride* + *T. virens* GV41; **15**: *T. asperellum* + *T. atroviride* + *T. virens* GV41; **16**: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin.

To obtain a multivariate overview of tomato fruit composition profiles and the influence of the *Trichoderma*–lignin formulations, a Principal Component Analysis (PCA) was carried out using the assessed qualitative and nutritional parameters (Figure 4). The first two principal components (PCs) explained 72% of the total observed variance, accounting for 61.4% (PC1) and 11.3% (PC2), respectively, with eigenvalues higher than 1.

**Table 6.** Effect of *Trichoderma*–lignin formulations on amino acid content. Values are expressed as mean ± standard deviation (micromole per gram of lyophilized tissue—μMol $g^{-1}$). Treatment (TRT) **1**: Control; **2**: *T. virens* GV41; **3**: *T. asperellum*; **4**: *T. atroviride*; **5**: lignin (1%); **6**: *T. virens* GV41 + lignin; **7**: *T. asperellum* + lignin; **8**: *T. atroviride* + lignin; **9**: *T. asperellum* + *T. atroviride* + lignin; **10**: *T. asperellum* + *T. virens* GV41 + lignin; **11**: *T. atroviride* + *T. virens* + lignin; **12**: *T. asperellum* + *T. atroviride*; **13**: *T. asperellum* + *T. virens* GV41; **14**: *T. atroviride* + *T. virens* GV41; **15**: *T. asperellum* + *T. atroviride* + *T. virens* GV41; **16**: *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin. Different letters highlight statistical differences according to one-way ANOVA followed by Ducan's multiple-range test ($p$-value $\leq 0.05$).

| TRT | Ala | | Arg | | Asn | | Asp | | Gln | | Glu | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 6.93 ± 1.87 | bcd | 17.24 ± 3.41 | efg | 14.89 ± 3.79 | e | 27.69 ± 7.70 | bc | 41.25 ± 14.09 | def | 235.41 ± 48.98 | bcd |
| **2** | 7.37 ± 0.90 | bcd | 14.90 ± 4.54 | fg | 17.65 ± 4.35 | e | 31.65 ± 9.98 | abc | 39.58 ± 12.10 | def | 239.56 ± 29.91 | bcd |
| **3** | 6.41 ± 1.23 | cd | 18.37 ± 4.00 | def | 24.14 ± 4.48 | de | 34.23 ± 10.87 | ab | 51.12 ± 14.10 | bcdef | 244.03 ± 80.58 | bcd |
| **4** | 4.52 ± 1.14 | d | 11.47 ± 2.95 | gh | 13.62 ± 2.58 | e | 22.64 ± 5.56 | bc | 32.26 ± 5.45 | ef | 157.17 ± 53.68 | d |
| **5** | 6.29 ± 1.71 | cd | 16.22 ± 4.45 | efg | 14.63 ± 5.06 | e | 26.12 ± 5.61 | bc | 31.84 ± 8.29 | ef | 199.62 ± 45.21 | cd |
| **6** | 5.46 ± 2.05 | cd | 14.46 ± 4.43 | fg | 15.05 ± 5.17 | e | 18.22 ± 8.75 | c | 29.15 ± 15.49 | f | 153.88 ± 58.26 | d |
| **7** | 8.00 ± 1.31 | bcd | 20.06 ± 0.20 | cdef | 14.66 ± 0.47 | e | 26.04 ± 3.84 | bc | 30.01 ± 2.71 | f | 203.53 ± 8.89 | cd |
| **8** | 8.82 ± 2.63 | bc | 30.40 ± 5.14 | b | 21.70 ± 5.60 | de | 35.51 ± 11.68 | ab | 52.55 ± 18.05 | bcdef | 257.83 ± 69.80 | bcd |
| **9** | 5.62 ± 1.45 | cd | 25.41 ± 6.22 | bcd | 15.02 ± 1.40 | e | 24.77 ± 5.58 | bc | 41.91 ± 11.03 | def | 211.72 ± 57.13 | cd |
| **10** | 10.99 ± 1.99 | ab | 37.96 ± 5.95 | a | 41.70 ± 5.36 | b | 46.88 ± 9.72 | a | 122.52 ± 25.25 | a | 338.11 ± 44.31 | ab |
| **11** | 8.23 ± 2.82 | bcd | 5.78 ± 0.62 | h | 30.46 ± 2.96 | cd | 29.60 ± 3.04 | bc | 68.35 ± 7.06 | bc | 215.15 ± 29.54 | cd |
| **12** | 6.16 ± 1.72 | cd | 5.42 ± 1.86 | h | 22.51 ± 3.37 | de | 31.56 ± 5.65 | abc | 61.57 ± 14.83 | bcd | 274.85 ± 72.78 | bc |
| **13** | 10.79 ± 3.83 | ab | 38.93 ± 7.57 | a | 28.14 ± 4.99 | d | 45.64 ± 7.19 | a | 70.41 ± 17.67 | bc | 389.70 ± 57.87 | a |
| **14** | 13.93 ± 4.00 | a | 30.64 ± 6.26 | b | 51.68 ± 14.66 | a | 46.27 ± 16.35 | a | 73.33 ± 9.99 | b | 395.57 ± 105.33 | a |
| **15** | 7.11 ± 1.17 | bcd | 27.59 ± 4.91 | bc | 39.14 ± 8.24 | bc | 26.13 ± 3.26 | bc | 47.38 ± 9.44 | cdef | 250.00 ± 55.27 | bcd |
| **16** | 8.68 ± 1.75 | bcd | 23.34 ± 2.67 | bcde | 23.15 ± 3.65 | de | 36.87 ± 7.18 | ab | 56.04 ± 16.21 | bcde | 271.71 ± 64.45 | bc |
| | **His** | | **Ile** | | **Leu** | | **Lys** | | **Met** | | **Phe** | |
| **1** | 5.09 ± 1.25 | d | 2.06 ± 0.44 | def | 3.73 ± 1.29 | cde | 2.09 ± 0.66 | de | 0.54 ± 0.15 | bcde | 8.51 ± 2.65 | cde |
| **2** | 6.96 ± 1.94 | cd | 2.58 ± 0.59 | abcde | 3.47 ± 0.95 | cde | 1.86 ± 0.42 | de | 0.53 ± 0.11 | bcde | 8.30 ± 1.50 | cde |
| **3** | 5.55 ± 1.55 | d | 3.12 ± 0.92 | abc | 4.35 ± 0.94 | bcde | 1.85 ± 0.62 | de | 0.79 ± 0.10 | ab | 7.73 ± 1.92 | de |
| **4** | 4.99 ± 0.59 | d | 1.75 ± 0.29 | ef | 2.61 ± 0.83 | e | 2.80 ± 0.06 | cde | 0.39 ± 0.07 | e | 6.52 ± 1.25 | e |
| **5** | 4.33 ± 1.30 | d | 1.74 ± 0.38 | ef | 3.00 ± 0.82 | de | 1.71 ± 0.86 | de | 0.44 ± 0.12 | de | 6.48 ± 1.92 | e |
| **6** | 4.89 ± 1.64 | d | 1.41 ± 0.49 | f | 2.66 ± 1.33 | e | 1.51 ± 0.72 | e | 0.37 ± 0.19 | e | 5.23 ± 3.40 | e |
| **7** | 7.63 ± 1.68 | bcd | 1.71 ± 0.13 | ef | 3.29 ± 0.29 | de | 1.82 ± 0.14 | de | 0.48 ± 0.05 | cde | 6.73 ± 0.86 | e |
| **8** | 9.34 ± 1.68 | abc | 2.43 ± 0.67 | bcde | 4.24 ± 0.98 | bcde | 2.26 ± 0.49 | de | 0.56 ± 0.11 | bcde | 9.62 ± 3.33 | bcde |
| **9** | 9.88 ± 2.40 | abc | 1.82 ± 0.36 | ef | 3.58 ± 1.18 | cde | 2.05 ± 0.62 | de | 0.55 ± 0.18 | bcde | 6.58 ± 1.11 | e |
| **10** | 10.76 ± 2.59 | ab | 2.83 ± 0.40 | abcd | 5.63 ± 0.79 | ab | 3.66 ± 0.39 | c | 0.72 ± 0.12 | abc | 11.94 ± 2.77 | abcd |
| **11** | 5.75 ± 0.87 | d | 2.27 ± 0.57 | bcdef | 4.59 ± 0.59 | bcd | 3.03 ± 0.24 | cd | 0.56 ± 0.10 | bcde | 7.95 ± 0.59 | cde |
| **12** | 7.59 ± 1.42 | bcd | 2.99 ± 0.72 | abcd | 5.31 ± 1.17 | abc | 3.93 ± 0.84 | bc | 0.76 ± 0.27 | ab | 8.43 ± 2.14 | cde |
| **13** | 12.54 ± 2.28 | a | 3.21 ± 0.58 | ab | 6.56 ± 1.17 | a | 5.00 ± 1.08 | ab | 0.92 ± 0.21 | a | 14.33 ± 4.31 | a |
| **14** | 11.45 ± 3.52 | a | 3.46 ± 0.58 | a | 6.58 ± 1.03 | a | 5.26 ± 1.64 | a | 0.90 ± 0.15 | a | 13.59 ± 3.34 | ab |
| **15** | 6.48 ± 1.92 | cd | 2.18 ± 0.25 | cdef | 4.35 ± 0.42 | bcde | 3.03 ± 0.23 | cd | 0.55 ± 0.08 | bcde | 7.26 ± 1.16 | e |
| **16** | 7.39 ± 1.37 | bcd | 2.44 ± 0.46 | bcde | 4.66 ± 1.58 | bcd | 2.35 ± 0.79 | de | 0.68 ± 0.23 | abcd | 12.20 ± 3.06 | abc |
| | **Thr** | | **Trp** | | **Tyr** | | **Val** | | **Gly** | | | |
| **1** | 2.70 ± 0.87 | c | 1.22 ± 0.38 | bcd | 2.31 ± 0.69 | cdef | 1.20 ± 0.27 | cdef | 3.33 ± 0.85 | ef | | |
| **2** | 2.55 ± 0.76 | c | 1.36 ± 0.17 | abc | 2.52 ± 0.67 | bcdef | 1.59 ± 0.23 | abcd | 2.03 ± 0.70 | fg | | |
| **3** | 2.11 ± 0.46 | c | 1.41 ± 0.26 | abc | 3.05 ± 0.62 | bcde | 1.77 ± 0.44 | abc | 0.73 ± 0.13 | g | | |
| **4** | 1.62 ± 0.50 | c | 0.52 ± 0.09 | e | 1.82 ± 0.22 | ef | 0.93 ± 0.14 | ef | 2.35 ± 0.14 | efg | | |
| **5** | 1.94 ± 0.59 | c | 0.59 ± 0.13 | e | 1.78 ± 0.44 | ef | 1.00 ± 0.23 | def | 2.83 ± 0.59 | efg | | |
| **6** | 1.62 ± 0.72 | c | 0.57 ± 0.42 | e | 1.66 ± 0.80 | f | 0.88 ± 0.33 | f | 4.10 ± 1.58 | def | | |
| **7** | 1.94 ± 0.23 | c | 0.90 ± 0.28 | cde | 1.94 ± 0.23 | def | 1.24 ± 0.25 | cdef | 4.58 ± 0.43 | de | | |
| **8** | 3.07 ± 1.10 | c | 1.38 ± 0.26 | abc | 3.57 ± 0.86 | bc | 1.55 ± 0.47 | abcde | 8.75 ± 2.21 | b | | |
| **9** | 1.93 ± 0.45 | c | 0.70 ± 0.22 | de | 2.23 ± 0.74 | cdef | 1.11 ± 0.22 | def | 2.52 ± 0.71 | efg | | |
| **10** | 3.14 ± 0.55 | c | 1.58 ± 0.50 | ab | 6.31 ± 1.55 | a | 2.00 ± 0.38 | ab | 15.20 ± 3.03 | a | | |
| **11** | 9.82 ± 1.43 | b | 1.08 ± 0.28 | bcde | 3.33 ± 0.19 | bc | 1.64 ± 0.43 | abcd | 10.56 ± 0.73 | b | | |
| **12** | 9.30 ± 2.40 | b | 1.52 ± 0.37 | abc | 2.90 ± 0.68 | bcdef | 1.81 ± 0.30 | abc | 8.39 ± 1.04 | bc | | |
| **13** | 7.79 ± 6.21 | b | 1.94 ± 0.56 | a | 3.68 ± 1.11 | b | 2.18 ± 0.62 | a | 10.34 ± 2.15 | b | | |
| **14** | 17.06 ± 3.92 | a | 1.94 ± 0.39 | a | 3.49 ± 0.48 | bc | 2.08 ± 0.46 | ab | 8.46 ± 1.57 | bc | | |
| **15** | 11.40 ± 2.53 | b | 1.31 ± 0.34 | bc | 1.95 ± 0.29 | def | 1.52 ± 0.20 | bcdef | 6.33 ± 1.44 | cd | | |
| **16** | 2.71 ± 0.52 | c | 1.22 ± 0.36 | bcd | 3.19 ± 0.68 | bcd | 1.54 ± 0.23 | abcde | 6.24 ± 0.48 | cd | | |

PC1 positively correlated to tryptophan, phenylalanine, MEA, methionine, alanine, lysine, glutamine, isoleucine, GABA, histidine, and asparagine (corr. > 0.8), to total (TAAs), essential (EAAs), and branched-chain amino acids (BCAAs), leucine, glutamic acid, aspartic

acid, and valine (corr. > 0.9), and negatively correlated to dry matter percentage (DM). Instead, PC2 was positively correlated to pH and EC (corr. ≥ 0.8) and negatively correlated to shape index (SI) and the amino acid threonine (Figure 4). In the loading plot of the PCA, single-component treatments appeared well distributed together with the water control, arranged along the negative quadrant of PC1, whereas the majority of the other treatments consisting of the *Trichoderma* combinations (containing 2 and 3 components) were distributed in the positive quadrants on the right. Instead, the separation of the treatment distribution along PC2 was attributed to the absence of lignin in the lower negative components and the presence of lignin in the upper positive quadrants. Clearly, the treatments with the fungal consortia (formulations 12, 13, 14, and 15) and the formulation with the three *Trichoderma* species in association with the lignin (formulation 16) had the greatest effects on the physiological–qualitative characteristics of the tomato fruits regarding the amino acids content (TAAs, BCAAs, EAAs) and the abundance of GABA and MEA. Formulation 10 containing *T. asperellum* + *T. virens* GV41 + lignin was found in a unique position in the PCA, at a dissociated distance from all other treatments, in the upper right coordinate. This can be associated with the significant accumulation of Gln, Orn, Tyr, Arg, and Gly in fruits.

## 4. Discussion

At present, hundreds of agricultural products based on antagonistic microorganisms are registered and applied as Plant Protection Products, used for the control of various plant diseases and crop pests. A multitude of other biological products are not registered but are marketed as bioprotectors, biostimulants, or biofertilizers, providing beneficial effects to cultivated crops [39]. A trend of great interest concerns the development of bioformulations based on synthetic microbial consortia, communities of different microorganisms with complementary or synergistic traits, able to carry out complex functions, precluded to monocultures, and positively affecting the soil microbiome [17]. The introduction of beneficial synthetic communities with functional and metabolic complementarity can modify the overall structure of the rhizosphere microbial composition and substantially improve plant productivity and pathogenic microorganism containment [40,41]. Several experimental studies evaluated the potential association of microbial consortia and bioactive molecules also belonging to the classes called "botanicals". These plant extracts are able to inhibit pathogen development, induce systemic resistance, and promote plant growth [42]. This research was aimed at developing new formulations based on multispecies consortia of *Trichoderma* spp. in combination with a commercial lignin-hydrolyzed extract (Solargo™, UPM-Kimmene Oyj, Finland), able to improve tomato plant growth and productivity, both in quantitative and qualitative terms.

Lignin is one of the most abundant natural polymers and represents the main "waste" of paper and biofuel industrial processes. Due to its antioxidant, antifungal, and antibiotic properties associated with its phenolic compositions, lignin can find value-added applications in a multitude of industrial sectors such as pharmacy, agrifood, nutrition, cosmetics, etc. [31]. In agriculture, lignin has been exploited to produce granular soil amendments with micronutrient-controlled timed release. In this regard, Ammonium Lignin Sulfonate (LSA) is a meso- and micro-element complexing agent that favors the metal adsorption by plant tissue, thus increasing its bioavailability and exhibiting antimicrobial and biostimulating properties similar to the effects noted by applications with humic substances [43,44]. The latter are known for their ability to stimulate the development and productivity of plants as well as increase resistance to biotic and abiotic stresses through different mechanisms [45–48].

In this work, a positive effect of lignin treatments on tomato seed germination was observed. In particular, the application of 0.3% lignin was the most effective, reaching a germination percentage of 67% compared to 18% of the control, 4 days after sowing. The highest concentration used (2% lignin) did not inhibit germination, which was comparable to the control for all the considered time points (D1, D4, D5, D7, and D10). However,

several other studies indicated that treatments with phenolic acids inhibit seed germination and plant development [49–51]. This inhibitory effect is strongly affected by different factors, such as the chemical nature of phenolic compounds, their concentration, and the "host" plant species [52,53]. Very often, a dose-effect correlation has been highlighted; for example, Kuiters et al. (1989) observed that p-coumaric acid stimulates herbaceous plant seed germination only at low concentrations [54]. Similar results were obtained by Reigosa et al. (1999) for ferulic acid, p-hydroxybenzoic acid, and p-vanillic acid [55]. The growth promotion activity of natural polyphenols has been extensively demonstrated [24,35]. Ertani et al., 2011, recorded a direct proportionality between the dry weight increase in root and leaves of corn seedlings and the content of phenolic compounds in the commercial biostimulants applied [56]. Furthermore, irrigation and foliar applications of polyphenolic mixtures derived from spruce bark (*Picea abes* L.) significantly increased sunflower plants (*Helianthus annuus* L.) root, stem, and leaf dry weights [25]. To date, the mechanisms underlying the induced plant growth promotion are still little known, principally due to the heterogeneous chemical nature of polyphenol mixtures [57]. Recent studies suggested that the plant growth promotion effect may be associated with the content of bioactive phenolic acids with hormone-like activity [58]. Despite their potential role as biostimulants, the use of lignin polyphenolic extracts in agriculture is still very limited, while the application of microbial-based biofertilizers or "growth promoters" is much more widespread. In the present work, strains belonging to three different *Trichoderma* species (*T. atroviride*, *T. virens*, and *T. asperellum*), selected for their beneficial abilities, including biological control and growth promotions, have been used [59–62]. In order to develop a new formulation based on a multispecies consortium of *Trichoderma* spp. with lignin, it was necessary to verify their compatibility both in vitro and in vivo. It is known that one of the crucial aspects in the assembly of microbial consortia lies in the selection of ecologically and physiologically compatible strains [17,63]. The results obtained in in vitro tests showed that lignin did not inhibit the fungal growth at all the concentrations applied. However, as the concentration in the medium increased, an early growth slowdown was recorded, which, in the following days, reached values comparable to the control (not-amended medium). *Trichoderma* spp. actively play in the delignification and biodegradation of lignocellulosic compounds in nature through the synthesis of ligninolytic enzymes such as laccase and peroxidase [64–70]. The ability of *Trichoderma* to tolerate the polyphenolic mixture and the ecological compatibility of the different strains used for the microbial consortium constitution was also confirmed by the in vivo assays. Once *Trichoderma*–lignin compatibility was verified, in planta assays were carried out, aimed at evaluating the growth promotion activity of formulations. *T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin (formulation 16) was the most effective in the tomato growth promotion assay, consistently increasing stem dry weight compared to the control and to all the single microbial components, except for treatment 2. The same formulation was found to be the most effective in field experiments, increasing yield compared to the control (+63%) and individual microbial components. The results suggested that the combination of all the assayed microorganisms in association with lignin can interact positively in promoting plant development and increasing marketable yield.

Tomato is a very important agricultural crop for its nutritional features, being one of the main sources of vitamins (A and K), minerals (potassium, phosphorus, calcium, zinc, and selenium), and antioxidants (phenolics, folic acid, vitamins C and E, lycopene, and β-carotene) [71]. In recent years, strategies aimed at increasing the content of these bioactive compounds, as well as improving the fruit flavor, have significantly increased [72]. This research also investigated the effect of biological treatments on the nutritional features of tomato fruits in consideration of the growing consumer demand for functional and palatable foods. Data indicated that plants treated with *T. asperellum* + *T. virens* GV41 + lignin (formulation 10), *T. asperellum* + *T. virens* GV41 (formulation 13), or with *T. atroviride* + *T. virens* GV41 (formulation 14) produced fortified fruits in terms of lycopene, GABA, MEA, EAAs, TAAs, BCAAs, ornithine, as well as free amino acid contents. The find-

ings of this research demonstrated that biological formulations can be applied to produce nutraceutical-enriched foods with fortified antioxidants or nutritional features. This effect would be especially remarkable for compounds whose production in vegetables is insufficient to meet human nutritional needs and for organisms unable to synthesize them, as it happens for essential amino acids [73,74]. Both the organic (lignin) and the microbial components (*Trichoderma* spp.) are known for their ability to affect plant metabolism by increasing the bioavailability of soil nutrients, in particular C and N, and favoring their absorption [23,75,76]. The formulations *T. asperellum* + *T. virens* GV41 + lignin (10), *T. asperellum* + *T. virens* GV41 (13), and *T. atroviride* + *T. virens* GV41 (14) could affect the nitrogen abundance in soil, increasing its bioavailability, with a consequent increase of free amino acids content in tomato fruits [77]. This hypothesis is supported by the increase in asparagine and glutamine content, the primary nitrogen transport compounds from source to sink organs, where they act as nitrogen reserves [78]. The observed increment in asparagine and glutamine could be associated with the simultaneous increment of ornithine, which can transfer its amino group to ketoglutarate, forming glutamate. Ornithine is derived from arginine through the action of arginine succinate synthetase 1, while in turn, arginine may derive directly from *Trichoderma* spp. since this amino acid is used as the main nitrogen translocation form by mycorrhizal fungi to plants [79,80]. Accordingly, Ruzicka et al., 2012, highlighted that glutamine synthetase and asparagine synthetase were significantly more expressed in tomato roots colonized by beneficial fungi, determining an increase of the related amino acid in plants [80]. In addition, a significant increase of GABA content was recorded for *T. asperellum* + *T. virens* GV41 (formulation 13)- and *T. atroviride* + *T. virens* GV41 (formulation 14)-treated plants, confirming that this non-protein amino acid could work as a temporary nitrogen storage to mitigate the accrual of ammonium [38].

*T. atroviride* + *T. virens* GV41 application (formulation 14) also determined a significant increase in the glutamic acid content compared to the control (untreated plants). Glutamic acid is the predominant amino acid in tomato fruit, and its content increases during the ripening process [81]. It is known to be a key compound in plant nitrogen metabolism and to play a fundamental role in the response mechanisms to biotic and abiotic stress [82,83]. Recently, it has been shown that this compound is able to influence and improve the flavor of tomato fruit, giving the characteristic "umami taste" typical of foods such as aged cheese [84]. The higher content of glutamic acid in tomato fruits can, therefore, enhance their flavor and increase their marketability, especially for fresh consumption [85,86].

## 5. Conclusions

The positive effects of *Trichoderma*-based microbial consortia in increasing the yield of different plant species are known. To date, few studies are available in the literature relating to the association between *Trichoderma* and hydrolyzed lignin-derived polyphenols. The results obtained in this work showed a positive interaction between the polyphenolic and microbial components, determining an improvement in the vegetative growth and productivity as was observed for formulation 16 (*T. virens* GV41 + *T. asperellum* + *T. atroviride* + lignin). On the other hand, the *Trichoderma*-based formulations 13 and 14 (*T. asperellum* + *T. virens* GV41 and *T. atroviride* + *T. virens* GV41, respectively) resulted in the best-performing treatments in terms of tomato fruit nutritional quality improvement. In this regard, bioformulations developed in this work represent a solid starting point to increase production in a sustainable manner and to produce functional foods and innovative products that can satisfy the growing demand for nutritional compound-enriched vegetables. The application of lignin "waste" matches the concept of a circular economy whereby resources, input, and emissions are minimized by reuse, recycling, or remanufacturing, enhancing the added value of by-products through their application in eco-sustainable agricultural production.

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