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Preliminary Results of the Impact of Beneficial Soil Microorganisms on Okra Plants and Their Polyphenol Components

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Abstract: Okra (*Abelmoschus esculentus* L.) is a highly nutritious vegetable rich in vitamins, minerals, and bioactive compounds, including polyphenols, offering numerous health benefits. Despite its nutritional value, okra remains underutilized in Europe; however, its cultivation and popularity may rise in the future with increasing awareness of its advantages. In agricultural practices, beneficial soil microorganisms, such as arbuscular mycorrhizal fungi (AMF), *Trichoderma* spp., *Streptomyces* spp., and *Aureobasidium* spp., play crucial roles in promoting plant health, enhancing agricultural productivity together with improved crop nutritional value. This study aimed to investigate the effects of individual and combined inoculation on the polyphenol content of okra fruits, as analyzed by HPLC. Moreover, growth parameters and glutathione-S-transferase enzyme (GST) activities of okra leaves were also estimated. Tested microorganisms significantly increased the yield of okra plants except for *A. pullulans* strain DSM 14950 applied individually. All microorganisms led to increased GST enzyme activity of leaves, suggesting a general response to biotic impacts, with individual inoculation showing higher enzyme activity globally compared to combined treatments. According to the polyphenol compound analysis, the application of tested microorganisms held various but generally positive effects on it. Only the combined treatment of *F. mosseae* and *Streptomyces* strain K61 significantly increased the coumaric acid content, and the application of *Aureobasidium* strain DSM 14950 had a positive influence on the levels of quercetin and quercetin-3-diglucoside. Our preliminary results show how distinct polyphenolic compound contents can be selectively altered via precise inoculation with different beneficial microorganisms.

Keywords: okra; *Funneliformis mosseae*; *Trichoderma* spp.; *Aureobasidium* spp.; *Streptomyces* spp.; polyphenol

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1. Introduction

Okra (*Abelmoschus esculentus* L.) is a significant member of the Malvaceae family. It originated in Ethiopia; today, it is still a widely cultivated and consumed vegetable plant

in tropical and sub-tropical regions [1]. Although the production of okra in Europe is not significant, it has increased in recent years, reaching 8094.37 t in 2022. Because of its origin, okra is frost-sensitive and vulnerable to drought conditions, and it thrives in regions characterized by warm climates and ample sunlight [2–4]. Its optimal germination temperature ranges between 28 and 32 °C; therefore, some warmer areas of Europe are also suitable for okra cultivation.

Okra pods have gastronomical and health benefits related to their rich nutrient content and essential mineral content [5–8]. They abound in microelements, like Fe, Zn, Mn, and Ni [9]; moreover, they are also rich in various vitamins, such as vitamins C, B, E, and K [10]. Both vitamins C and E maintain antioxidant functions and play a vital role in reducing and controlling oxidative stress [11,12]. In addition, okra fruits are also rich in polyphenols [13], which represent a diverse group of naturally occurring compounds found abundantly in plants, characterized by the presence of multiple phenolic rings, and are known for their antioxidant properties [14,15]. Polyphenols have been extensively studied for their potential health benefits, including anti-inflammatory, anticancer, antimicrobial, and cardiovascular protective effects. Their antioxidant attribution and their abundance establish them as one of the major bioactive compounds of okra fruits [16–19].

Okra plants are not widely known and consumed in Europe; however, due to the beneficial properties of this vegetable previously described, its popularity and relevant outcomes will increase in the future. This opportunity requires the gathering of information about the cultivation of okra plants, and considering global climatic trends, it is pivotal to broaden the knowledge about the application of sustainable methods. Among these technologies, there is a popular method of using beneficial soil microorganisms which play crucial roles in promoting plant health and enhancing agricultural productivity. These microorganisms have an impact on plants through various mechanisms, both direct and indirect ways to influence nutrient acquisition, disease suppression, and stress tolerance. Among the beneficial fungi, arbuscular mycorrhizal fungi (AMF) represent a major part of plant–microbe interactions according to their abundance and great beneficial impacts [20]. Furthermore, they also improve the secondary metabolite production and nutritional values of different crops [21]. Another beneficial fungus, *Trichoderma asperellum*, generally occurs in soil, as does *Streptomeyces griseoviridis*, a Gram-positive bacterium; besides their antagonistic trait, their saprophytic nature is also well-known. Both these genera are characterized by the production of secondary metabolites, which provides opportunities for practical applications [22,23]. In addition to these biocontrol microorganisms, *Aureobasidium pullulans* has also emerged as a new alternative, further expanding the existing possibilities [24].

In our study, we inquire if these microorganisms' combined application with AMF shows possible synergistic combinations despite the fact that a major obstacle to beneficial impacts may be their antagonistic properties. Moreover, we aimed to gain deeper insight into how different inoculants influence the quantity of polyphenol components.

2. Materials and Methods

2.1. Plant Material and Maintaining the Strains

The commercial seeds of okra (*Abelmoschus esculentus* L. 'Moench' var. Lady Finger F1) originated from a company (Agrimax group S.L.U, Barcelona, Spain).

Arbuscular mycorrhizal (AM) fungi, based on spore morphology and molecular methods, were identified as *Funneliformis mosseae* (Glomerales, Glomeraceae). This strain originated from the collection of the Department of Microbiology and Applied Biotechnology of the Hungarian University of Agriculture and Life Sciences, Hungary. *F. mosseae* was propagated on maize (*Zea mays* L. 'Golda F1') growing on sterilized peat (Klasmann TS3, 100 mg L^{-1} P_2O_5) and sand 1:3 (*v*/*v*) substrate for three successive propagation cycles, each lasting 5 months. The most probable number (MPN) of infective propagules (approximately 35 infective propagules g^{-1}) was determined following the method of Feldmann and Idczack [25].

Trichoderma asperellum strain T34 of a filamentous fungus, a commercial product named Xilon (Kwizda Agro GmbH, Wien, Austria), was selected for this research. It was cultured on Potato Dextrose Agar medium (Difco PDA, 20 g/L dextrose, 15 g/L agar, and 4 g/L potato starch) at 25 °C (ambient temperature and illumination) for a week.

Aureobasidium pullulans strain DSM 14950 originated from Blossom Protect (SAN Agrow Holding GmbH, Herzogenburg, Austria), a commercial product. It was cultured on Potato Dextrose Agar medium (Difco PDA, 20 g/L dextrose, 15 g/L agar, and 4 g/L potato starch).

Streptomyces strain K61, previously *S. griseoviridis*, was from Lalstop K61 WP (formerly Mycostop, Danstar Ferment AG, Zug, Switzerland), which is also a commercial product. We used dextrose, 15 g agar, and 4 g potato starch in 1 L of distilled and bacterial media, Nutrient Agar (NA), 5 g of peptic digest of animal tissue, 3 g of beef extract, and 15 g of agar in 1 L of distilled media to subculture it.

2.2. Experimental Design

The experiments were conducted during the summer of 2022 in the experimental station belonging to the Plant Protection Institute—Hungarian University of Agriculture and Life Sciences (MATE), located at 2100 Gödöllő, Pest, Hungary (coordinates: 47.594315, 19.368984).

The okra plants were initially cultivated in a greenhouse environment, which is renowned for maintaining temperature conditions typically ranging between 18 and 35 °C, and the duration of illumination was 12–14 h of light per day in a greenhouse. The applied soil type in this experiment was sterilized sand mixed with horticultural soil (Klasmann TS3, 100 mg L^{-1} P_2O_5) 3:1 (*v*/*v*); the juvenile plants were cultivated in small pots, and subsequently, they were transferred to bigger pots containing 2 kg of sterilized soil mixture with one plant in each pot. Soil pH was 6.7. Pots were placed in glasshouse benches in a completely randomized design for each treatment. The plants were subjected to a watering regimen, with irrigation occurring every two days or as needed, by monitoring the moisture content of the soil.

Plants were treated with three microorganism strains and AMF individually and in combination. The experiment setup contained 11 replicates of okra plants per treatment.

AMF inoculum was introduced to okra plants through an inoculation process involving the addition of 10 g mycorrhizal inoculum containing approximately 350 infective propagules per plant. The control plants received the same amount of inoculum after it was sterilized three times.

Spore suspensions (4.5×10^6 spores mL^{-1}, 8.8×10^6 spores mL^{-1}, and 1.1×10^6 spores mL^{-1}, respectively) of *T. asperellum* strain (T34), *A. pullulans* strain (DSM 14950), and *S. griseoviridis* (K61) were prepared after cultivation on PDA medium or Nutrient Agar at 25 °C for 7 days. Plants were irrigated with the respective suspensions at 20 D when they had at least nine or more unfolded leaves on the main shoot.

Plants were harvested after 75 days.

2.3. Determination of the Root Colonization by Arbuscular Mycorrhizal Fungi

After the 75-day-old plants were harvested, root samples of 5 plants per treatment were randomly taken to identify mycorrhiza colonization of the root. Root samples of okra plants were washed thoroughly with tap water and then cut into 1 cm long pieces. The roots were placed in a 10% aqueous solution of KOH (*w*/*v*). Roots and KOH were heated in a water bath at 90 °C for 60 min; then, the solution was decanted, and the roots were washed with running water; after that, the roots were washed with 5% vinegar (acetic acid) for 1–2 min and put in 5% ink (Pelikan blue), and then the roots and the ink were boiled for 2 min [26]. Root colonization was evaluated through visual observation using a stereomicroscope with a magnification of 100×. To determine the colonization, the gridline intersection method was used [27]. This involved observing the presence or absence of mycorrhizal structures at the intersections between the root fragments and the gridlines.

2.4. Assessment of the Growth Parameters

The shoot and root fresh weight (FW) of each sample were weighed after harvesting (75D), and then each sample was dried in a hot-air oven at 70 °C for 2 days to determine its dry weight (DW).

The yield of okra can be measured in terms of the quantity of pods harvested per treatment. Fruit was collected from the plants between the 54th and 75th days.

2.5. Determination of Glutathione-S-Transferase Enzyme Activity

During the sampling process (D60) for measuring glutathione-S-transferase (GST) enzyme activity, plant leaves (the third leaf from the top of the plant, which is healthy) were collected and stored at −80 °C until utilization.

Leaf tissue (500 mg) was suspended in 100 µL of Cell Lysis Buffer; then, leaves were homogenized with a mortar and pestle. Samples were centrifuged for 10 min at 4 °C and 13,300 rpm using a cold microcentrifuge to remove any insoluble material. The supernatant was transferred into a clean tube and kept on ice. GST activity was measured based on the method of Habig et al. [28]. The soluble protein level of all extracts was determined according to the method of Bradford [29].

2.6. HPLC Analysis of the Polyphenol Compounds

For the purpose of conducting phenol analysis, the fruit of the okra plants was physically harvested by handpicking every two to three days, starting from the commencement of the flowering and fruiting period. It was then preserved in a biotechnology laboratory of MATE at a temperature of −20 degrees Celsius.

Then, 300 mg of lyophilized (freeze-dried) whole okra fruits was taken and crushed in a crucible mortar in the presence of 1–2 g of quartz sand. The phenolic compounds were extracted by adding methanol containing 2% orthophosphoric acid. The macerate was then transferred to a centrifuge tube and subjected to ultrasonication for 15 min at 40 °C in a water-bath ultrasonic device (model RK-165-BH Bendelin Sonorex, Berlin, Germany) followed by mechanical shaking at room temperature for 20 min. The extract was centrifuged for 5 min at 5000 rpm (M-Universal, MPW Med. Instrument, Warsaw, Poland). The supernatant was decantated into a round-bottom flask, and the solvent was evaporated to dryness under vacuum at 45 °C. The residues were redissolved in 5 mL of 1:1 methanol/L% orthophosphoric acid and finally purified through a 0.45 µm, 25 mm cellulose acetate syringe filter before injection into the HPLC apparatus.

A Hitachi Chromaster HPLC instrument (Tokyo, Japan) containing a Hitachi Chromaster Model 5160 gradient pump (Tokyo, Japan), a Hitachi Chromaster Model 5260 autosampler, a Hitachi Chromaster Model 5310 column oven (Tokyo, Japan), and a Hitachi Chromaster Model 5430 diode-array detector (Tokyo, Japan) was used with Agilent OpenLab EZChrom A.04.10 software (Santa Clara, CA, USA) for operation and data processing.

The separation of phenolic compounds was performed on an Ascentis phosphor-conditioned C18 phase (C18-PCP, from Supelco, Bellefonte, PA, USA) with gradient elution of 1% ortho-phosphoric acid (A) and acetonitrile (B) according to a recently developed protocol (under publication). The gradient elution started with 1% B in A, changed to 20% B in 20 min, stayed isocratic for 10 min, changed to 30% B in 5 min, stayed isocratic for 10 min, and finally turned to 1% B in 5 min. The DAD detection was between 190 nm and 700 nm. The quantification was based on recording the area at the maximum absorbance wavelength of each compound and relating it to that of the standard solution.

Stock solutions for different phenolics (Sigma-Aldrich via Merck, Budapest, Hungary) were prepared by dissolving 2–3 mg in 10 mL absolute ethanol or methanol and diluted 10 times with 40% ethanol in 1% ortho-phosphoric acid. The working solutions were used for calibration curves, identification, and quantification of phenolic compounds; in case no standard was available, the compounds were tentatively identified based on a comparison of their spectral characteristics and chromatographic behavior with literature data [30–32].

2.7. Statistical Analysis

RStudio 2024.04.0+735 software was used for statistical analysis. All data were evaluated by one-way analysis of variance (ANOVA) with various microorganism applications. Means were compared by Tukey post hoc test at $p < 0.05$, and Pearson correlation coefficients (R) among physiological parameters, GST enzyme activity, and polyphenol contents of okra fruits were calculated. In addition, PCA was carried out to identify patterns, i.e., interactions among the studied variables and treatments, in the polyphenolic data of okra fruits treated with different beneficial microorganisms and their combinations. The results of the statistical analysis are presented in Tables A1–A5 in the Appendix A.

3. Results

3.1. AMF Colonization

The plants inoculated with arbuscular mycorrhizal fungi showed an appropriate level of colonization (Figure 1); applying *T. asperellum* strain T34 (M + T) or *A. pullulans* strain DSM 14950 (M + A) slightly decreased the colonization level; however, the application of *S. griseoviridis* strain K61 (M + S) did not influence mycorrhizal presence. The highest colonization level was reached by using AMF individually (M) and AMF combined with *S. griseoviridis* strain K61 and was 84.68% and 85.16%, respectively.

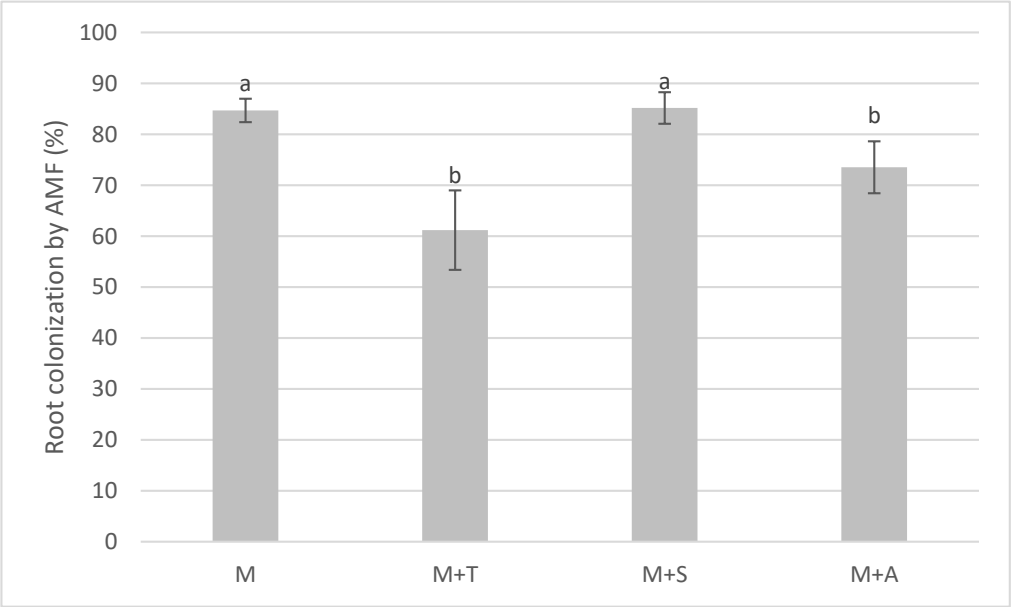

Figure 1. Root colonization level of the okra plants inoculated with arbuscular mycorrhizal fungi. M: *F. mosseae*; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

3.2. Fresh and Dry Weight of Plant and Yield Quality

According to our results, the treatments in which we applied AMF increased the plants' fresh and dry weight (Figure 2). When we applied only *T. asperellum* strain T34 (T), *S. griseoviridis* strain K61 (S), or *A. pullulans* strain DSM 14950 (A), the treatment did not have a significant impact on the weights compared to the control plants (C). In the case of the combined treatments, the beneficial effect of the AMF prevailed; when it was applied with *T. asperellum* strain T34 (M + T) or *A. pullulans* strain DSM 14950 (M + A), the measured shoot and root weights were the same as when we applied AMF individually (M); therefore, we did not observe the antagonist effect of the other fungi. When we applied AMF and *S. griseoviridis* strain K61 (M + S) in combined treatment, we recorded lower weight data compared to the plants that were inoculated only with AMF.

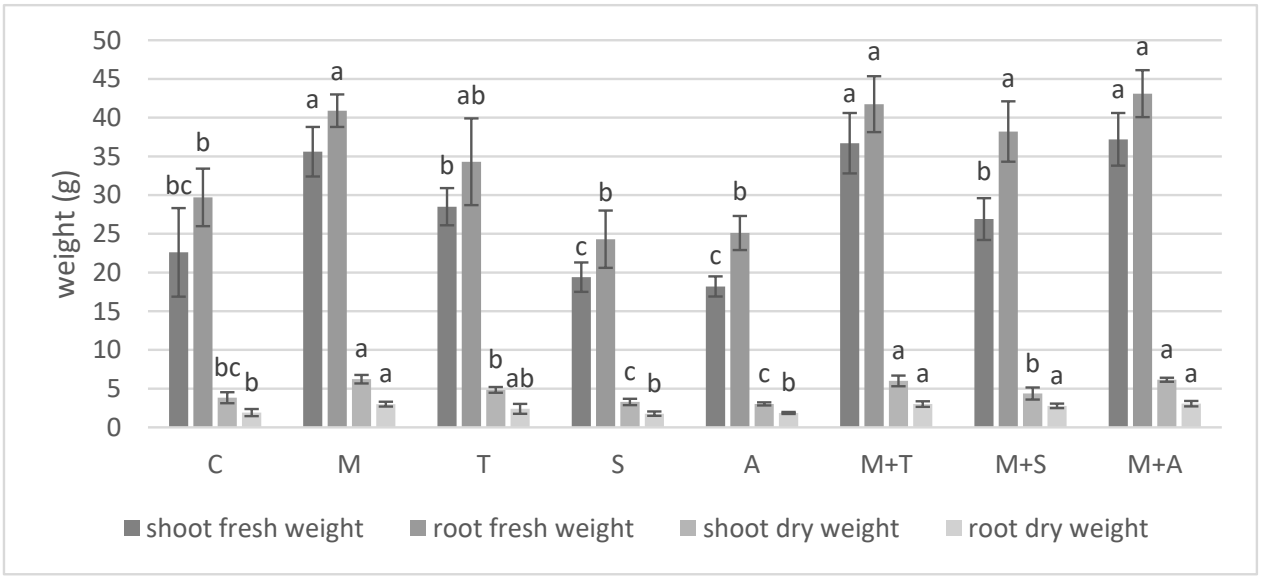

Figure 2. Average fresh and dry weight of the okra plants' shoots and roots. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

According to our results, the yield of the okra plants is significantly elevated by the applied microorganisms (Figure 3), except for the treatment in which we applied *A. pullulans* strain DSM 14950 individually (A). This yeast-like fungus also had a negative effect on the beneficial impact of the AMF; in the combined treatment (M + A), we did not record as high a yield as the other cases.

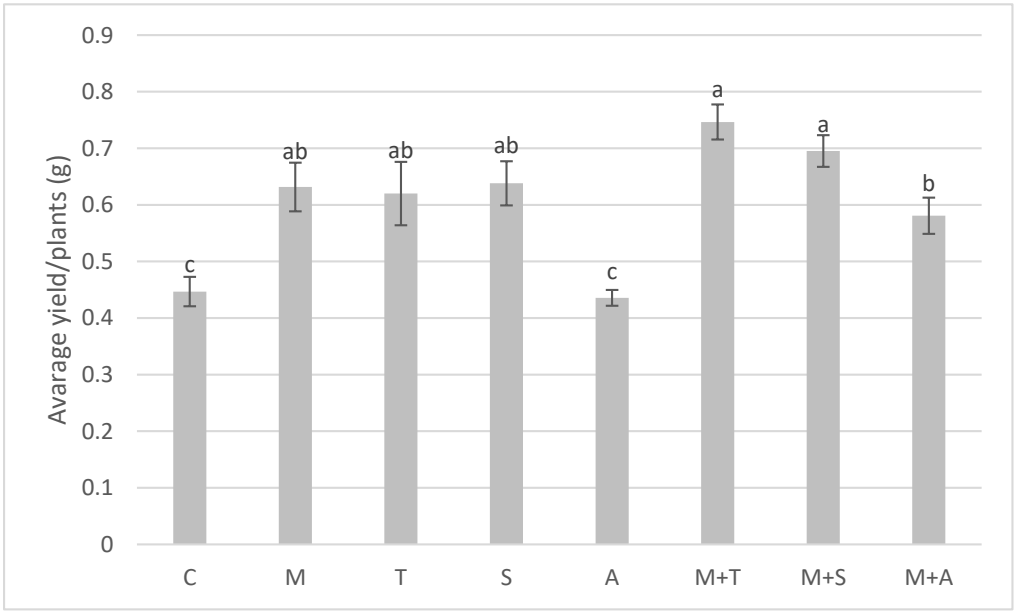

Figure 3. The average yield of the okra fruits/plants. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

3.3. GST Enzyme Activity

We observed elevated levels of GST enzyme activity when we applied beneficial microorganisms (Figure 4). Combined treatments also increased GST enzyme activity, although when we applied AMF and *S. griseoviridis* strain K61 together, they did increase the GST enzyme activity compared to the control plants (C) but not as much as other treatments. Further studies are needed to broaden the knowledge about the interaction between the applied microorganisms and their effect on the GST enzyme activity.

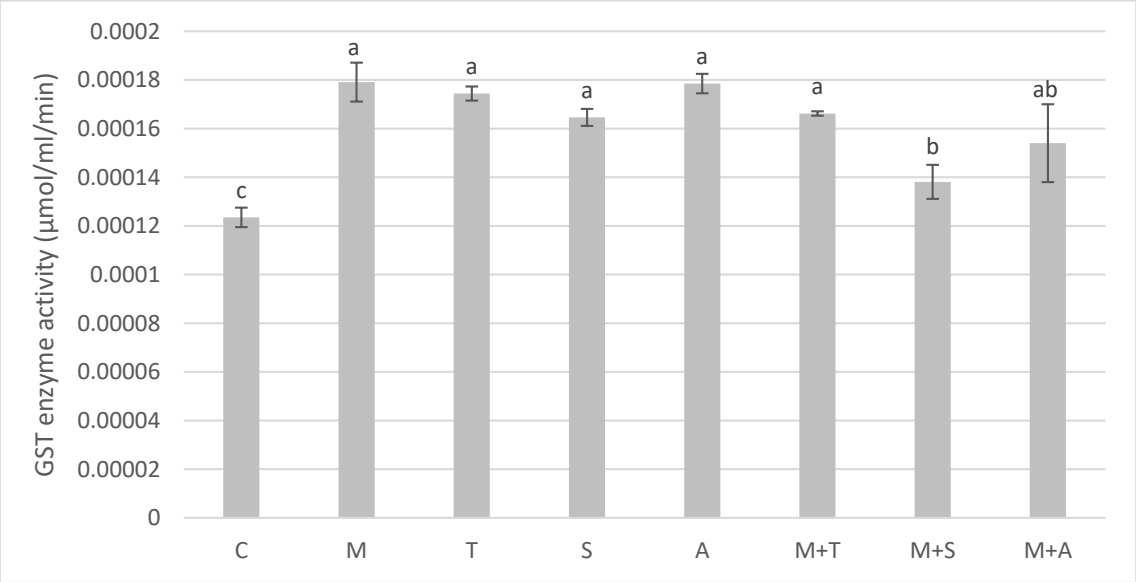

Figure 4. GST enzyme activity in the leaf of the okra plants. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

3.4. Effects of Biotic Treatments on Phenolic Compounds of Fruit

One of the important objectives of the present work is to investigate the effect of mycorrhiza alone and in combination with microbes on the content of polyphenols in okra fruits. The HPLC protocol applied to analyze polyphenols from the whole okra fruit allowed for excellent separation of the main compounds and their derivatives, mainly dimers and glycosides (Figure 5). To make the discussion easier and more meaningful, the obtained results are arranged in groups for the main compounds.

Figure 6 shows the effect of mycorrhiza and antagonistic microorganisms on the content of kaempferol derivatives in the whole okra fruit. The combination of arbuscular mycorrhizal fungi with other microorganisms in different treatments (M, T, S, M + T, M + S, M + A) caused a significant increase in the level of okra fruit phenols as compared to the control treatment (C). The results presented in Figure 6 demonstrate the effects of various treatments on kaempferol content in okra fruit, in conjunction with arbuscular mycorrhizal fungi and other microorganisms. Figure 6 shows the impact of the combination of mycorrhiza and microbe strains on the okra in increasing the percentage of fruit phenols (kaempferol derivatives). The inoculation of the plants with *T. asperellum* strain T34 (T), mycorrhiza fungi (M), *Streptomyces* strain K61 (S), M + S, M + T, M + A, and *A. pullulans* strain DSM 14950 (A) increased the content of kaempferol by 38%, 29%, 27%, 22%, 20%, 17%, and 14%, respectively, compared with the control, and these increases were highly significant. The highest increase in the content of the kaempferol derivatives was recorded for T treatment.

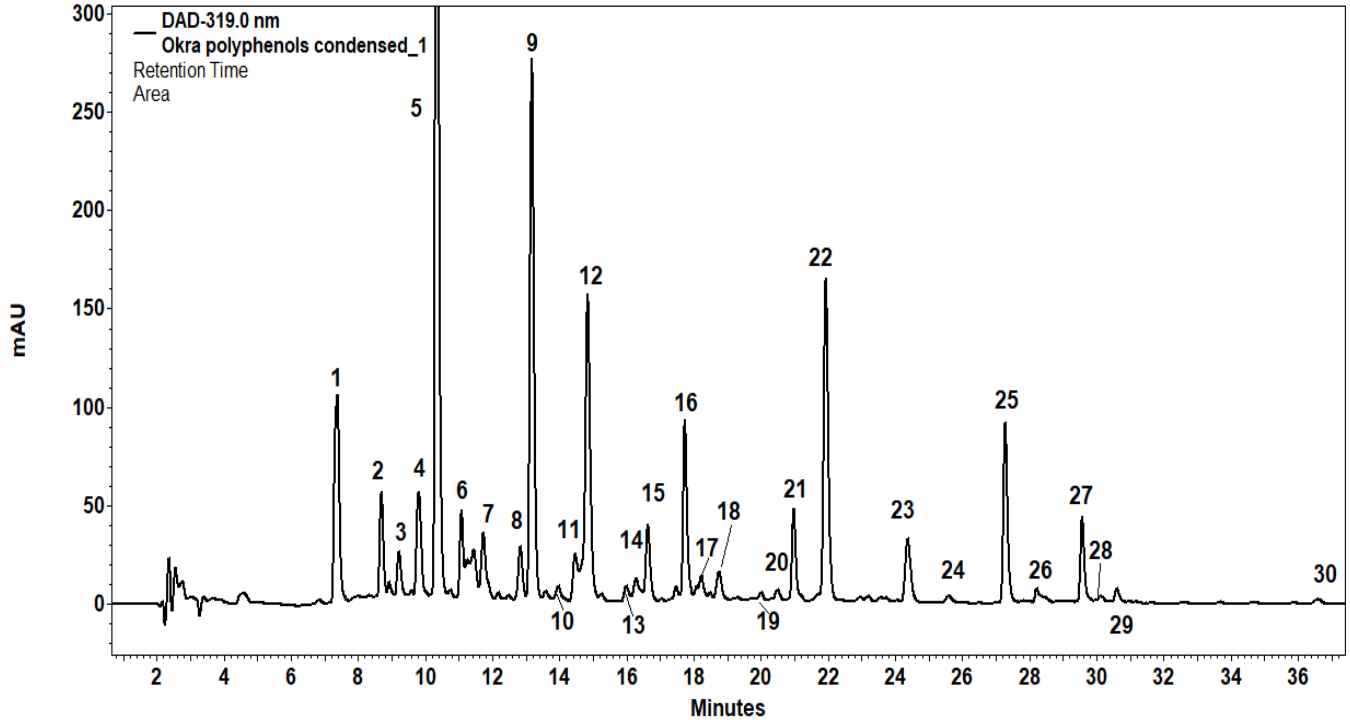

Figure 5. HPLC profile of phenolic compounds extracted from lyophilized okra fruits and separated on C18-PCP column with gradient elution of acetonitrile in 1% orthophosphoric acid. Detection was at 319 nm. Peak identification is shown in Table A6 in the Appendix A.

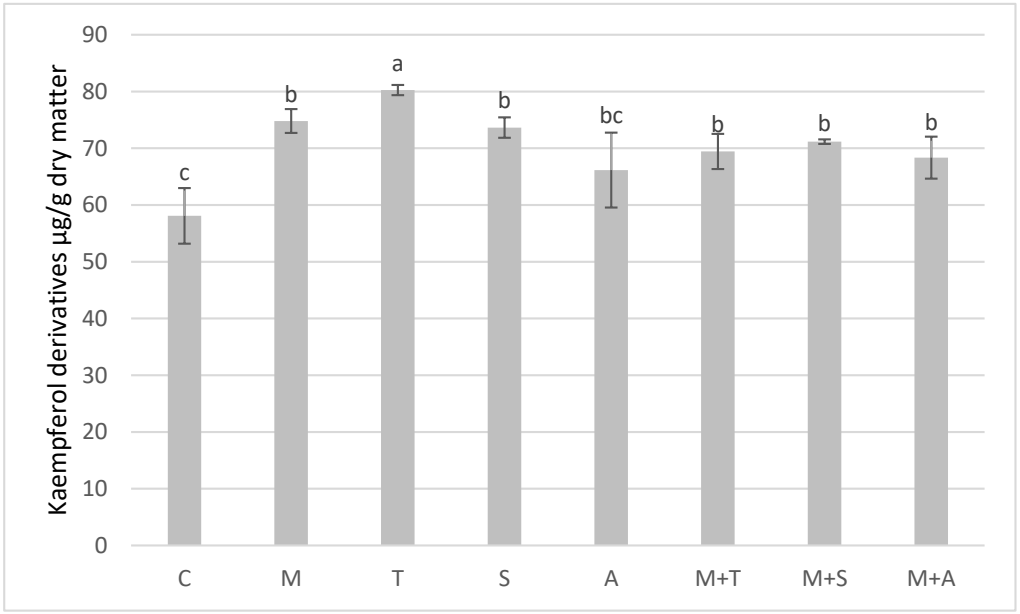

Figure 6. Effect of AMF and antagonistic microorganisms on kaempferol derivatives in okra fruit. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

The results presented in Figure 7 demonstrate the response of coumaric acid derivatives to various treatments. Such important polyphenolic compounds exist in okra fruits in a free form and as coumaroyl-hexoside. The treatments of M + S and M + A showed

an increase in the content of the two derivatives as compared to the control group. It is evident that a highly significant increase of coumaric acid and coumaroyl hexoside was recorded for treatments of M + A and M + S, respectively. As compared to the control, other treatments either decreased or had no significant effect on the level of coumaric acid derivatives. The results showed that the plants' inoculation with microbes increased the level of coumaroyl derivatives by 43%, 22%, 12%, 9%, and 7% with M + A, M + S, M, S, and A, respectively. As for *T. asperellum* strain T34, no significant effect on coumaric acid in the okra plant was noticed.

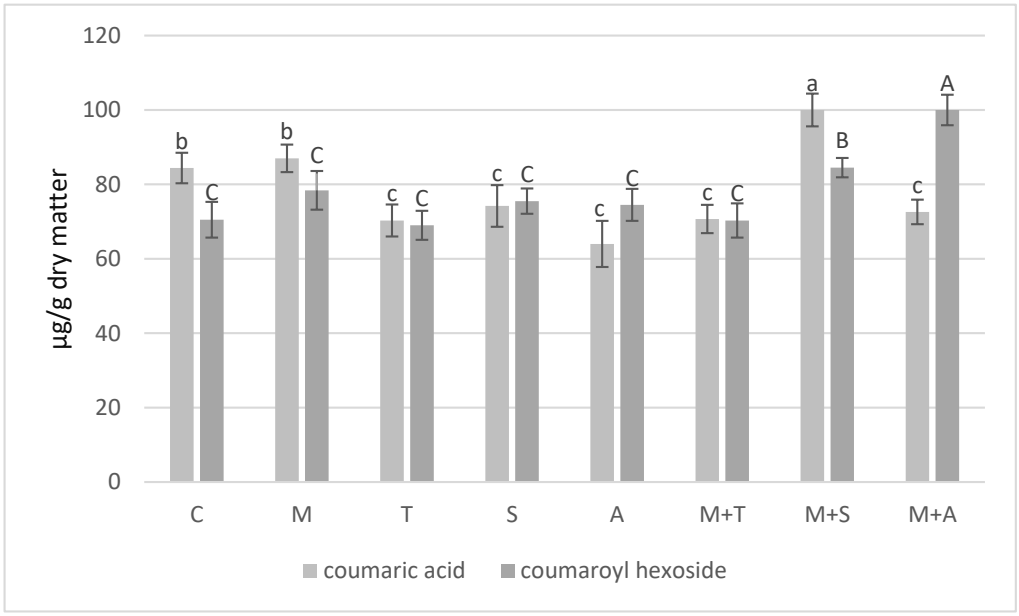

Figure 7. Effect of AMF and antagonistic microorganisms on coumaric acid and coumaroyl hexoside content in okra fruit. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

Quercetin was found to exist in the extract of the whole okra fruit as quercetin-3-o-glucoside, quercetin-di-glucoside, and quercetin-3-O-(melanoyl)glucoside. The impact of the different treatments on the quantity of quercetin derivatives is shown in Figure 8. It is of interest that *A. pullulans* strain DSM 14950 (A) treatment resulted in a highly significant increase in the average content of quercetin, which was found to be due to higher activation of quercetin-3-diglucoside biosynthesis. Interestingly, the combination of mycorrhiza with *A. pullulans* strain DSM 14950 (M + A) yielded fruits with significantly higher levels of quercetin 3-diglucoside as compared to other treatments, but not as high as those recorded for *A. pullulans* strain DSM 14950 alone.

Distinct patterns were found in the accumulation of chlorogenic acid–catechin–glucoside phenols in okra fruit under the influence of arbuscular mycorrhizal fungi and antagonistic microorganisms M, T, A, and S and the combination of AMF with other microorganisms: M + T, M + A, and M + S. Quantitative measurements allowed for the characterization of phenolic content, with specific attention to variations induced by the microbial treatments. The biotic treatments with M, M + T, M + S, Sg, M + A, T, and A increased the level of these polyphenols by 163%, 116%, 86%, 79%, 63%, 29%, and 5%, respectively, and it was found that M and M + T treatments significantly increased the content of chlorogenic acid–catechin dimers in okra fruits as compared to the control (Figure 9).

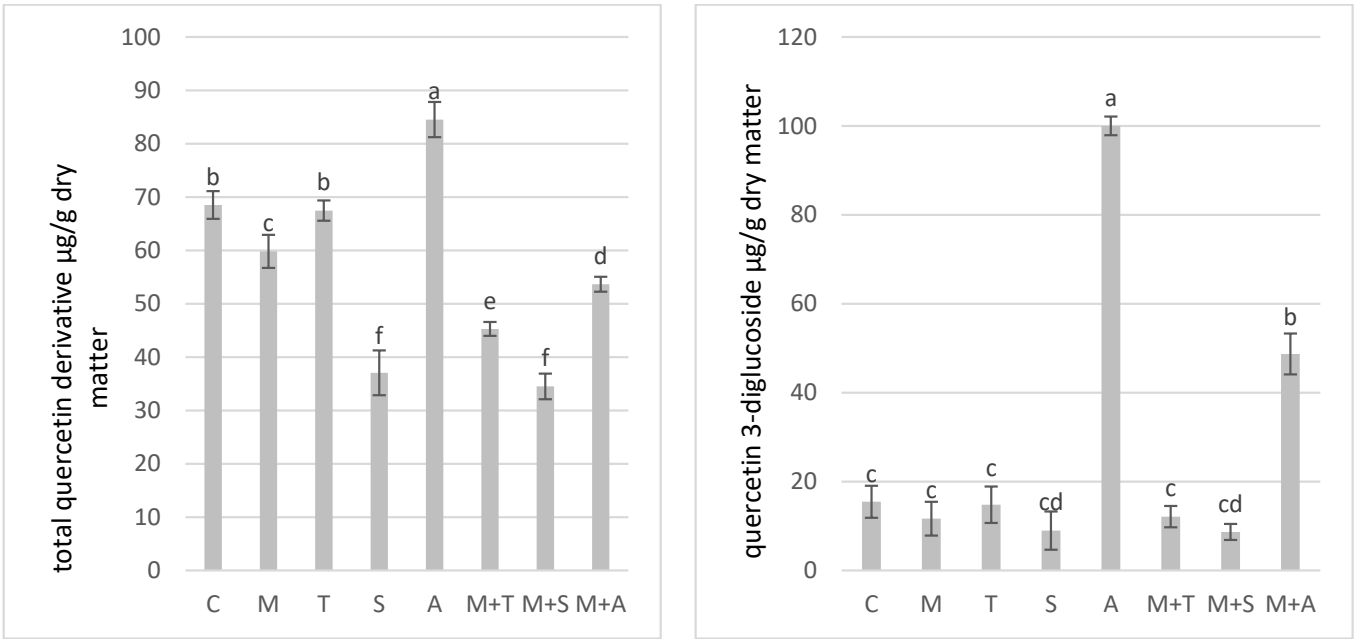

Figure 8. Impact of different biotic treatments on the content of total quercetin derivatives (**a**) and quercetin 3-diglucoside (**b**) in okra fruits. C: no treatment; M: *F. mosseae;* T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

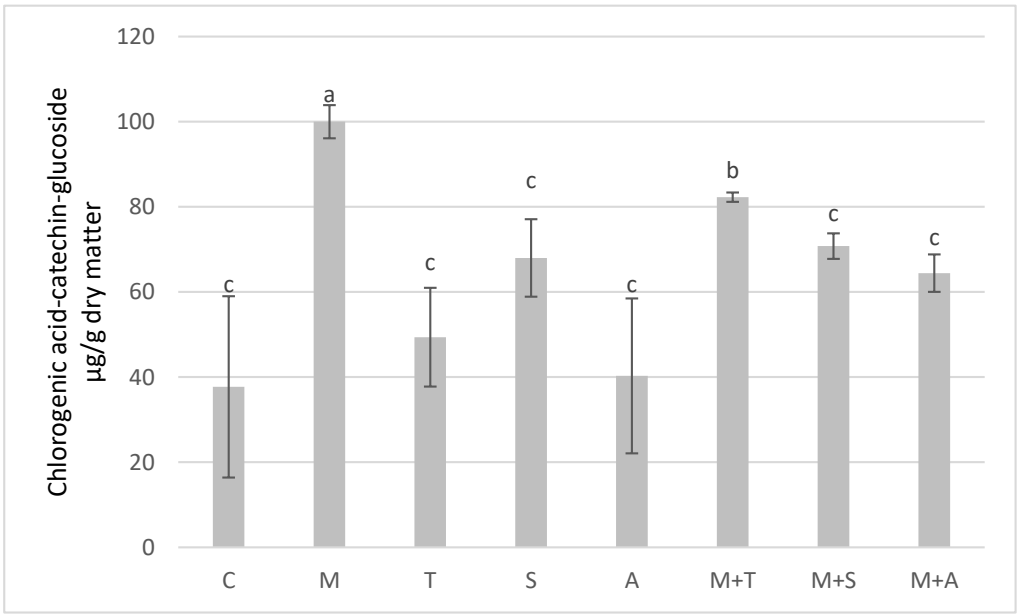

Figure 9. Effect of AMF and antagonistic microorganisms on chlorogenic acid–catechin–glucoside in okra fruit. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

It is evident from the results presented in Figure 10 that all treatments of mycorrhiza alone or combined with other microbial strains, except for the combination of AMF with *S. griseoviridis* strain K61 (M + S), promoted the biosynthetic pathways of sinapic acid derivatives as compared to the control treatment. The highest increase in the content of such dimers was found in fruits of okra treated with AMF combined with *A. pullulans* strain DSM 14950.

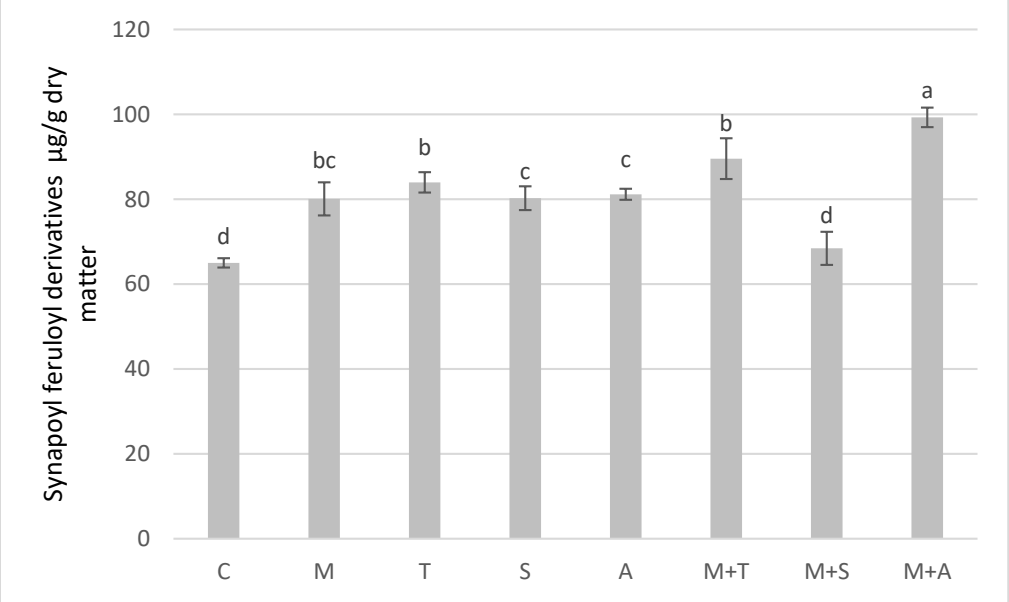

Figure 10. Effect of AMF and antagonistic microorganisms on total content of sinapoyl feruloyl derivatives in okra fruit. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

In Figure 11, the synergistic impact of the combined application of mycorrhiza and other microorganisms on di-caffeoylquinic acid levels in okra fruits is depicted. All treatments except S and M + S treatments showed significantly higher contents of di-caffeoylquinic acid in okra fruits. The highest increase in the concentration of this polyphenol was found in M, M + T, and M + A with no significant variation between them in their impact on di-caffeoylquinic acid. In particular, the S-type bacterial inoculation had a remarkable negative impact on the metabolic pathway of this polyphenol, most probably due to the partial inhibition of the enzymes involved in the biosynthesis processes of di-caffeoylquinic acid. The combination of S with AMF slightly moderated the negative effect of S on di-caffeoylquinic acid formation in okra fruit; however, the difference was not significant.

Principal component analysis of individual polyphenols was performed, and its result is shown in Figure 12. The first two principal components (PC1 and PC2) explain 59.7% of the total variation. Three components showed eigenvalues higher than 1 (Table A4 in the Appendix A). PC1, covering 33.5% of the total variation, had negative associations with quercetin and quercetin-3-diglucoside and positive associations with coumaric acid and chlorogenic acid. In addition, 26.2% of the total variance was covered by PC2, which was positively influenced by kaempferol, coumaroyl hexoside, chlorogenic acid, sinapoyl, and di-caffeylquinic acid (Table A5 in the Appendix A).

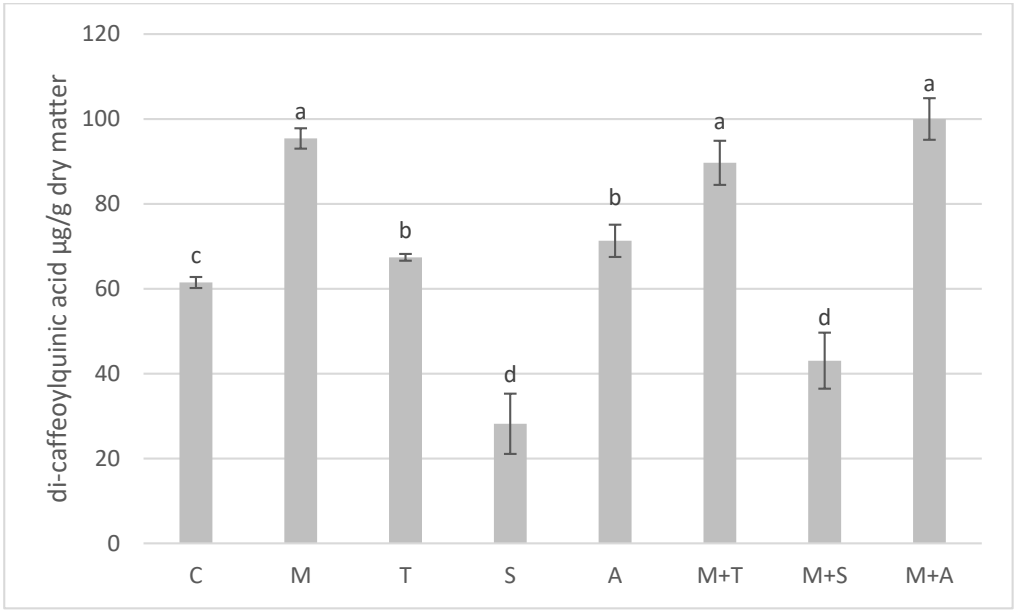

Figure 11. Effect of AMF and antagonistic microorganisms on di-caffeoylquinic acid in okra fruit. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950. Mean values followed by the same alphabets did not differ significantly based on Tukey's test ($p < 0.05$).

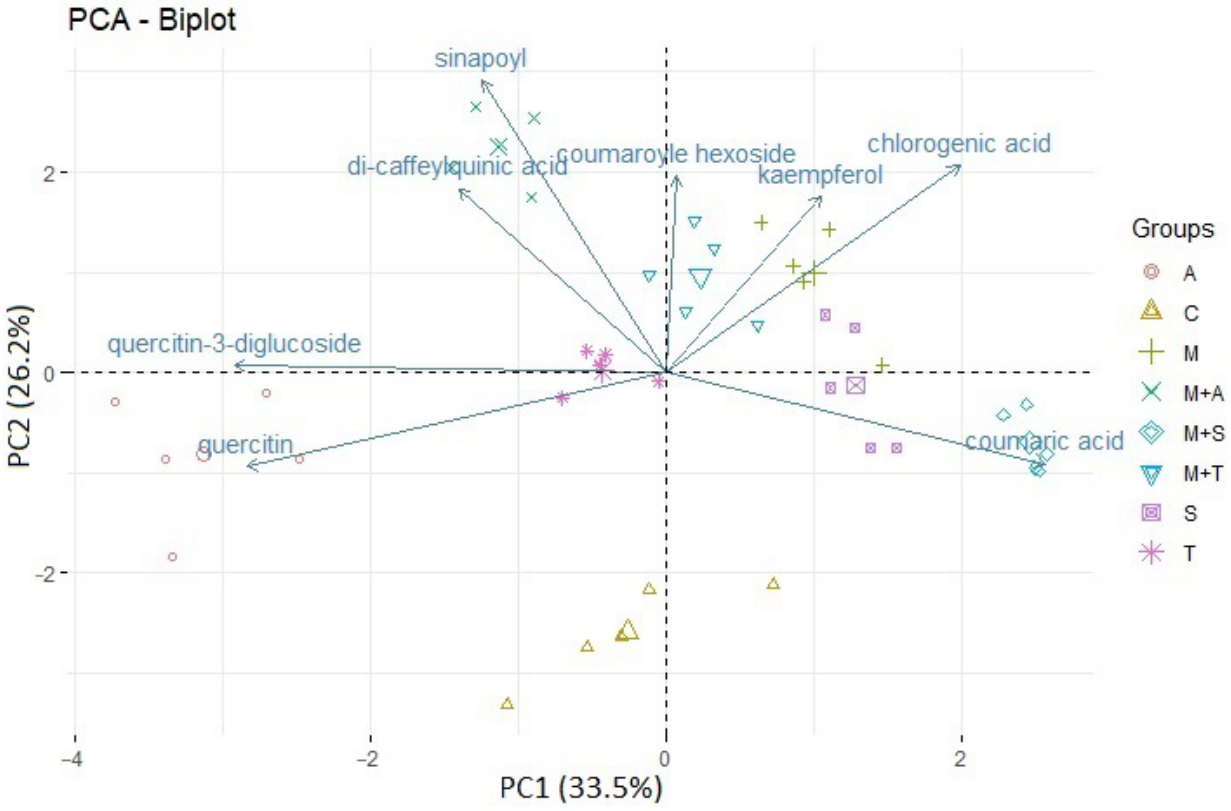

Figure 12. Principal component analysis of individual polyphenols in the fruits of okra plants. C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950.

The biplot demonstrated relatively clear discrimination among the groups of the control treatment and the treatments with beneficial microorganisms. Differences among A, C, and M + S groups were discriminated by PC1, while PC2 distinguished between the beneficial microbes and the control treatment.

Coumaric acid content showed a strong negative correlation with the sinapoyl, quercetin-3-diglucoside, and quercetin contents of the okra fruits according to the Pearson correlation analysis (Table A3 in the Appendix A). There were positive correlations between kaempferol and chlorogenic acid, as well as between sinapoyl and coumaroyl hexoside.

Some of the polyphenolic compounds of the okra fruit showed a strong correlation with the yield (Table A3 in the Appendix A); kaempferol and chlorogenic acid had a positive association; meanwhile, quercetin-3-diglucoside and quercetin had a negative connection with it. The kaempferol and the sinapoyl content showed a positive correlation with the GST enzyme activity, while in the case of coumaric acid content, their connection was described negatively. Obviously, strong positive relationships were also found between all the growth parameters.

4. Discussion

Okra is a highly nutritious vegetable due to its rich vitamin and mineral content. Furthermore, the presence of bioactive compounds, including polyphenols, offers numerous advantageous impacts on human health. However, despite its nutritional value and potential benefits, okra is not widely consumed in Europe, but its cultivation and popularity may increase in the future with growing awareness of its opportunities; therefore, it is pivotal to gather more scientific results about how cultivation methods influence its contents of beneficial secondary metabolites. Vegetable plants like okra are exposed to several biotic and abiotic factors that influence their secondary metabolic pathways and thereby alter the quantity and quality of their bioactive compounds [33]. In our work, well-known beneficial microorganisms like *F. mosseae*, *T. asperellum* strain T34, *S. griseoviridis* strain K61, and *A. pullulans* strain DSM 14950 and their combinations were applied to test their influence on okra's growth and polyphenol content.

Among the applied microorganisms, *F. mosseae* and *T. asperellum* strain T34 showed the best plant-growth-promoting effect, similar to the work of Ali et al. and Mwangi et al. [34,35]. These tendencies were manifested both in individual and combined treatments. Interestingly, mixed inoculation always caused higher growth responses than separate inoculation, and a synergistic relationship between inoculants could be observed, but not for M + T. Some references also highlight that microorganisms living in the rhizosphere could influence the expression of plant–AMF symbiosis [36–38]. Estimating the root colonization together with the growth parameters of okra, our results confirmed that the antagonism of *Trichoderma* strain T34 against AMF could be compensated for by the saprophytic ability of the mentioned fungi. The degradation of organic matter in the soil and the release of nutrients by *Trichoderma* are well-known [39,40], and the bridge offered by the external mycelium of AMF between the plant and nutrient-rich patches provides support for the plant. In this way, even the putative negative effects of biocontrol strains, like *Trichoderma* spp., can be compensated for.

Different classes of polyphenols, including flavonoids (e.g., flavonols, flavanols, flavones, flavanones, anthocyanins), phenolic acids (e.g., hydroxybenzoic acids, hydroxycinnamic acids), and other polyphenols (e.g., lignans, stilbenes), are important components of okra fruits [41]. Although there are some studies about the polyphenol content of okra fruits inoculated with or without AMF [42–44], our work is novel in the scope of changes in various polyphenols in the fruit of okra under the influences of different microorganisms.

Our preliminary results, based on PCA, clearly showed that the estimated polyphenol profile can be altered by the applied microorganisms. Coumaric acid, a well-known antioxidant, increased significantly only with the combined treatment of *F. mosseae* and *S. griseoviridis* strain K61. This finding is contradictory to other studies that reported a positive effect of AMF on coumaric acid [45,46], where different plant species, like cucumber (*Cucumis sativus* L.) or strawberry (*Fragaria* × *ananassa* Duch.), and AMF were

used. This result highlights that different plant and AMF species isolates can modulate plant metabolites on distinct levels.

Another important polyphenol is quercetin, which has antioxidant characteristics and is also influenced by AMF [47]. Eftekhari et al. presented a generally positive effect on quercetin using three different AMF strains and their combinations on grapes [48]. We were not able to confirm these results for okra in the presence of one AMF strain. But at the same time, the combined treatment of *F. mosseae* and *A. pullulans* strain DSM 14950 increased significantly the level of quercetin-3-diglucoside compared to other treatments. Furthermore, the individual application of *A. pullulans* strain DSM 14950 led to more elevated quercetin and quercetin-3-diglucoside contents.

As it is known, secondary metabolites in general are essential for the defense mechanism of plants and can be influenced by microorganisms, like AMF [49]. These compounds, released by both plants and AMF, act as signaling molecules during the symbiotic interaction between plants and AMF. Plants' responses to different stress factors cause secondary metabolism alterations, resulting in changes not only in polyphenol compounds but also in other metabolites, such as terpenoids [50,51]. Our GST enzyme activity measurements prove that the okra plants detected the presence of all the applied microorganisms as a slight biotic stress factor that resulted in elevated enzyme concentrations compared to the control treatment. The observed negative correlation between GST enzyme activity and coumaric acid indicates that the cause of this is the antioxidant activity of coumaric acid or the possibility that one monomer from the enzyme family of GST (4-CA) binds p-coumaric acid [52].

Our results highlight the potential benefits of utilizing these microorganisms in combination with mycorrhiza as a means of enhancing okra fruit phenolic content and productivity.

5. Conclusions

The aim of this study was to assess how the tested biocontrol microorganisms affect okra growth with a specific focus on the production of polyphenols in the fruit. Our preliminary results indicate that all tested microorganisms had various but generally positive effects on plant growth and yield, with the exception of *A. pullulans* strain DSM 14950 and *S. griseoviridis* strain K61 applied individually.

We were able to assign different microorganisms and their combinations to specific polyphenol compounds and determine the relationships between them. These results provide insights into how the content of specific polyphenol compounds can be selectively influenced through targeted inoculation with different microorganisms.

Our results highlight the potential of tested microorganisms in agricultural practices for improving crop quality and quantity. Further investigation in this field is warranted to fully elucidate the scope of their potential applications and optimize their utilization.

Author Contributions: Supervision, conceptualization, formal analysis, writing—original draft, writing—review and editing, resources, K.P. and G.T.; formal analysis, A.L.S.; formal analysis, A.I.A.Y.; writing, data curation, formal analysis, L.L.; data curation, formal analysis, investigation, methodology, methods, A.A.A.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study did not involve human participants or animals. The current study complies with relevant institutional, national, and international guidelines and legislation for experimental research and field studies on plants (either cultivated or wild) and fungi, including the collection of plant and fungal materials.

Data Availability Statement: The associated dataset of this study is available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Results of one-way ANOVA.

	F Value	P
Colonization	25.223	2.643×10^{-6} ***
Shoot FW	27.161	9.537×10^{-12} ***
Shoot DW	29.249	3.523×10^{-12} ***
Root FW	21.455	2.083×10^{-10} ***
Root DW	10.937	5.755×10^{-7} ***
Yield	47.42	4.1×10^{-15} ***
GST	297.68	2.2×10^{-16} ***
Kaempferol	17.36	2.933×10^{-9} ***
Coumaric acid	33.488	5.545×10^{-13} ***
Coumaroyle hexoside	30.239	2.243×10^{-12} ***
Quercetin	202.9	$<2.2 \times 10^{-16}$ ***
Quercetin-3-diglucoside	422.88	$<2.2 \times 10^{-16}$ ***
Chlorogenic acid	17.245	3.181×10^{-9} ***
Sinapoyl	63.176	$<2.2 \times 10^{-16}$ ***
Di-caffeyl quinic acid	8.7901	5.327×10^{-6} ***

*** significant at $p < 0.001$.

Table A2. Results of Tukey's post hoc test (COL: colonization level; SFW: shoot fresh weight; SDW: shoot dry weight; RFW: root fresh weight; RDW: root dry weight; Y: yield; GST: gluthatione-S-transferase enzyme activity; KMP: kaempferol; CA: coumaric acid; CHex: coumaroyl hexoside; QCT: quercetin; Q3DG: quercetin-3-diglucoside; CGA: chlorogenic acid; SIN: sinapoyl DCQH: di-caffeyl quinic acid; C: no treatment; M: *F. mosseae*; T: *T. asperellum* strain T34; S: *S. griseoviridis* strain K61; A: *A. pullulans* strain DSM 14950; M + T: *F. mosseae* and *T. asperellum* strain T34; M + S: *F. mosseae* and *S. griseoviridis* strain K61; M + A: *F. mosseae* and *A. pullulans* strain DSM 14950).

	COL	SFW	SDW	RFW	RDW	Y	GST	KMP	CA	CHex	QCT	QDG	CGA	SIN	DCQH
C − A		0.438	0.296	0.496	0.999	0.999	0.000	0.069	0.000	0.080	0.000	0.000	0.999	0.000	0.041
M − A		0.000	0.000	0.000	0.001	0.000	0.999	0.072	0.000	0.815	0.000	0.000	0.000	0.999	0.037
M + A − A		0.000	0.000	0.000	0.000	0.000	0.064	0.974	0.084	0.000	0.000	0.000	0.087	0.000	0.022
M + S − A		0.005	0.009	0.000	0.011	0.000	0.000	0.353	0.000	0.137	0.000	0.000	0.064	0.000	0.031
M + T − A		0.000	0.000	0.000	0.001	0.000	0.053	0.817	0.303	0.754	0.000	0.000	0.000	0.003	0.027
S − A		0.999	0.995	0.999	0.999	0.000	0.062	0.381	0.092	0.999	0.000	0.000	0.056	0.999	0.003
T − A		0.001	0.000	0.072	0.371	0.000	0.203	0.000	0.376	0.449	0.000	0.000	0.908	0.038	0.999
M − C		0.000	0.000	0.001	0.002	0.000	0.000	0.000	0.983	0.088	0.000	0.673	0.000	0.000	0.008
M + A − C		0.000	0.000	0.000	0.001	0.000	0.000	0.046	0.005	0.000	0.000	0.000	0.074	0.000	0.006
M + S − C		0.467	0.752	0.016	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.027	0.066	0.648	0.022
M + T − C		0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.001	1.000	0.000	0.794	0.000	0.000	0.001
S − C		0.789	0.735	0.299	0.999	0.000	0.000	0.000	0.023	0.568	0.000	0.048	0.055	0.000	0.000
T − C		0.128	0.090	0.496	0.496	0.000	0.000	0.000	0.001	0.999	0.998	0.999	0.739	0.000	0.002
M + A − M	0.014	0.994	0.999	0.977	0.999	0.348	0.064	0.107	0.000	0.000	0.020	0.000	0.000	0.000	0.999
M + S − M	0.999	0.005	0.000	0.934	0.982	0.124	0.000	0.728	0.002	0.039	0.000	0.872	0.007	0.000	0.003
M + T − M	0.000	0.999	0.998	0.999	1.000	0.051	0.071	0.272	0.000	0.075	0.000	0.999	0.025	0.001	0.999
S − M		0.000	0.000	0.000	0.000	0.999	0.056	0.999	0.002	0.953	0.000	0.921	0.002	1.000	0.000
T − M		0.036	0.006	0.112	0.214	0.999	0.086	0.025	0.000	0.071	0.002	0.840	0.000	0.494	0.031
M + S − M + A	0.010	0.001	0.000	0.419	0.935	0.000	0.224	0.905	0.000	0.000	0.000	0.000	0.986	0.000	0.000
M + T − M + A	0.057	0.999	0.999	0.999	0.999	0.000	0.636	0.999	0.997	0.000	0.001	0.000	0.025	0.000	0.988
S − M + A		0.000	0.000	0.000	0.000	0.140	0.481	0.287	0.999	0.000	0.000	0.000	0.999	0.000	0.000
T − M + A		0.005	0.011	0.056	0.132	0.662	0.601	0.000	0.991	0.000	0.000	0.000	0.448	0.000	0.016
M + T − M + S	0.000	0.001	0.001	0.782	0.977	0.336	0.000	0.993	0.000	0.000	0.000	0.770	0.023	0.000	0.001
S − M + S		0.023	0.042	0.000	0.004	0.218	0.000	0.949	0.000	0.034	0.803	0.999	0.999	0.000	0.916
T − M + S		0.994	0.857	0.686	0.731	0.054	0.000	0.006	0.000	0.000	0.000	0.137	0.094	0.000	0.008
S − M + T		0.000	0.000	0.000	0.000	0.053	0.978	0.569	0.919	0.520	0.001	0.840	0.031	0.001	0.000
T − M + T		0.010	0.033	0.050	0.198	0.051	0.727	0.001	0.999	0.999	0.000	0.921	0.002	0.110	0.005
T − S		0.003	0.001	0.054	0.198	0.992	0.093	0.033	0.866	0.250	0.000	0.179	0.202	0.046	0.001

Table A3. Pearson correlation coefficient values (R: Pearson correlation; P: *p*-value; KMP: kaempferol; CA: coumaric acid; CHex: coumaroyl hexoside; QCT: quercetin; Q3DG: quercetin-3-diglucoside; CGA: chlorogenic acid; SIN: sinapoyl DCQH: di-caffeyl quinic acid; SFW: shoot fresh weight; SDW: shoot dry weight; RFW: root fresh weight; RDW: root dry weight; Y: yield; GST: gluthation-S-transferase enzyme activity). * moderate degree of correlation at R > ±0.30, ** high degree of correlation at R > ±0.50.

		KMP	CA	CHex	QCT	QDG	CGA	SIN	DCQH	SFW	SDW	RFW	RDW	Y	GST
KMP	R	1.00	−0.07	0.01	−0.23	−0.26	0.42 **	0.28	−0.02	0.17	0.24	0.19	0.23	0.51 **	0.57 **
	P		0.66	0.97	0.15	0.10	0.01	0.08	0.90	0.29	0.13	0.24	0.14	0.00	0.00
CA	R	−0.07	1.00	0.13	−0.45 **	−0.52 **	0.28	−0.58 **	−0.22	0.06	0.07	0.22	0.21	0.24	−0.53 **
	P	0.66		0.42	0.00	0.00	0.08	0.00	0.18	0.70	0.69	0.17	0.18	0.14	0.00
CHex	R	0.01	0.13	1.00	−0.27	0.18	0.19	0.41 **	0.23	0.39 *	0.36 *	0.42 **	0.40 **	0.07	−0.13
	P	0.97	0.42		0.09	0.26	0.25	0.01	0.16	0.01	0.02	0.01	0.01	0.69	0.43
QCT	R	−0.23	−0.45 **	−0.27	1.00	0.66 **	−0.52 **	−0.02	0.31	−0.23	−0.21	−0.27	−0.31	−0.75 **	0.24
	P	0.15	0.00	0.09		0.00	0.00	0.90	0.05	0.14	0.19	0.09	0.05	0.00	0.13
Q3DG	R	−0.26	−0.52 **	0.18	0.66 **	1.00	−0.41 **	0.27	0.21	−0.29	−0.30	−0.28	−0.22	−0.61 **	0.29
	P	0.10	0.00	0.26	0.00		0.01	0.09	0.20	0.07	0.06	0.08	0.17	0.00	0.07
CGA	R	0.42 **	0.28	0.19	−0.52 **	−0.41 **	1.00	0.24	0.14	0.50 **	0.58 **	0.51 **	0.56 **	0.61 **	0.27
	P	0.01	0.08	0.25	0.00	0.01		0.14	0.39	0.00	0.00	0.00	0.00	0.00	0.10
SIN	R	0.28	−0.58 **	0.41 **	−0.02	0.27	0.24	1.00	0.47 **	0.56 **	0.52 **	0.38 *	0.38 *	0.25	0.48 **
	P	0.08	0.00	0.01	0.90	0.09	0.14		0.00	0.00	0.00	0.01	0.01	0.13	0.00
DCQH	R	−0.02	−0.22	0.23	0.31	0.21	0.14	0.47 **	1.00	0.58 **	0.61 **	0.45 **	0.41 **	0.02	0.21
	P	0.90	0.18	0.16	0.05	0.20	0.39	0.00		0.00	0.00	0.00	0.01	0.92	0.19
SFW	R	0.17	0.06	0.39 *	−0.23	−0.29	0.50 **	0.56 **	0.58 **	1.00	0.89 **	0.83 **	0.76 **	0.51 **	0.13
	P	0.29	0.70	0.01	0.14	0.07	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.42
SDW	R	0.24	0.07	0.36 *	−0.21	−0.30	0.58 **	0.52 **	0.61 **	0.89 **	1.00	0.80 **	0.68 **	0.47 **	0.16
	P	0.13	0.69	0.02	0.19	0.06	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.32
RFW	R	0.19	0.22	0.42 **	−0.27	−0.28	0.51 **	0.38 *	0.45 **	0.83 **	0.80 **	1.00	0.75 **	0.54 **	−0.02
	P	0.24	0.17	0.01	0.09	0.08	0.00	0.01	0.00	0.00	0.00		0.00	0.00	0.90
RDW	R	0.23	0.21	0.40 **	−0.31	−0.22	0.56 **	0.38 *	0.41 **	0.76 **	0.68 **	0.75 **	1.00	0.46 **	0.10
	P	0.14	0.18	0.01	0.05	0.17	0.00	0.01	0.01	0.00	0.00	0.00		0.00	0.55
Y	R	0.51 **	0.24	0.07	−0.75 **	−0.61 **	0.61 **	0.25	0.02	0.51 **	0.47 **	0.54 **	0.46 **	1.00	0.14
	P	0.00	0.14	0.69	0.00	0.00	0.00	0.13	0.92	0.00	0.00	0.00	0.00		0.39
GST	R	0.57 **	−0.53 **	−0.13	0.24	0.29	0.27	0.48 **	0.21	0.13	0.16	−0.02	0.10	0.14	1.00
	P	0.00	0.00	0.43	0.13	0.07	0.10	0.00	0.19	0.42	0.32	0.90	0.55	0.39	

Table A4. Eigenvalues of the principal component analysis.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigenvalue	2.682	2.094	1.170	0.832	0.551	0.408	0.136	0.127
Variability (%)	33.529	26.181	14.620	10.406	6.882	5.096	1.697	1.590
Cumulative %	33.529	59.710	74.330	84.736	91.617	96.713	98.410	100.000

Table A5. Correlations between variables and factors of the principal component analysis.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Kaempferol	0.186	0.350	−0.571	−0.117	−0.617	0.130	0.098	0.110
Coumaric acid	0.455	−0.183	0.407	0.254	−0.371	0.051	0.450	−0.433
Coumaroyl hexoside	0.012	0.394	0.651	−0.341	−0.268	0.198	−0.436	0.058
Quercetin	−0.504	−0.189	−0.111	0.312	−0.405	−0.063	−0.440	−0.489
Quercetin-3-diglucoside	−0.519	0.014	0.173	−0.233	−0.367	−0.446	0.498	0.255
Chlorogenic acid	0.352	0.414	−0.066	0.269	−0.062	−0.756	−0.228	0.017
Sinapoyl	−0.221	0.587	−0.072	−0.171	0.322	0.037	0.286	−0.621
Di-caffeylquinic acid	−0.249	0.368	0.179	0.743	0.058	0.291	0.151	0.328

Table A6. Peak identification of the HPLC profile of phenolic compounds.

Peak No.	Rt	Absorption Maxima (nm)				Compound's Name
1	7.372	297	326			Feruloyl dihexose
2	8.692	281	313			Coumaroyl dihexose
3	9.211	281	300	328		Sinapoyl di-glucoside
4	9.803	299	325			Feruloyl hexose
5	10.202	216	242	299	331	Sinapoyl hexose
6	10.854	279	302	331		Sinapoyl catechoyl derivative
7	11.714	302	329			Sinapoyl derivative
8	12.801	289	312			Coumaroyl glucoside
9	13.116	210	230	314		Caffeoylquinic acid derivative
10	14.442	311				Coumaric acid
11	14.843	216	238	297 (sh)	330	Sinapic acid derivative
12	15.982	298	327			Neochlorogenic acid
13	16.311	284	328			Chlorogenic acid isomer
14	16.643	294	327			Chlorogenic acid
15	17.341	300	330			Sinapic acid
16	17.764	282	326			Ferulic acid derivative
17	18.132	282	325			Feruloyl catechin derivative
18	18.442	282	322			Caffeic acid
19	20.053	282	311			Di-coumaroylquinic acid-glucose
20	20.69	255	266	354		Quercetin.3-o-glucose-xylose
21	21.471	255	266	318	362	Isorahmnetin-3-coffeoly di-glucoside
22		255				
23	24.317	297	329			Di-coffeoylquinic acid
24	25.634					
25	27.482	255	266	355		Rutin
26	28.227	268	348			Kaempferoyl-3-glucoside
27	29.416	222	266	355		Quercetin-3-glucoside
28	30.112					
29	30.604	298	332			Miric
30	36.354	241	297	322		Di-coffeoyl derivative

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